TARGETING THE METAL CHELATOR D-PENICILLAMINE TO EXPLOIT THE ELEVATED COPPER AND OXIDATIVE STRESS ASSOCIATED WITH CANCER

Anshul Gupte
University of Kentucky

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TARGETING THE METAL CHELATOR D-PENICILLAMINE TO EXPLOIT THE ELEVATED COPPER AND OXIDATIVE STRESS ASSOCIATED WITH CANCER

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By
Anshul Gupte
Lexington, Kentucky

Co-Directors: Dr. Russell Mumper, Professor of Molecular Pharmaceutics, University of North Carolina at Chapel Hill, North Carolina and Dr. Michael Jay, Professor of Pharmaceutical Sciences, Lexington, Kentucky

2008

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ABSTRACT OF DISSERTATION

TARGETING THE METAL CHELATOR D-PENICILLAMINE TO EXPLOIT THE ELEVATED COPPER AND OXIDATIVE STRESS ASSOCIATED WITH CANCER

The significantly increased copper and oxidative stress levels are characteristic hallmarks of cancer cells. These differences provide a unique opportunity for selective targeting of cancer cells. D-penicillamine (D-pen) has been proposed to generate reactive oxygen species (ROS) in presence of copper. Therefore, these studies were aimed at investigating the potential application of a currently marketed copper chelator, D-pen, as a novel cytotoxic anti-cancer agent.

D-pen was shown to produce ROS, specifically hydrogen peroxide (H$_2$O$_2$), in the presence of cupric sulfate through a copper catalyzed oxidation reaction. During this process D-pen was converted to D-pen disulfide. The experimental proof of the H$_2$O$_2$ generation was conclusively shown with the aid of a novel High Performance Liquid Chromatography (HPLC) assay.

The in-vitro cytotoxicity of D-pen co-incubated with cupric sulfate was examined in human breast cancer (MCF-7 and BT474) and leukemia cells (HL-60, HL-60/VCR, and HL-60/ADR). D-pen was shown to cause concentration dependent cytotoxicity in both leukemia and breast cancer cells. A direct correlation between the detection of intracellular ROS and cytotoxicity was established. The treatment of D-pen plus cupric sulfate resulted in a significant reduction in the intracellular thiol content.

D-pen is highly hydrophilic and is rapidly eliminated from the body; therefore to improve the intracellular uptake and to protect the thiol group of D-pen, we carried out the synthesis and the in-vitro characterization of a novel gelatin-D-pen conjugate. It was shown that D-pen alone does not enter cells. Confocal microscopy was employed to exhibit the uptake of the novel gelatin-D-pen conjugate by cancer cells.

As the cancer cells in-vitro do not accumulate the same levels of copper as reported for cancer cells in-vivo, cancer cells were pre-treated with cupric sulfate to simulate the elevated copper levels. The cupric sulfate pretreatment resulted in reduced thiol level and significantly increased cellular copper content compared to untreated cells. Whereas both free D-pen and gelatin-D-pen conjugate lacked cytotoxicity in un-treated cells, both agents caused concentration dependent cytotoxicity in cupric sulfate pre-treated leukemia cells.

Therefore, it was shown that the administration of D-pen as polymer conjugate would potentially provide cytotoxicity and specificity in the treatment of cancer.
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To my mother, brother, wife
and in the loving memory of my father, Prafull
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# TABLE OF CONTENTS

Acknowledgements ............................................................................................................ iii  
List of Tables ................................................................................................................... viii  
List of Figures ...................................................................................................................... x  
List of Files ...................................................................................................................... xiv

Chapter 1: Introduction and statement of the problem ........................................................1  
Chapter 2: Plan of research .................................................................................................5  
  2.1 An *in-vitro* investigation into copper catalyzed D-penicillamine oxidation  
    and the subsequent hydrogen peroxide generation ..................................................6  
  2.2 An *in-vitro* investigation of D-penicillamine cytotoxicity in breast cancer and  
    leukemia cells as a result of intracellular reactive oxygen species generation ....6  
  2.3 Enhanced intracellular delivery of the reactive oxygen species (ROS)-    
    generating copper chelator D-penicillamine via a novel gelatin-D-penicillamine  
    conjugate ..................................................................................................................7  
  2.4 Copper pre-treatment of human leukemia cells augments the cytotoxicity  
    resulting from D-penicillamine chelation of copper .................................................8

Chapter 3: Background and significance ...........................................................................10  
  3.1 Copper and cancer .............................................................................................10  
    3.1.1 Copper ......................................................................................................10  
    3.1.1 a Ceruloplasmin ....................................................................................15  
    3.1.2 Copper levels in the serum and tumor of cancer patients .......................16  
    3.1.3 Copper and angiogenesis .......................................................................21  
    3.1.4 Copper chelators as anti-angiogenic agents ............................................23  
  3.2 D-penicillamine (D-pen) ..................................................................................25  
    3.2.1 D-pen in the treatment of Wilson’s disease and rheumatoid arthritis ......28  
    3.2.2 Copper chelation by D-pen .....................................................................30  
    3.2.3 Metal catalyzed D-pen oxidation ..............................................................33
3.2.4 Quantitative analysis of D-pen .................................................................35
3.2.5 D-pen as an anti-angiogenic agent ...........................................................37
3.2.6 D-pen as cytotoxic anti-cancer agent .......................................................38
3.3 Cancer and oxidative stress ............................................................................42
3.3.1 Reactive oxygen species (ROS) ...............................................................46
  3.3.1 a Hydrogen peroxide (H₂O₂) .................................................................51
  3.3.1 b Superoxide radical (O₂⁻) ....................................................................55
3.3.2 Antioxidants .............................................................................................56
  3.3.2 a Glutathione .........................................................................................64
3.3.3 ROS and cancer ........................................................................................68
  3.3.3 a Leukemia and oxidative stress ...........................................................75
  3.3.3 b Breast cancer and oxidative stress .....................................................78
3.3.4 ROS in cancer treatment ..........................................................................83
3.4 Intracellular delivery of hydrophilic anticancer drugs ...................................89
  3.4.1 Polymer drug conjugates .........................................................................90
    3.4.1 a Gelatin conjugates .............................................................................95

Chapter 4: An in-vitro investigation into copper catalyzed D-pen oxidation and the
subsequent hydrogen peroxide generation ............................................................96
  4.1 Summary ....................................................................................................96
  4.2 Introduction ................................................................................................98
  4.3 Materials and methods .............................................................................101
  4.4 Results and discussion .............................................................................106

Chapter 5: An in-vitro investigation of D-pen cytotoxicity in breast cancer and
leukemia cells as a result of intracellular reactive oxygen species generation ..........125
  5.1 Summary ..................................................................................................125
  5.2 Introduction ..............................................................................................126
  5.3 Materials and methods .............................................................................129
  5.4 Results .....................................................................................................133
  5.5 Discussion ...............................................................................................137
Chapter 6: Enhanced intracellular delivery of the reactive oxygen species (ROS) generating copper chelator D-penicillamine via a novel gelatin-D-penicillamine conjugate .......................................................... 154

6.1 Summary ...................................................................................................... 154
6.2 Introduction .............................................................................................. 156
6.3 Materials and methods ............................................................................. 159
6.4 Results and discussion ............................................................................ 166
6.5 Conclusion ............................................................................................... 170

Chapter 7: Copper pre-treatment of human leukemia cells augments the cytotoxicity resulting from D-penicillamine chelation of copper ......................................................... 182

7.1 Summary .................................................................................................. 182
7.2 Introduction ............................................................................................ 183
7.3 Materials and methods .......................................................................... 187
7.4 Results .................................................................................................... 191
7.5 Discussion ............................................................................................... 194

Chapter 8: Summary and conclusions .................................................................. 203

Appendices ............................................................................................................. 208

Appendix A: Illustrations and chemical structures of compounds employed in the dissertation .......................................................................................................................... 209

Appendix B: Development of HPLC-Fluorescence assay for determination of D-pen in biological samples .................................................................................................................. 215

Appendix C: Preparation and characterization of D-pen nanoparticles .................. 221

Appendix D: Synthesis of monostearyl ester of D-pen disulfide, a prodrug approach for the potential D-pen delivery ............................................................................................................. 229

Appendix E: Preparation and characterization of Herceptin-D-pen immunoconjugate ............................................................................................................................... 233
LIST OF TABLES

Table 3.1. Copper-dependent enzymes in mammals .............................................................14
Table 3.2. Copper levels in the serum of cancer patients .......................................................18
Table 3.3. Copper levels in the tumor tissue of cancer patients ..............................................20
Table 3.4. Classification of biologically important ROS .......................................................48
Table 3.5. Intracellular functions and targets of ROS ............................................................49
Table 3.6. Classification of antioxidants in the human body .................................................63
Table 3.7. Lipid peroxidation and antioxidant system status in plasma of oral cancer patients vs. control ..................................................................................................................73
Table 3.8. Lipid peroxidation and antioxidant system status in tissue of stomach cancer patients vs. control ................................................................................................................74
Table 3.9. Antioxidant levels in leukemia patients compared to controls .........................76
Table 3.10. Oxidants and antioxidants in leukemia patients compared to controls ..........77
Table 3.11. Glutathione status in breast cancer patients ......................................................81
Table 3.12. Antioxidant status in breast cancer patients ......................................................82
Table 3.13. Polymer-drug conjugates in clinical trials .........................................................94
Table 4.1. Effect of D-pen added in succession on the copper catalyzed D-pen oxidation at pH 6.2 and 7.4 .........................................................................................................................117
Table 6.1. The effect of increasing SPDP/gelatin on the D-pen conjugation to gelatin.171
Table 7.1. The effect of cupric sulfate pretreatment on HL-60 cells .................................197
Table 7.2. In-vitro cytotoxicity of D-pen in HL-60 cells.........................................................198
Table 7.3. a) In-vitro cytotoxicity of the gelatin-D-pen conjugate in HL-60 cells ..........199  
             b) In-vitro cytotoxicity of the gelatin plus D-pen in HL-60 cells ..................200
Appendices

Table C1. Stability of D-pen-NPs

a) Temperature dependent stability of D-pen-NPs ........................................228

b) Stability of disulfide bond between D-pen and NPs after exposure to DTT .................................................................228
LIST OF FIGURES

Figure 3.1. Structure of D-pen and D-pen disulfide ........................................................26

Figure 3.2. Mechanism of copper catalyzed D-pen oxidation and hydrogen peroxide
generation...........................................................................................................................40

Figure 3.3. Mechanism of action of D-pen as a cytotoxic anti-cancer agent.................41

Figure 3.4. Reactive oxygen species (ROS) exposure and cancer cells ......................45

Figure 3.5. Kinetics of hydrogen peroxide (H₂O₂) entry into cells ..............................54

Figure 3.6. Redox couples in the human body.................................................................67

Figure 3.7. Mechanism of ROS based anti-cancer therapies ...........................................88

Figure 4.1. Mechanism of copper catalyzed D-pen oxidation .......................................118

Figure 4.2. HPLC chromatogram showing the elution of H₂O₂, D-pen disulfide, and
D-pen..................................................................................................................................119

Figure 4.3. Copper catalyzed D-pen oxidation to D-pen disulfide and the generation
of H₂O₂
   a) Effect of incubation time .......................................................................................120
   b) Effect of cupric sulfate concentration.................................................................120

Figure 4.4. Effect of pH and copper and iron concentration on D-pen oxidation.........121

Figure 4.5. Effect of temperature on copper catalyzed D-pen oxidation.......................122

Figure 4.6. Cytotoxicity of copper catalyzed D-pen oxidation.......................................123

Figure 4.7. Copper catalyzed D-pen oxidation results in the generation of intracellular
reactive oxygen species.................................................................................................124
Figure 5.1. The concentration dependent *in-vitro* cytotoxicity of H$_2$O$_2$ alone compared to D-pen plus cupric sulfate in HL-60, HL-60/VCR and HL-60/ADR leukemia cells.

a) H$_2$O$_2$ alone .................................................................141
b) D-pen plus cupric sulfate .....................................................141

Figure 5.2. The concentration dependent *in-vitro* cytotoxicity of H$_2$O$_2$ alone compared to D-pen plus cupric sulfate in MCF-7 and BT474 breast cancer cells.

a) H$_2$O$_2$ alone .................................................................143
b) D-pen plus cupric sulfate .....................................................143

Figure 5.3. Catalase inhibits the D-pen plus cupric sulfate cytotoxicity in breast cancer and leukemia cells.

a) MCF-7 cells .................................................................145
b) HL-60 cells .................................................................145

Figure 5.4. D-pen in the presence of cupric sulfate generates intracellular ROS in breast cancer and leukemia cells.

a) MCF-7 cells .................................................................147
b) HL-60 cells .................................................................147

Figure 5.5. The correlation between D-pen concentration and the intracellular ROS generation in breast cancer and leukemia cells.

a) MCF-7 cells .................................................................149
b) HL-60 cells .................................................................149

Figure 5.6. D-pen in the presence of cupric sulfate causes the reduction in intracellular thiol levels in leukemia cells .................................................................151

Figure 5.7. Intracellular superoxide anion generation in HL-60 cells ..................153
Figure 6.1. Schematic illustration of gelatin-D-pen conjugate synthesis.................172
Figure 6.2. Effect of increasing SPDP concentration on the modification of gelatin.....174
Figure 6.3. Release of D-pen from the gelatin-D-pen conjugate under reducing conditions.................................................................175
Figure 6.4. HPLC chromatogram showing glutathione and D-pen elution ...............177
Figure 6.5. Cell uptake of D-pen ........................................................................178
Figure 6.6. Intracellular uptake of gelatin-D-pen conjugate with confocal microscopy.................................................................179
Figure 6.7. Sustained cytotoxicity of the gelatin-D-pen conjugate in HL-60 cells ......181
Figure 7.1. The intracellular copper and thiol status after cupric sulfate pre-treatment .................................................................201
Figure 7.2. In-vitro cytotoxicity of D-pen plus cupric sulfate in HL-60 cells..........202

Appendices
Figure A1. Structure of thiols and disulfides employed in these studies.............209
Figure A2. Structure of chelators and reducing agents used these studies ..........210
Figure A3. Structure of cross-linker and lipid used in these studies ....................211
Figure A4. Mechanism of Ellman’s and trinitrobenzenesulfonic acid (TNBS) assay...212
Figure A5. Mechanism of intracellular ROS and superoxide anion assay .............213
Figure A6. Structure of the amino reactive fluorescein (NHS-Fluorescein) ..........214
Figure B1. Structure of N-pyrenyl maleimide (NPM)...........................................218
Figure B2. HPLC-Fluorescence assay of D-pen
  a) HPLC chromatograph of NPM-D-pen derivative elution.........................219
b) *In-vitro* recovery of D-pen spiked in brain homogenate .......................219

Figure B3. Stability of NPM-D-pen adduct
   a) Stability of NPM-D-pen adduct ..............................................220
   b) Characterization of incubation time of D-pen with NPM ...............220

Figure C1. Formulation of D-pen nanoparticles
   a) Schematic of the formulation of D-pen nanoparticles ..................224
   b) Schematic showing the conjugation of D-pen to PDP-NPs ...............224

Figure C2. Effect of PDP incorporation on the size and PI of NPs
   a) Effect of increasing PDP (% w/w) loading on NPs size (nm) ..........225
   b) Effect of increasing PDP loading on particle size (nm) and PI ........225

Figure C3. Gel permeation chromatography (GPC) separation of free D-pen from D-pen-NPs ..............................................................226

Figure C4. Short term stability of D-pen-NPs in biological media ...............227

Figure D1. Schematic of the synthesis of monostearyl ester of D-pen disulfide ........231

Figure D2. Mass spectrum of the purified monostearyl ester of D-pen-disulfide ........232

Figure E1. Schematic of the synthesis of the Herceptin-D-pen immunoconjugate ......238

Figure E2. GPC separation of Herceptin, thiolated Herceptin or the Herceptin-D-pen immunoconjugate
   a) Coomassie assay of fractions showing the elution of Herceptin .........239
   b) Ellman’s assay on all fractions showing thiolated Herceptin ..........239

Figure E3. *In-vitro* release of D-pen from the Herceptin-D-pen immunoconjugate in non-reducing and reducing conditions .................................................240
Chapter 1

Introduction and statement of problem

As we gain better understanding of the factors affecting cancer etiology, we can design improved treatment strategies. Over the past three to four decades, there have been successful efforts in identifying several important cellular proteins which might be involved in cancer growth and hence numerous studies have investigated the targeting of these proteins as an anti-cancer strategy. However, studies have shown that targeting one or two proteins in the complex cancer cascade may not be sufficient in controlling and/or inhibiting cancer growth. Therefore, there is a need to develop ‘smart cancer treatment strategies’, which target multiple facets of cancer development.

Angiogenesis is simply defined as ‘the development of new blood vessels’ [1]. While angiogenesis is a normal phenomenon in the body, tumors are known to employ this process for their benefit. Tumors can only grow to about 1 to 2 mm in diameter without recruiting blood vessels that supply essential nutrients [1]. Cells employ a number of endogenous angiogenic stimulators and inhibitors in the process of angiogenesis [1]. It is known that in a normal cell there exists a balance between the endogenous stimulators and inhibitors, and thus the process of angiogenesis is controlled [1]. Copper is an important trace metal needed for the normal physiological and biochemical functions in the body, by acting as a co-factor for many important enzymes [2, 3]. Copper has also been established to be an endogenous angiogenic stimulator, by acting as a co-factor for a number of important pro-angiogenic molecules such as Vascular Endothelial Growth Factor (VEGF), basic-Fibroblast Growth Factor (β-FGF),
and Angiogenin [4, 5]. Several in-vitro and in-vivo studies have shown that cancer cells in high copper environment find it easy to proliferate into a solid tumor [6, 7] and copper reduction has been shown to inactivate the functions of structurally diverse angiogenic factors, cytokines and prostaglandins [8].

Both the serum and tumor copper levels have been shown to be elevated in a variety of malignancies, including both solid tumor and blood cancer [9-12]. Additionally, the copper levels have been shown to be directly correlated to cancer progression [13]. As copper has been established to be essential for angiogenesis, copper chelators such as D-penicillamine (D-pen), tetrathiomolybdate, clioquinol, trientine have been extensively examined for their potential use as anti-angiogenic agents [8, 14-16].

 Reactive oxygen species (ROS) can be defined as a collective term that includes both oxygen radicals and non-radicals that are oxidizing agent and/or easily converted into radicals [17]. ROS has been implicated both in the etiology and the treatment of cancer [18]. ROS includes radicals such as superoxide (O$_2^-$), hydroxyl (OH) radical and non-radicals such as hydrogen peroxide (H$_2$O$_2$). Enhanced levels of intrinsic ROS stress has been shown in variety of tumors, possibly due to the combination of factors such as elevated active metabolism, mitochondrial mutation, cytokines, and inflammation [18, 19]. The proof of elevated ROS stress in cancer cells has been shown directly with the measurements of the increase in ROS production in cancer cells compared to normal cells, and indirectly through the accumulation of oxidative products, and the overexpression of anti-oxidant enzymes (the consequences of adaptation to the excess ROS in cancer cells) [19]. Cellular proliferation, DNA damage, genetic instability, cellular injury, cell death, and alteration in drug sensitivity have been known to be
associated with the ROS stress in cancer cells [19]. Thus, the cancer cells under sustained ROS stress tend to heavily utilize adaptation mechanisms and may exhaust cellular ROS-buffering capacity [20, 21]. The cellular protection against ROS includes antioxidant scavengers such as glutathione, ascorbate, thioredoxin and antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase [22].

D-pen is a highly hydrophilic aminothiol. D-pen has been approved by the Food and Drug Administration (FDA) for over 40 years for the treatment of Wilson’s disease and Rheumatoid Arthritis. The use of D-pen in the treatment of the aforementioned disorders is based on its well known copper chelating ability; however, the exact mechanism of copper chelation (specifically its interaction with copper during copper chelation) is poorly understood. Additionally, Starkebaum et al. has previously proposed that H₂O₂ and other ROS are produced during the D-pen interaction with copper [23, 24]; however, the qualitative and quantitative experimental proof of ROS generation from D-pen in the presence of copper still remains lacking and further investigation is warranted.

Therefore, the present research was focused both on the proof of concept of the use of widely established copper chelator D-pen as a novel cytotoxic copper chelating anticancer agent and on the investigation of the enhanced intracellular delivery of D-pen with the aid of novel delivery strategies including nanoparticles, immunoconjugates, and polymer-drug conjugates. These studies are of great potential significance since elevated copper levels in an assortment of malignancies have been clearly documented. Therefore, D-pen shown to produce ROS in the presence of copper then the ROS production from D-pen could be targeted to cancer cells. The use of D-pen in the treatment of cancer may potentially provide dual anti-cancer effects: 1) cytotoxic, through the copper catalyzed
ROS (H$_2$O$_2$) generation from D-pen and 2) anti-angiogenic, through the copper chelation. Several *in-vitro* studies were carried out to experimentally prove the quantitative H$_2$O$_2$ generation as result of D-pen oxidation. Additionally, direct evidence of intracellular ROS generation was shown in human breast cancer and leukemia cells after treatment with D-pen in the presence of cupric sulfate. The cytotoxicity resulting from D-pen in the presence of cupric sulfate was compared with that caused by H$_2$O$_2$ alone.

Further, to improve the inherent physicochemical properties of D-pen such as hydrophilicity, short *in-vivo* half-life, and the liability of spontaneous oxidation, a novel disulfide gelatin-D-pen conjugate was synthesized. *In-vitro* studies evaluating parameters such as release of D-pen in non-reducing/reducing conditions, qualitative and quantitative uptake, and cytotoxicity were conducted with the gelatin-D-pen conjugate. Given the significantly elevated copper levels in cancer patients compared to normal individuals these studies have important implications for the potential use of D-pen as a novel, selective and potent agent in cancer treatment.
Chapter 2

Plan of research

The overall goal of this research was to investigate the cytotoxic anti-cancer properties of the copper chelating agent, D-penicillamine (D-pen), through the generation of hydrogen peroxide (H$_2$O$_2$) and other reactive oxygen species (ROS) and to develop novel delivery strategies for enhancing its intracellular uptake in order to potentially improve its in-vivo therapeutic efficacy as an anti-cancer agent. This research was guided by four fundamental hypotheses:

**Hypothesis 1.** Copper catalyzed D-pen oxidation will lead to the concentration dependent generation of H$_2$O$_2$.

**Hypothesis 2.** H$_2$O$_2$ generated from D-pen in the presence of cupric sulfate will cause ROS stress in human cancer cells and result in cytotoxicity.

**Hypothesis 3.** A novel polymer-D-pen conjugate could be synthesized and the conjugate would be able to deliver D-pen intracellularly and cause cytotoxicity in cancer cells.

**Hypothesis 4.** Copper pre-treatment of human cancer cells in culture would simulate the elevated in-vivo copper status and augment the cytotoxicity of D-pen.

To evaluate these hypotheses, the research plan described in sections 2.1 to 2.4 was carried out.
2.1 An *in-vitro* investigation into copper catalyzed D-pen oxidation and the subsequent hydrogen peroxide (H$_2$O$_2$) generation

The main objectives of this section were: 1) to develop a simple, sensitive and rapid high performance liquid chromatography (HPLC) assay to simultaneously detect and quantify D-pen, D-pen disulfide and H$_2$O$_2$ in a single run; 2) to provide experimental evidence that concentration dependent H$_2$O$_2$ is generated *in-vitro* as a result of copper catalyzed D-pen oxidation; and 3) to investigate the mechanism of copper catalyzed D-pen oxidation and simultaneous H$_2$O$_2$ production as a function of time, concentration of cupric sulfate or ferric chloride, temperature, pH, anaerobic condition and chelators such as ethylenediaminetetraacetic acid (EDTA) and bathocuproinedisulfonic acid (BCS). The biological implications of cytotoxic H$_2$O$_2$ generation as a result of chelation of D-pen with copper was examined against MCF-7 cells, the human breast cancer cells and compared to the cytotoxic effects of D-pen, cupric sulfate and H$_2$O$_2$ alone.

2.2 An *in-vitro* investigation of D-pen cytotoxicity in human breast cancer and leukemia cells as a result of intracellular reactive oxygen species generation

The main objectives of this section were: 1) to investigate the *in-vitro* cytotoxicity of D-pen in the presence and absence of cupric sulfate and/or catalase compared to H$_2$O$_2$ alone in human breast cancer (HER2 positive vs. HER2 negative) and leukemia cells (wild type vs. anthracycline resistant cells); 2) to examine if the cytotoxicity of D-pen in the presence of cupric sulfate correlated to the *in-vitro* non-cell based molar ratio of D-pen oxidized to H$_2$O$_2$ generated; 3) to investigate the intracellular H$_2$O$_2$, superoxide anion ($O_2^{-}$), and total cellular thiol (glutathione) content as result of treatment with D-pen plus
cupric sulfate compared to H₂O₂ alone. Breast cancer cell lines differing in HER2 expression: MCF-7 (HER2 negative) and BT474 (HER2 positive), and leukemia cell lines differing based on their anthracycline sensitivity: HL-60 (wild type), HL-60/VCR (P-gp) and HL-60/ADR (MRP-1) were used in these studies to ascertain differences in H₂O₂ and ROS cytotoxic effects. The cell viability was measured using 3-(4, 5-dimethyl-2-yl)-2, 5-diphenyleraolium bromide (MTT) assay. 2’-7’-dichlorodihydrofluorescein diacetate (H₂DCFDA) was used as an indicator of intracellular ROS generation. H₂DCFDA is a cell permeable probe, it enters the cell and is deacetylated to a non-fluorescent product, 2’-7’-dichlorodihydrofluorescein (H₂DCF) by cellular esterases and is oxidized by ROS to a fluorescent product, 2’-7’-dichlorofluorescein (DCF). The intracellular reduced thiol levels were measured with 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB).

2.3 Enhanced intracellular delivery of the reactive oxygen species (ROS)-generating copper chelator D-penicillamine via a novel gelatin-D-penicillamine conjugate

The overall objectives of this study were: 1) to synthesize and characterize a novel bioactive disulfide gelatin-D-pen conjugate; 2) to synthesize fluorescein-gelatin (fluorescein labeled gelatin), fluorescein-gelatin-D-pen conjugate for cell uptake studies; 3) to evaluate the biological reversibility of the disulfide bond between gelatin and D-pen through the in-vitro release of D-pen from the gelatin-D-pen conjugate in simulated physiological conditions in the presence of glutathione; 4) to perform the in-vitro intracellular uptake of fluorescein labeled conjugates in human leukemia cells (HL-60).
with confocal microscopy; 4) to evaluate the *in-vitro* cell cytotoxicity of the conjugate compared to D-pen, gelatin alone and a physical mixture of D-pen plus gelatin. D-pen was covalently coupled with a reversible disulfide bond to gelatin with the aid of a heterobifunctional cross linker, (N-Succinimidyl-3-(2-pyridyldithio)-propionate) (SPDP). Gelatin was coupled with SPDP through the amino group of gelatin and the amine-reactive N-hydroxysuccinimide (NHS) ester of SPDP to form a stable amide bond. D-pen was then conjugated to the SPDP modified gelatin through thiol exchange with D-pen. Thus, D-pen was efficiently conjugated to gelatin with a potentially reversible disulfide bond. The conjugate was incubated at pH 6.2 (to simulate the early endosomal pH~5.5-6.5) and pH 7.4 (to simulate the cytoplasmic pH~7.4) in the presence of various concentrations of glutathione for specific time to simulate intracellular release of D-pen from the conjugate. Gelatin-D-pen conjugate was fluorescently labeled to determine the intracellular uptake of the conjugate with the human leukemia cells. The cytotoxicity of the conjugate was evaluated in human leukemia cells and compared to gelatin, D-pen alone and also to a physical mixture of gelatin with D-pen.

### 2.4 Copper pre-treatment of human leukemia cells augments the cytotoxicity resulting from D-penicillamine chelation of copper

The main objectives of this section were: 1) pre-treat cancer cells for predetermined time with various concentrations of cupric sulfate to increase the intracellular copper levels and simulate elevated *in-vivo* copper levels. 2) Assess the total cell number, viability, intracellular glutathione status and intracellular copper amounts after the cupric sulfate pre-treatment and compare these values to un-treated (naïve) cancer cells. 3)
Assess the *in-vitro* cytotoxicity of free D-pen and the novel gelatin-D-pen conjugate in naïve and copper pretreated cancer cells. Leukemia cells were used as model cell line for these studies. Cultured cells *in-vitro* do not accumulate the same levels of copper compared to cells *in-vivo*; therefore, cells were pre-treated with cupric sulfate. The total cell number, cellular thiol status, cellular protein status, intracellular copper content was analyzed after the copper pre-treatment. Additionally, the *in-vitro* cytotoxicity of a copper chelator, D-pen and novel gelatin-D-pen conjugate on the naïve and the cupric sulfate pre-treated cancer cells was investigated.
Chapter 3

Background and significance

3.1 Copper and cancer

3.1.1 Copper

Copper (Cu) is an essential trace element that plays a central role in the biochemistry of every living organism [3]. In the body copper exists in both oxidation states, oxidized Cu(II) and reduced Cu(I) [2, 3]. The unique electronic structure of copper allows it to serve as a co-factor in redox reactions of enzymes that carry out fundamental biological functions required for normal growth and development such as cytochrome c oxidase (involved in the mitochondrial electron transport chain), lysyl oxidase (involved in the cross-linking of elastin and collagen), and superoxide dismutase (involved in the detoxification of reactive oxygen species (ROS)) [2]. A comprehensive list of the major copper dependent enzymes and their physiological functions is listed in Table 3.1.

The recommended daily copper intake in healthy adults is 0.9 mg/day [25, 26]. The absorption of copper into the human body is a complicated process and depends on various factors and dietary components [27]. Copper is largely absorbed in the small intestine through the amino acid transporters, mainly containing methionine, histidine and cysteine. Copper absorbed in the intestine is transported to the liver bound primarily to serum albumin, and to some extent transcuprein [28]. During the process of cellular copper uptake, Cu(II) is reduced to Cu(I) and copper enters the cell through various transmembrane transporters such as human copper transporter (hCtr1), which belongs to the family of high affinity copper transporters [29, 30]. The intracellular transport of
Copper is performed with the aid of either glutathione as the Cu(I)-Glutathione complex or a class of small cytosolic proteins known as copper chaperons such as antioxidant (Atox 1), cytochrome c oxidase (Cox 17) and copper chaperon for superoxide dismutase (CCS) [28, 29, 31, 32]. The Cu(I)-Glutathione complex serves as a vehicle for delivering copper to metallothioneins (MT), a family of proteins that serve the purpose of intracellular metal detoxification. In contrast, the copper chaperons shuttle the copper to specific compartments inside the cell [28, 33, 34]. Copper is mainly excreted via the biliary pathway (~80%) [33, 34].

Ceruloplasmin is the major copper carrying protein in human plasma and contains ≥ 75% of total plasma copper [2, 35]. Albumin and transthyretin are known to carry the remaining plasma copper. The copper bound to these carriers has been reported to be the less tightly bound (exchangeable) copper [36, 37]. The concentration of free copper in human plasma is reported to be ~10^{-13} M [2]. The chemical properties which make copper biologically useful can also potentially lead to toxic effects, namely, copper induced oxidative stress. Therefore, the metabolism and compartmentalization of copper is a highly regulated process [3]. The complex mechanisms of copper transport and metabolism in order to maintain copper homeostasis are underscored by two inherited disorders, Wilson’s disease and Menke’s disease.

Wilson’s disease is autosomal recessive disorder resulting from the mutation in the ATP7B gene [38, 39]. The Wilson’s protein is encoded by the ATP7B gene. The protein is homologous to the P-type ATPases and is predominately expressed in the liver and the brain [38]. Wilson’s disease results in copper accumulation mainly in the liver and brain causing in liver cirrhosis and neurological degeneration [38, 39]. A characteristic
hallmark of the neurological form of Wilson’s disease is the presence of ‘Kayser-Fleischer ring’ which forms due to copper deposits in the cornea [38]. The clinical features of Wilson’s disease include decreased serum copper and ceruloplasmin levels and increased liver copper levels [3, 38, 40, 41]. The frequency of the disease has been estimated to be around 1 in 30,000 and the carrier frequency to be approximately 1 in 90 [3]. The Long Evans Cinnamon rat and the toxic milk mouse have been used as the animal model to study Wilson’s disease [39]. The current clinical treatment of Wilson’s disease includes low copper diet and the use of copper chelators such as tetrathiomolybdate and D-pen [38, 39].

Menke’s disease is an X-linked disorder resulting from the mutation in the ATP7A gene [3]. The Menke’s protein is encoded by the ATP7A gene. The Menke’s protein is more widely expressed throughout the body compared to its counterpart the Wilson’s protein [3, 42]. Menke’s disease is characterized by severe neurodegeneration, hypothermia and connective tissue abnormalities due to deficient copper-dependent enzyme activity [3, 36]. The clinical features of Menke’s disease include decreased serum copper, ceruloplasmin and liver copper levels [3, 36]. The frequency of the disease has been estimated to be around 1 in 300,000 [3]. The mottled mouse has been used as the animal model to study Menke’s disease [42, 43].

Although copper is known to play an important functional role in the brain as a cofactor of enzymes, it is also known to contribute to brain disorders such as Alzheimer’s disease and Parkinson’s disease. Alzheimer’s disease is a neurodegenerative disorder with no effective cure or treatment [44]. The distribution of copper has been reported to be severely altered in Alzheimer’s disease patients, copper levels are significantly
elevated (~400 µM) compared to healthy brain (~70 µM) [45, 46]. Additionally, the β-amyloid protein, which has been proposed to be responsible for the formation of the damaging plaques in Alzheimer’s disease, is known to avidly bind copper [47-50].

As discussed earlier, copper is highly regulated in the biological system. Excess copper has been known to be a potent oxidant causing the generation of ROS in the cells. The role of copper in both the etiology and growth of tumors has been extensively studied for the past two decades [4, 51-53]. This was based on reports that copper distribution was altered in studies of tumor bearing mice [54, 55], rats [54] and in humans [56-58]. Additionally, serum and tumor copper levels have been reported to be significantly elevated in cancer patients compared to healthy individuals [9, 59, 60].
### Table 3.1 Copper-dependent enzymes in mammals. (Adapted from Tapiero [2])

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c-oxidase</td>
<td>Electron transport in mitochondria</td>
</tr>
<tr>
<td>Cu/Zn-SOD</td>
<td>Free radical detoxification</td>
</tr>
<tr>
<td>Metallothioneins</td>
<td>Storage of excess Cu</td>
</tr>
<tr>
<td>Ceruloplasmin (extracellular)</td>
<td>Ferroxidase</td>
</tr>
<tr>
<td>Lysine-6-oxidase</td>
<td>Cross-linking of collagen and elastin</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Formation of melanin</td>
</tr>
<tr>
<td>Dopamine-β-monoxygenase</td>
<td>Catecholamines production</td>
</tr>
<tr>
<td>Diamine oxidase</td>
<td>Inactivation of histamine</td>
</tr>
<tr>
<td>Amine Oxidase</td>
<td>Inactivation of histamine and dopamine</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Induction of blood vessel formation</td>
</tr>
</tbody>
</table>
3.1.1.a Ceruloplasmin

The protein ‘ceruloplasmin’ was first isolated from plasma and characterized as a copper containing protein by Holmberg and Laurell in 1948. Ceruloplasmin belongs to a family of multicopper oxidases [61]. This group of proteins is characterized by the presence of three types of spectroscopically distinct copper sites [35]. The structural model of human ceruloplasmin based on X-ray crystallographic data has confirmed the presence of three type I copper sites (charge transfer between copper and the sulfur in cysteine results in strong absorption at 600 nm conferring intense blue color to the protein), combined with the type II and III coppers it forms a trinuclear copper cluster that is the site of oxygen binding [35, 61]. Ceruloplasmin contains six integral copper atoms together with an additional (loosely bound) copper atom. This loosely bound copper atom has been suggested to be responsible for the pro-oxidant nature of ceruloplasmin [62-64].

Ceruloplasmin is a 1046 amino acid serum glycoprotein normally synthesized in the liver [65]. The normal human plasma concentration of ceruloplasmin is between 18-35 mg/dL [35]. Ceruloplasmin is a multifunctional protein; 1) It is involved in copper transport to various tissues and its subsequent incorporation in copper containing enzymes, 2) It has ferroxidase activity (based on its ability to oxidize Fe(II) to Fe(III) and its subsequent incorporation into transferrin, 3) It is involved in the oxidation of aromatic phenols and amines, and 4) It aids in the antioxidant defense as a result of the removal of superoxide anion [66]. Due to the above stated multifunctional aspect of ceruloplasmin, it has been suggested to be a ‘moonlighting’ protein. This implies that ceruloplasmin has the capacity to switch between functions in response to changes in the concentration of its
ligand/substrate, to be differentially localized and/or be differentially expressed [66]. This might be the reason for conflicting reports about anti-oxidant and pro-oxidant effect of ceruloplasmin in literature [35, 62-64].

In Wilson’s disease, the levels of ceruloplasmin and plasma copper levels are reduced, despite the excess copper levels in various organs such as liver and brain [3, 40].

Aceruloplasminemia is an autosomal recessive disorder, which causes the lack of ceruloplasmin generation. The symptoms of aceruloplasminemia include dementia, dystonia, and retinal degeneration [35]. The absence of ceruloplasmin does not produce marked changes in copper metabolism; however this absence does affect iron levels. This is due to the previously mentioned ferroxidase activity of ceruloplasmin. Iron levels in the liver are gradually reduced leading to liver degeneration [35].

The levels of ceruloplasmin have also shown to be elevated in various forms of cancers such as lymphoma [67], breast cancer [68], and gastrointestinal tract cancer [69]. Ceruloplasmin has also been proposed to be an endogenous angiogenic stimulator used by the cancer cells for development of new blood vessels [4].

3.1.2 Copper levels in the serum and tumor tissue of cancer patients

Several reports in the literature have shown that both serum and tumor copper levels in cancer patients are significantly elevated. A comprehensive literature review of copper levels reported in serum and tumor tissue of cancer patients compared to that in the healthy individuals is documented in Table 3.2 and 3.3, respectively. A major percentage of these studies have focused on determining the concentrations of four important elements; copper, zinc, iron and selenium. The studies showed that while the zinc, iron
and selenium concentrations where significantly lower in cancer patients, the copper concentrations were almost always found to be either elevated or significantly elevated (upto 2-3 fold) compared to age matched samples from normal tissue [9, 70]. Furthermore, it has also been shown that the Cu/Zn, Cu/Se and Cu/Fe ratios are almost always higher in malignant patients compared to normal individuals [9]. It should be noted that the elemental concentration in these studies were determined with highly specific and sensitive elemental techniques such as atomic absorption spectroscopy or X-ray fluorescence (XRF).

Additionally, these elevated copper levels documented in cancer patients have been reported i) in a wide spectrum of tumors including breast [9, 12, 59, 60], cervical [71], ovarian [71], lung [69, 72], prostate [73], stomach [10, 11, 69, 74], reticulo-endothelial system [75] and leukemia [70, 76], ii) in different age groups of patients (10-50 and >50 years), iii) in both gender (male and female) [75, 77], iv) patients in different geographical location (Asia, Europe, and Americas) [9, 59, 60, 70, 72, 78], and most striking v) to correlate with cancer stage and/or progression. [9, 72, 79].
Table 3.2 Copper levels in serum of cancer patients

<table>
<thead>
<tr>
<th>Study</th>
<th>Cancer</th>
<th>Healthy (µg/dL ± SD)</th>
<th>Patients (µg/dL ± SD)</th>
<th>Number of subjects (significance compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carpentieri et al. 1986 [76]</td>
<td>Acute lymphocytic leukemia</td>
<td>114 ± 29</td>
<td>328 ± 74</td>
<td>21 (p&lt;0.01)</td>
</tr>
<tr>
<td>Huang et al. 1999 [12]</td>
<td>Breast cancer</td>
<td>115 ± 20</td>
<td>131 ± 20</td>
<td>35 (p&lt;0.01)</td>
</tr>
<tr>
<td>Chan et al. 1993 [71]</td>
<td>Cervical</td>
<td>92.9</td>
<td>129.3</td>
<td>19 (p&lt;0.001)</td>
</tr>
<tr>
<td>Chan et al. 1993 [71]</td>
<td>Ovarian</td>
<td>92.9</td>
<td>139.5</td>
<td>4 (p&lt;0.001)</td>
</tr>
<tr>
<td>Cohen et al. 1994 [80]</td>
<td>Non-hodgkin’s lymphoma</td>
<td>120.4 ± 23.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yucel et al. 1982 [60]</td>
<td>Breast cancer</td>
<td>122.4 ± 15.8</td>
<td>222.7 ± 44.4 (Stage I)</td>
<td>35</td>
</tr>
<tr>
<td>Diez et al. 1989 [72]</td>
<td>Lung cancer</td>
<td>100 ± 18.2</td>
<td>125 ± 20.2 (Stage I and II)</td>
<td>65 (p&lt;0.001)</td>
</tr>
<tr>
<td>Habib et al. 1980 [73]</td>
<td>Prostate cancer</td>
<td>84.1 ± 6.27</td>
<td>124 ± 8.3</td>
<td>44 (p&lt;0.01)</td>
</tr>
<tr>
<td>Rajput et al. 1975 [75]</td>
<td>Reticulo-endothelial system</td>
<td>115.8 ± 13.9 (males)</td>
<td>228.0 ± 52.4 (males)</td>
<td>70 (p&lt;0.001)</td>
</tr>
<tr>
<td>Scanni et al. 1977 [69]</td>
<td>1) Lung cancer</td>
<td>143.0 ± 3.2</td>
<td>188.2 ± 14.8 (Lung)</td>
<td>20 (p&lt;0.01)</td>
</tr>
<tr>
<td>Study</td>
<td>Cancer Type</td>
<td>Comparison</td>
<td>p-Value</td>
<td>n</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------</td>
<td>------------</td>
<td>----------</td>
<td>---</td>
</tr>
<tr>
<td>Gupta et al. 1993 [81]</td>
<td>Colorectal cancer</td>
<td>98.8 ± 24.3</td>
<td>165 ± 33.9</td>
<td>30 (p&lt;0.001)</td>
</tr>
<tr>
<td>Zuo et al. 2006 [70]</td>
<td>Leukemia</td>
<td>86.7 ± 25.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jayadeep et al. 1997 [77]</td>
<td>1) Oral leukoplakia</td>
<td>66.9 ± 22.0 (male)</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>2) Squamous cell carcinoma</td>
<td>66.8 ± 14.0 (female)</td>
<td></td>
<td>22 (p&lt;0.001)</td>
</tr>
<tr>
<td>Lightman et al. 1986 [79]</td>
<td>Ovarian cancer</td>
<td>133 ± 17.0 (Benign, n = 42)</td>
<td>160 ± 17.0 (Stage I)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>190 ± 6.0 (Stage II)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>179 ± 18.0 (Stage III)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>210 ± 23.0 (Stage IV)</td>
<td>11</td>
</tr>
<tr>
<td>Kuo et al. 2002 [9]</td>
<td>Breast cancer</td>
<td>96.5 ± 7.3 (control) n=26</td>
<td>125.2 ± 15.0</td>
<td>25 (p&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>103.8 ± 8.3 (Benign) n=43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharma et al. 1994 [59]</td>
<td>Breast cancer</td>
<td>100.7 ± 40.5</td>
<td>172.8 ± 12.2</td>
<td>50 (p&lt;0.01)</td>
</tr>
</tbody>
</table>
Table 3.3 Copper levels in the tumor tissue of cancer patients

<table>
<thead>
<tr>
<th>Study</th>
<th>Tumor Type</th>
<th>Healthy (µg/g ± SD)</th>
<th>Patients (µg/g ± SD)</th>
<th>Number of subjects (significance compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuo et al. 2002 [9]</td>
<td>Breast cancer</td>
<td>6.13 ± 4.32 (normal) 6.51 ± 5.33 (benign)</td>
<td>11.08 ± 4.98 (Stage I) 10.10 ± 5.61 (Stage II) 17.18 ± 11.83 (Stage III)</td>
<td>25</td>
</tr>
<tr>
<td>Rizk et al. 1984 [82]</td>
<td>Breast cancer</td>
<td>9.3 ± 2.3</td>
<td>21.0 ± 10.7</td>
<td>22 (p&lt;0.0001)</td>
</tr>
<tr>
<td>Yaman et al. 2007 [74]</td>
<td>Ovarian cancer</td>
<td>0.2-0.9</td>
<td>0.4-2.8</td>
<td>10</td>
</tr>
<tr>
<td>Gupta et al. 1991 [81]</td>
<td>Colorectal cancer</td>
<td>1.79 ± 0.57</td>
<td>2.78 ± 0.84</td>
<td>30 (p&lt;0.001)</td>
</tr>
<tr>
<td>Santoliquido et al. 1976 [83]</td>
<td>Breast cancer</td>
<td>2.38</td>
<td>3.25</td>
<td>20</td>
</tr>
<tr>
<td>Geraki et al. 2002 [84]</td>
<td>Breast cancer</td>
<td>0.29 ± 0.29</td>
<td>0.89 ± 0.56</td>
<td>40</td>
</tr>
<tr>
<td>Margaliot et al. 1983 [11]</td>
<td>Large bowel</td>
<td>1.53 ± 0.35</td>
<td>1.90 ± 0.6</td>
<td>24 (p&lt;0.05)</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>1.44 ± 0.38</td>
<td>2.09 ± 0.52</td>
<td>7 (p&lt;0.02)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>1.26 ± 0.45</td>
<td>2.16 ± 0.63</td>
<td>5 (p&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>1.58 ± 0.62</td>
<td>1.91 ± 0.56</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.80 ± 0.42</td>
<td>1.61 ± 0.25</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>1.54</td>
<td>2.80 ± 0.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Testis</td>
<td>1.48 ± 0.7</td>
<td>1.43</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hodgkin’s</td>
<td>1.42 ± 0.44</td>
<td>3.18</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total cases</td>
<td>1.42 ± 0.44</td>
<td>2.08 ± 0.76</td>
<td>53 (p&lt;0.001)</td>
</tr>
<tr>
<td>Mulay et al. 1971 [85]</td>
<td>Breast cancer</td>
<td>1.47 ppm</td>
<td>5.12 ppm</td>
<td>15 (p&lt;0.01)</td>
</tr>
<tr>
<td>Yaman et al. 2007 [10]</td>
<td>Stomach cancer</td>
<td>1.1 ± 0.4</td>
<td>1.7 ± 0.1</td>
<td>18 (p&lt;0.01)</td>
</tr>
<tr>
<td>Lightman et al. 1986 [79]</td>
<td>Ovarian cancer</td>
<td>1.95 ± 0.64</td>
<td>2.17 ± 0.64</td>
<td>40</td>
</tr>
<tr>
<td>Carpentieri et al. 1986 [76]</td>
<td>Leukemia</td>
<td>15 ± 4 µg/10^6 cells</td>
<td>52 ± 16 µg/10^6 cells</td>
<td>12 (p&lt;0.01)</td>
</tr>
</tbody>
</table>
3.1.3 Copper and angiogenesis

‘Angiogenesis’ is defined as the process of development of new blood vessels. The concept that ‘tumor growth is dependent on angiogenesis’ was pioneered by Judah Folkman in the 1970-80’s. Tumor cells cannot grow more than 1-2 mm in diameter without angiogenesis, which supplies the tumor with oxygen and nutrients [1]. Angiogenesis in a normal cell involves a complex interaction and balance between endogenous stimulators and inhibitors [1]. Cancer cells have developed ways to either synthesize their own angiogenic stimulators or to recruit endothelial cells to synthesize them [51-53]. These endogenous angiogenesis stimulators include growth factors such as the Vascular Endothelial Growth Factor (VEGF), Angiogenin, Fibroblast Growth Factor (β-FGF), Tumor Necrosis Factor (TNF-α), Epidermal Growth Factor (EGF), cytokines such as Interleukin (IL) 1, 6 and 8, trace elements such as copper. The endogenous angiogenesis inhibitors include cytokines such as Interleukin 10 and 12 and modulators such as Angiotensin, Endostatin and Angiopoietin-2 [4, 5, 86, 87].

Based on the discoveries regarding the importance of angiogenesis in tumor development, the concept of anti-angiogenic therapy has gained tremendous interest, and several antiangiogenic agents are currently in clinical trials [4, 86]. These antiangiogenic agents in clinical trials belong to the following categories: i) protease inhibitors, ii) endothelial cell proliferation/migration inhibitors, iii) antagonists of angiogenic factors, iv) endothelial cell specific integrin/survival signaling inhibitors, v) copper chelators and vi) agents with unspecific mechanism [86]. Bevacizumab, a monoclonal antibody against the angiogenic stimulator VEGF, is currently on the market for the treatment of colorectal cancers. Although, anti-angiogenic agents targeted to one specific angiogenic stimulator
such as Bevacizumab (anti-VEGF) have shown improvements when used in combination with chemotherapeutic agents such as Paclitaxel, 5-Fluorouracil, and Doxorubicin against certain tumors such as colorectal, but their effectiveness seems to decrease in other cancer types namely breast cancer and non-small cell lung cancer [51, 53]. This might simply be due to the availability of a host of other angiogenic factors (as described above) to the tumor cell (by either synthesizing themselves or by recruiting endothelial cells) for angiogenesis. Therefore, a successful anti-angiogenic therapy would either target multiple angiogenic factors or an angiogenic factor which is critical to angiogenesis or specifically controls a host of other angiogenic factors.

In 1980, McAuslan and Reilly, in an attempt to isolate a peptide ‘endothelial stimulating growth factor’, noted that copper salts were the simplest angiogenic component of the tumor extract, stimulating the migration of endothelial cells [52, 53, 88, 89]. It has been reported that rabbits fed with copper deficient diets, caused the reduction of their serum copper levels by half and resulted in the inability of an angiogenic response regardless of the angiogenic stimulus in the rabbit cornea angiogenesis model [90, 91]. Copper ions have been shown to stimulate the motility of endothelial cells in-vitro [91]. Copper salts in-vitro have been shown to induce the synthesis of fibronectin, a matrix glycoprotein associated with angiogenesis [92, 93]. The copper levels have been shown to locally regulate the growth or regression of new blood vessels [94, 95]. Copper has also been shown to stimulate angiogenesis in chick embryo chorioallantoic models [94]. In addition, structurally diverse but vital angiogenic cytokines/growth factors such as IL-1, 8 and 6, β-FGF, TNF-α and VEGF have been shown to be inactivated with copper withdrawal [5, 86]. Further proof of the involvement of copper in tumor growth is
suggested from the elevated copper levels in the serum and tumor of cancer patients compared to healthy individuals [73, 84]. Serum copper levels have been correlated to tumor burden, progression, incidence, regression, and reoccurrence [51]. Ceruloplasmin expression has also been reported to be elevated in tumors [68, 69]. Ceruloplasmin levels have been reported to be increased four to eight fold during malignant progression and return to normal levels after tumor regression [86, 96]. Further, X-ray fluorescence microscopy (XFM) has recently revealed large scale relocalization and extracellular translocation of cellular copper during angiogenesis [97].

All these findings suggest that copper plays a central role in angiogenesis and therefore controlling copper levels with the aid of chelators would result in anti-angiogenic activity.

3.1.4 Copper chelators as anti-angiogenic agents

Copper reduction as an anti-cancer strategy is currently under intense investigation [98, 99]. Copper chelators such as D-pen [5], tetrathiomolybdate (TM) [100, 101], clioquinol [102], and trientine [103, 104] have been shown to inhibit angiogenesis both in-vitro and in-vivo. Several animal studies have supported the hypothesis of employing copper chelators as anti-angiogenic agent. Pan et al. [105] reported a synergistic tumor growth reduction effect of tetrathiomolybdate plus doxorubicin in a SUM149 murine xenograft. Khan et al. [106] showed a reduction in tumor mass and synergistic effects of radiotherapy with tetrathiomolybdate in a murine lung cancer model. Moriguchi et al. [107] described a decrease in tumor growth and IL-8 production with trientine treatment in hepatocellular carcinoma. Yoshida et al. [108] reported a reduction in the tumor weight and vascular density after low copper diet plus D-pen treatment of gliosarcoma.
xenografts. Brem et al. [109] described the reduced tumor growth and vascularization after low copper diet and D-pen treatment in glioma implanted intracerebrally in rabbits. Additionally, Pan et al. [100] published that copper deficiency induced by tetrathiomolybdate resulted in impairment of tumor growth and angiogenesis in two animal models of breast cancer; an inflammatory breast cancer xenograft model in nude mice and HER2/neu cancer prone transgenic mice.

Some clinical trials with copper chelators such as D-pen and tetrathiomolybdate to determine their antiangiogenic activity have been conducted. A phase II trial of copper depletion and penicillamine therapy as antiangiogenesis therapy for glioblastoma reported an effective ceruloplasmin depletion (ceruloplasmin levels decreased from 130 µg/dL to <50 µg/dL) after two months of combination therapy of penicillamine and a low copper diet, but the achievement of hypocupremia was reported not to significantly increase survival in glioblastoma patients [8]. A phase I clinical trial to investigate tetrathiomolybdate as an anticopper antiangiogenic agent against metastatic cancer reported the reduction of ceruloplasmin without added toxicity, with five of six patients with stable disease [14]. A phase II trial with tetrathiomolybdate in patients with advanced kidney cancer concluded that tetrathiomolybdate was well tolerated and consistently depleted copper in all patients and 31% patients exhibited stable disease for at least 6 months [110]. Other phase II clinical trials are currently underway to further investigate use of copper chelators are anti-angiogenic agents.
3.2 D-penicillamine (D-pen)

D-penicillamine (D-pen) is an aminothiol, which was first identified as a product of penicillin hydrolysis in 1943 by Abraham [111]. D-pen was introduced in the clinic as a copper chelating agent for the treatment of Wilson’s disease by Walshe in 1956 [112, 113]. After more than 50 years of clinical use in the treatment of Wilson’s disease, D-pen is currently also used in the treatment of cystinuria, rheumatoid arthritis and heavy metal intoxication. D-pen is currently approved by the Food and Drug Administration (FDA) and is marketed as Cuprimine® (250 mg capsules) and Depen® (250 mg tablets). D-pen (3-mercapto-D-valine, 3,3-dimethylcystiene or D-(-)-2-amino-3-mercapto-3-methylbutyric acid; m.w. 149.2) is the S enantiomer of the racemic DL-penicillamine [114]. The L-isomer is not used due to toxic effects [114]. D-pen possesses three prominent functional groups: an amine, a carboxyl and a sulfhydryl (as shown in Figure 3.1) [30]. The pharmacokinetics and pharmacology of D-pen is regulated by the reactions of these functional groups with endogenous compounds [115]. The pKa of D-pen functional groups (amino, sulphydryl and carboxyl) are 10.5, 7.9, and 1.8, respectively [116]. D-pen is known to react with, i) metals to form complexes and/or chelates, ii) with carbonyl compounds to form thiazolidine rings, iii) thiols or disulfides to form mixed disulfides [116, 117].

Several initial pharmacokinetic studies with D-pen had reported a problem of incomplete D-pen recovery. For example, only between 47 to 85% of D-pen was recovered after oral administration in a short term (5 day) human study [118, 119].
Figure 3.1 Structure of D-pen and D-pen disulfide
D-pen *in-vivo* is known to be rapidly transformed into: i) low molecular weight disulfides (through the interaction of D-pen with the endogenous thiols), ii) S-methylpenicillamine, and iii) form disulfide with plasma albumin [119-121]. The low molecular weight disulfides have relatively short plasma half life. It is now believed that the incomplete recovery for D-pen is mainly due to its stable binding with plasma albumin [116]. It has been estimated that *in-vivo* upto 80% of D-pen is bound to plasma proteins, 7% of D-pen exist as cysteine-D-pen disulfide, 5% as D-pen disulfide and only 6% as free D-pen [116]. Orally administered D-pen is rapidly absorbed by the small intestine, with drug detectable in the plasma within 20 min after administration [116, 122]. The peak plasma concentrations of D-pen have been reported to range from 4 µM after 150 mg to 27.5 µM after 800 mg oral administration. The time to peak plasma concentration ranges between 1.5-4 h after oral administration. While the oral bioavailability is 50-70%, and the volume of distribution is between 57-93 L. The terminal elimination half life has been shown to be 1 h (monoexponential) and 7.5 h (biexponential) and the clearance rate between 561-727 mL/min (independent of dose) has been published [114, 116, 122-125]. D-pen has very low lipid solubility owing to the ionization of its functional groups. Additionally there is no established or known mechanism of active uptake of D-pen into cells [114, 126]. It has been shown that the cellular uptake of D-pen is very limited in mucosal cells [127], lymphocytes, macrophages [128] and several mammalian cell lines [126]. Lodemann et al. reported that while L-Pen was able to accumulate into cells to some extent and also compete with most of the natural amino acids (valine, leucine, alanine, threonine, phenylalanine, methionine, praline, arginine, and histidine) and reduce their cellular uptake between ~20-60%, D-pen
did not accumulate in cells and did not compete with natural amino acids for cell uptake [126]. Despite the limited entry into cells, the volume of distribution of D-pen exceeds the total body water volume, which can be explained by its binding to tissues such as skin and aorta and to plasma proteins (such as albumin). Binding to albumin occurs rapidly but is only slowly reversible [129]. The reaction of poorly soluble cystine (cysteine disulfide) with D-pen to form a more soluble mixed disulfide is the basis of the use of D-pen in the treatment of cystinuria [114, 117].

3.2.1 D-pen in the treatment of Wilson’s disease and rheumatoid arthritis

The treatment of Wilson’s disease has two main aims: i) minimize the dietary intake and absorption of copper and ii) to promote the excretion of copper deposited in tissues. D-pen is an orally effective copper chelating agent, which is used in combination with reduced copper diet as a treatment of Wilson’s disease [38]. The optimum dose of D-pen is generally determined by measurement of the urinary copper excretion and the determination of free copper in the serum [115]. The actual doses of D-pen range between 250 mg to 1.5 g/day [39]. The side effects of chronic D-pen use have been reported to be as aplastic anemia, agranulocytosis, thrombocytopenia, goodpasture’s syndrome and myasthenia gravis [39, 115]. D-pen as a new oral therapy in the treatment of Wilson’s disease was introduced by Walshe et al. in 1956. D-pen essentially replaced 2, 3-dimercaptopropanol (BAL) and became the gold standard in the treatment of Wilson’s disease [112, 130]. Although new copper chelators such as tetrathiomolybdate have been introduced, D-pen still remains one of the primary choices in the treatment of Wilson’s disease [38].
Gooneratne et al. investigated the copper excretion capacity of established copper chelating agents such as EDTA, BAL, D-pen and TM from sheep following a high and low copper diet [131]. D-pen administration was shown to increase the urinary copper excretion by 266% and 196% in the low and high copper diet sheep respectively, compared to 254, 11, 46% and 354, 13, 20% excretion in low and high copper diet sheep after administration of TM, EDTA, and BAL, respectively. Therefore, the authors concluded that TM and D-pen were the most efficient decoppering agents in the group of copper chelating agents examined. However, it was also reported that TM and D-pen actually remove copper through different excretion sites (TM through bile, while D-pen through urine) [131].

Rheumatoid arthritis is an autoimmune disorder and is characterized by chronic inflammation of the synovial tissues [132, 133]. The goals of treatment of rheumatoid arthritis are to prevent or control joint damage, prevent the loss of function, and decrease the pain [132]. D-pen has been used in the treatment of rheumatoid arthritis for over 40 years [115, 134]. D-pen belongs to the second line treatment group, known as the disease modifying anti-rheumatic drugs (DMARD). The mechanism of action of D-pen against rheumatoid arthritis is still largely unknown. A number of potential mechanisms have been proposed such as dissociation of immunoglobulin (IgM) in-vivo [134], interference with the collagen cross-linking in the synovial junctions [135, 136], an anti-inflammatory agent [137, 138], interference with the chemotaxis of the leukocytes [139, 140], and that D-pen might express immunosuppressive activity [24]. The property of D-pen to produce hydrogen peroxide (H₂O₂) in the presence of copper has been investigated as an additional mode of action in its activity in rheumatoid arthritis [141]. It is interesting to
note that D-pen shares a reactive thiol group with other DMARD’s like captopril, thiopyridoxine, and thiopronine [114]. D-pen has been shown to suppress human fibroblast proliferation in the presence of cupric sulfate in-vitro [142], and inhibit the T-cell function in-vitro in the presence of cupric sulfate [24] Also, the pre-incubation of D-pen with cupric sulfate was shown to directly effect the ability of the T lymphocytes to respond to mitogens and their subsequent proliferation [133].

3.2.2 Copper chelation by D-pen

Although the copper chelating ability of D-pen has long been established, the exact mechanism of copper chelation by D-pen has remained the focus of immense controversy. The copper chelation mechanism of D-pen has revolved around the in-vivo formation and existence of the multivalent cluster complex \((\text{Cu}^{II}_6\text{Cu}^{I}_8(\text{D-pen})_{12}\text{Cl})^\delta^-\) shown to be formed between D-pen and copper in-vitro [143, 144]. The multivalent cluster complex has been shown to be formed in-vitro under simulated physiological conditions, but it has never been found in the urine of patients due to its apparent instability in urine [144].

Walshe et al. [112] proposed several possible mechanism of copper chelation by D-pen, i) a single copper being bound to a single thiol group (C-S-Cu\(^+\)), ii) a copper atom bound on both sides with the thiol groups of two D-pen molecules (D-pen-Cu-D-pen), or iii) a ring compound between a copper atom and the thiol and amino groups of D-pen. It should be noted that the hypothesis of a ring compound involving both the thiol and amino groups was put forth specifically because it was known that ethylmercaptan does not function as a copper chelator [112].
In 1976 Birker et al. [145] isolated and described the crystal structure of a purple color multivalent cluster complex, \((\text{Cu}^{\text{II}}_6\text{Cu}^{\text{I}}_8\text{(D-pen)}_{12}\text{Cl})^5^-\). D-pen in the cluster complex was reported to bind copper in both oxidation states, \(\text{Cu(II)}\) and \(\text{Cu(I)}\) [143]. The \(\text{Cu(II)}\) was described to be in equilibrium with the surrounding media although strongly chelated with the nitrogen and sulfur group of D-pen, while \(\text{Cu(I)}\) was proposed to be removed from equilibrium [143]. The methyl groups provided steric hindrance to the complex, thus protecting the \(\text{Cu(I)}\) against oxidation [143]. The solubility of the complex was proposed to be due to the presence of ionized carboxyl groups on the surface of the complex. The complex was reported to degrade in urine at aerobic conditions, but to be highly stable \textit{in-vitro} in the simulated physiological pH and saline conditions [143].

In 1977 May et al. [146] introduced the term, plasma mobilization index (PMI), which was defined as the relative ability of chelating agent to compete for a metal ion. Based on the computer modeling with the aid of PMI, May et al. predicted that D-pen would be unable to compete with plasma proteins for \(\text{Cu(II)}\) chelation. It was instead proposed that D-pen could liberate copper from metalloproteins by reduction to \(\text{Cu(I)}\). Other copper chelating agents such as EDTA and Trien were shown to effectively chelate \(\text{Cu(II)}\) [146]. In 1977 Tran-Ho et al. [147] with the aid of the PMI showed that D-pen had much lower capacity to remove copper compared to another copper chelator, trien [147]. Thus, the authors proposed that the mobilization of copper by D-pen in Wilson’s disease is unlikely to be explained by complexation of D-pen with \(\text{Cu(II)}\) alone [147]. These studies agree with the initial observation by Walshe et al. that trien and D-pen might act on different copper pools within the body [130].
In 1979 Laurie et al. [148] showed that D-pen is unable to compete for copper bound to albumin. The authors showed that the rate of formation of the D-pen-copper cluster complex was dependent on a host of different factors including copper concentration, Cu-D-pen ratio, chloride ion concentration, pH and temperature [148], and thus the authors concluded that the multivalent cluster complex might not be the mechanism of copper removal by D-pen [148].

In 1999 Kato et al. [144] with the aid of the $^1$H-NMR studied the reactions of Cu(I) and Cu(II) with D-pen in the presence of glutathione. The data supported the existence of the cluster species ($\text{Cu}_6^{\text{II}}\text{Cu}_8^{\text{I}}(\text{D-pen})_{12}\text{Cl})^{5-}$. Kato et al. concluded that the coexistence of D-pen, D-pen disulfide, Cu(II), and/or Cu(I) leads to the formation of the stable cluster species regardless of the presence or absence of other thiols such as glutathione [144]. The cluster species was proposed to be thermodynamically favored compared to other copper-containing complexes such as D-pen$^+$Cu$^+$, Glutathione$^{\text{Cu}^+}$, D-pen disulfide-Cu$^{2+}$, and Glutathione disulfide-Cu$^{2+}$ [144].

The stability constant (log $K_{\text{app}}$) for EDTA with Cu(II) at pH 7.0 has been reported to be 15.5, while that of D-pen with Cu(I) is 18.8 [47, 48]. It has been shown that D-pen-Cu(I) complexes have very poor aqueous solubility in the pH range 1.9-7.6, due to the formation of neutral complexes [147]. However, the D-pen-copper cluster complex which is proposed to be formed in-vivo would have enhanced solubility due to the earlier described negative charge on the complex [144].
3.2.3 Metal catalyzed D-pen oxidation

Thiol oxidation to disulfides is a two electron oxidation process, where the electron donor and acceptor in this process typically are other thiols, disulfides, or oxygen:

$$2 \text{RSH} \leftrightarrow \text{RSSR} + 2e^- + 2\text{H}^+$$

The reversible thiol/disulfide exchange reactions are typically initiated by the thiolate anion [149]. Glutathione is the major biological intracellular thiol and therefore the interaction of any other thiol and glutathione is one of the pathways for thiol oxidation with simple thiol exchange reaction

$$K_{\text{mix}} = \frac{(\text{PSSG}) (\text{GSH})}{(\text{PSH}) (\text{GSSG})}$$

where PSH is D-pen, GSH is glutathione, GSSG is glutathione disulfide, and PSSG is the D-pen-glutathione mixed disulfide. The equilibrium constant ($K_{\text{mix}}$) for the formation of glutathione mixed disulfides of D-pen has been reported to be 3.0, which is higher than other thiols such as cysteine ($K_{\text{mix}}$: 1.1), cysteamine ($K_{\text{mix}}$: 2.4) [150].

The direct oxidation of thiols by oxygen is very slow; however the rate of oxidation is increased in the presence of some transition metals, as these metals act as efficient electron carriers between thiols and oxygen [151, 152]. Copper and iron are the biologically relevant transition metals which are known to catalyze thiol oxidation [152]. The oxidation of thiols is generally initiated by the electron transfer from the thiolate anion to metal, a subsequent transfer of electron from the metal to oxygen results in the formation of superoxide radical and hydrogen peroxide [151]. The superoxide radical and hydrogen peroxide can then react to form the extremely harmful hydroxyl radicals through the well known Haber-Weiss reaction [153]. Thus, the formation of the thiolate anion is the rate limiting step in the autoxidation reaction of thiols, implying that thiol
oxidation would be highly dependent on the ionization of the thiol group [154]. The pKa of the thiol group of some important endogenous and exogenous thiol compounds are as follows: D-pen (pK$_{\text{SH}}$: 7.9), cysteine (pK$_{\text{SH}}$: 8.3), cysteamine (pK$_{\text{SH}}$: 8.3), glutathione (pK$_{\text{SH}}$: 8.8), dithiothreitol (pK$_{\text{SH}}$: 9.1), N-acetylcysteine (pK$_{\text{SH}}$: 9.5), captopril (pK$_{\text{SH}}$: 9.8) [154]. As can be seen, the pKa of D-pen is physiologically significant compared to other exogenous thiols. Therefore, D-pen would undergo the highest rate of oxidation in the presence of transition metals compared to the other listed thiol compounds. However, it should be noted that at sufficiently high concentrations, the thiolate anion of D-pen can also react with the H$_2$O$_2$ which is present in the surrounding solution or that has been generated in the autoxidation process. The rate of reaction of thiolate anion with H$_2$O$_2$ and superoxide radical has been shown to be inversely proportional to the pKa of the thiol group [154]. Thus, at sufficiently high concentrations (millimolar) D-pen could act as antioxidant compared to its oxidative nature at low concentrations (micromolar). D-pen like other thiols also reacts with free radicals to form thiy1 radical (PS$^\cdot$). The interaction of two thiy1 radicals forms disulfides. Listed below are some of the possible reactions of D-pen [155, 156]

The possible reactions of the thiolate anion of D-pen with other radical and non-radical species:

\begin{align*}
(Radical)$^\cdot$ + PSH & $\leftrightarrow$ Radical-H + PS$^\cdot$ \\
PS$^\cdot$ + PS$^\cdot$ & $\leftrightarrow$ PSSP \\
PSH & $\leftrightarrow$ PS$^-$ + H$^+$ \\
PS$^\cdot$ + PS$^\cdot$ & $\leftrightarrow$ (PSSP)$^-$ \\
(PSSP)$^-$ + O$_2$ & $\rightarrow$ PSSP + O$_2^-$
\end{align*}

(a) (b) (c) (d) (e)
\[
\text{PS}^- + \text{H}_2\text{O}_2 \rightarrow \text{PSOH} + \text{HO}^-
\]  \text{(f)}

\[
\text{PSOH} + \text{PS}^- \rightarrow \text{PSSP} + \text{HO}^-
\]  \text{(g)}

where D-pen (PSH), D-pen radical (PS\(^{-}\)), D-pen disulfide (PSSP), D-pen disulfide radical (PSSP\(^{-}\)), superoxide radical (O\(_2^{-}\)), hydroxyl radical (HO\(^{\cdot}\)).

### 3.2.4 Quantitative analysis of D-pen

Compounds containing thiol functional group are usually difficult to assay due to the rapid oxidation of the thiol group to the respective disulfide \textit{in-vitro} and the formation of mixed disulfides \textit{in-vivo}. Several methods have been reported in the past for the quantitative analysis of thiols such as ion exchange chromatography, gas chromatography, capillary electrophoresis and high performance liquid chromatography (HPLC) with fluorescence (FL), electrochemical and ultraviolet (UV) detection.

HPLC with either pre or post-column derivatization of the endogenous thiols and thiol containing drugs such as Homocysteine, N-acetyl-cysteine, Cysteine, Glutathione, Bucillamine, Cysteamine, Captopril, and D-pen have been assayed with the aid of fluorescence compounds such as 5,5’-dithio-(bis-2-nitrobenzoic acid) [157], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [158-163], 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [164], N-(p-(2-benzoxazolyl)-phenyl) maleimide (BOPM) [165], \(\alpha\)-phthalaldehyde [166], monobromobimane [167], and N-(1-pyrenyl)maleimide) (NPM) [168-171]. The derivatization of thiol compounds with these derivatizing agents results in the formation of fluorescent adducts, which are then detected using fluorescence chromatography. The formation of these adducts usually employ the sulphydryl functional group of the thiol compounds also resulting in the protection of these thiols from oxidation. However, this strategy also restricts the
detection of only reduced thiols and not their disulfides. Thus, there is often the problem of a lack of mass balance of the thiol compound. The solution to this problem is to employ a reduction step before the derivatization process to convert the disulfides to thiols that may then react with the derivatizing agent.

The quantitative analysis of D-pen in biological samples (plasma, tissue homogenates) is complicated given the occurrence of D-pen in various forms such as free D-pen, mixed disulfide with cysteine or glutathione, D-pen disulfide, and S-methyl-D-pen, and also D-pen bound to plasma proteins [171]. A number of methods have been reported in the literature in order to analyze D-pen such as HPLC-electrochemical detection [172-174], gas chromatography [175, 176], radioimmunoassay [177], cation exchange chromatography [178, 179], capillary electrophoresis [180-182], chemiluminescence [183], and HPLC-Fluorescence detection [165, 171, 184]. Almost all of these methods detect D-pen only in the reduced form, as the reduction of D-pen disulfide to D-pen has been shown to be very limited even in the presence of high concentrations of reducing agents such as dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine (TCEP) [185, 186]. Therefore, the D-pen assay methods have to account for D-pen conversion to its disulfide or mixed disulfides either by detecting D-pen disulfide or with inhibiting D-pen oxidation. We have recently reported on a HPLC-UV assay for the simultaneous detection and quantitation of both D-pen and D-pen disulfide in a single run [187]. This is the first assay method that can quantify both D-pen and D-pen disulfide together in a single run. This assay method provides the simple, sensitive, accurate and simultaneous determination of D-pen and D-pen disulfide concentration in samples.
3.2.5 D-pen as an anti-angiogenic agent

As reviewed earlier, copper has been established as an endogenous angiogenic stimulator. As copper is now known to control a variety of other endogenous stimulators by acting as a co-factor, strategies to chelate copper and thus cause anti-angiogenesis are being extensively investigated. D-pen and Tetrathiomolybdate are two copper chelators currently being investigated as anti-angiogenic agents in clinical trials.

D-pen is an effective copper chelator and has been shown in in-vivo studies to remove copper, Goonerante et al. have shown that D-pen is as effective as tetrathiomolybdate in removing excess copper through urinary excretion in both low and high copper diet sheep [131]. D-pen has been shown to cause inhibition of the human endothelial cell proliferation and neovascularization in-vitro [6]. D-pen was also shown to suppress the growth of 9L gliosarcoma tumor implanted in rats [7]. Yoshida et al. published that D-pen caused copper depletion and the reduction in the copper/zinc ratio in a rat tumor model which might have lead to the reduction in the tumor weight [7, 108]. Brem et al. concluded that treatment of D-pen resulted in the inhibition of pseudopodial protrusion and the invasive spread of 9L gliosarcoma cells in the rat brain [188]. Hourani et al. have shown the inhibition of S-91 mouse melanoma metastases and growth by D-pen [13].

In addition, D-pen has also been shown to have properties other than copper chelation for it to be an effective anti-angiogenic agent, such as the inhibition of several important growth factors (VEGF, FGF) that require copper as a co-factor, dose dependent inhibitor of urokinase-type plasminogen activator [189], and activator of angiostatin [86]. A phase II clinical trial was recently conducted to investigate the anti-angiogenic activity
of D-pen against glioblastoma [8]. D-pen was able to remove copper and reduced the ceruloplasmin levels from 130 µg/dL to <50 µg/dL [8] after two months of oral D-pen administration.

3.2.6 D-pen as cytotoxic anti-cancer agent

As described earlier, thiols can generate hydrogen peroxide (H_2O_2) and other ROS through a metal (copper or iron) catalyzed oxidation process. The incubation of D-pen with copper in the presence of endothelial cells [6] and lymphocytes [23] have been previously reported to result in cell death proposed to be due to the generation of ROS. Starkebaum et al. had proposed a mechanism of a copper catalyzed H_2O_2 generation from D-pen and cytotoxicity in lymphocytes [23].

The mechanism of copper catalyzed D-pen oxidation and subsequent H_2O_2 formation is thought to be a free radical based process [23, 190]. As shown in Figure 3.2 Starkebaum et al. postulated that D-pen initially reduces Cu(II) to Cu(I) presumably during copper chelation, and that this process leads to the generation of D-pen radical. The Cu(I) then reduces oxygen to superoxide anion followed by reduction of superoxide anion into H_2O_2. During this reduction of oxygen and superoxide anion, Cu(I) is oxidized back to Cu(II). The D-pen radicals are proposed to react to form D-pen disulfide. The net reaction mechanism shows the reduction to 2 moles of D-pen by 1 mole of O_2 leading to the formation of 1 mole H_2O_2 and 1 mole of D-pen disulfide [23, 190].

Kato et al. [144] has recently performed ^1H-NMR studies which support the H_2O_2 formation during D-pen interaction with copper during copper chelation and the formation of the multivalent cluster complex. It should also be pointed out that these
studies were conducted in the presence of glutathione. Additionally, we have recently conducted both non-cell based and cell based studies to conclusively show the generation of H$_2$O$_2$ and subsequent oxidation of D-pen to D-pen disulfide using a novel HPLC assay [187, 190].

We have also shown that D-pen was able to cause concentration dependent cytotoxicity only in the presence of copper in human breast cancer and leukemia cells [187, 190]. The D-pen cytotoxicity was inhibited in the presence of catalase. Intracellular generation of H$_2$O$_2$ was shown in both breast cancer and leukemia cells [190].

Thus as shown in Figure 3.3, D-pen targeted to the tumor in presence of the excess copper in the tumor microenvironment and/or cells would cause cytotoxicity. The elevated oxidative stress in cancer cells compared to normal cells would result in selective cytotoxicity in cancer cells compared to normal cells. D-pen could thus be employed as a dual anti-cancer agent exhibiting both anti-angiogenic properties due to copper chelation and cytotoxic properties due to generation of ROS.
\[
2\text{PSH} + 2\text{Cu}^{2+} \rightarrow 2\text{PS}^- + 2\text{Cu}^+ + 2\text{H}^+\\
\text{Cu}^+ + \text{O}_2 \rightarrow \text{Cu}^{2+} + \text{O}_2^-\\n\text{Cu}^+ + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2\\n\text{PS}^- + \text{PS}^- \rightarrow \text{PSSP}\\n\text{2PSH} + \text{O}_2 \rightarrow \text{PSSP} + \text{H}_2\text{O}_2
\]

Figure 3.2 Mechanism of copper catalyzed D-pen oxidation and hydrogen peroxide generation. Adapted from Starkebaum et al. [23]
Figure 3.3 Mechanism of action of D-pen as a cytotoxic anti-cancer agent. The mechanism of D-pen activity selectively against cancer cells is based on the difference in the oxidizing-reducing balance in cancer vs. normal cells.
3.3 Cancer and oxidative stress

Each cell in the human body maintains a homeostasis between the oxidant and antioxidant species. Both of these species are very important for the normal metabolism, signal transduction and regulation of cellular functions [191]. Oxidative stress could be simply defined as the disturbance in the oxidant-antioxidant balance, favoring the oxidant environment [192]. Oxidative stress associated cellular damage has been indicated in a range of disorders such as cancer [193, 194], diabetes mellitus [195], atherosclerosis [21, 196], neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease [197, 198], autoimmune disorders such as arthritis [21, 199] and aging [200-202]. The relationship between cancer and oxidative stress has been the subject of intense debate; mainly due to the well documented fact that the cancer cells are under high levels of oxidative stress compared to normal cells [203, 204]. In the past decade oxidative stress has been linked both in the etiology and the potential treatment of cancer [203, 204].

The elevated oxidative stress in cells can lead to modification of number of cellular targets and cause cell damage and death. The most important of these targets include:  

1) DNA (nuclear and mitochondrial) leading to single strand breaks,  
2) mitochondrial inner membrane leading to the loss of cellular stores of ATP, and  
3) membrane phospholipids by means of lipid peroxidation [205]. The cell damage and the subsequent lack of cellular repair processes due to the constant oxidative damage have been associated with carcinogenesis [206-208].

The oxidation of guanine nucleotide base in DNA, through reaction with oxidative species results in the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG) [193, 200, 209]. The interaction of ROS with DNA has been reported to result in fragmentation of
DNA with the loss of bases and leading to strand breaks [205, 210, 211]. These DNA strand breaks can accumulate in mammalian cells leading to oncogenic transformation and/or cell killing, depending on the capacity of cellular repair processes to overcome them [206]. Mammalian cells in culture when exposed to ROS have been shown to have a direct relationship between accumulation of strand breaks and cell killing [212].

Lipid peroxidation takes place in the polyunsaturated fatty acids; the process is generally initiated by the extraction of hydrogen from a methylene carbon by ROS such as hydroxyl radical [209]. Under aerobic conditions, the fatty acid with unpaired electron undergoes oxidation to form the peroxyl radical. This radical can then extract hydrogen from the adjacent fatty acids to form lipid peroxides and thus a chain reaction is initiated and propagated. The simple schematic example of the mechanism of lipid peroxidation with hydroxyl radical is shown below [209]:

\[
\begin{align*}
\text{LH} + \cdot \text{OH} &\rightarrow \text{L'} + \text{H}_2\text{O} & (a) \\
\text{L'} + \text{O}_2 &\rightarrow \text{LOO'} & (b) \\
\text{LOO'} + \text{LH} &\rightarrow \text{LOOH} + \text{L'} & (c)
\end{align*}
\]

where LH: polyunsaturated fatty acid, L': alkyl radical, LOOH: lipid hydroperoxide

Fruehauf et al. has proposed a model of transformation of normal cells into malignant cells based on the exposure to ROS. The model is shown in the Figure 3.4 and depicts that low level of ROS stress (normally observed in all cells) could cause DNA damage, this damage is normally fixed with the aid of specialized DNA repair enzymes present in the nucleus. However, constant ROS stress results in loss of efficiency of the repair process and may lead to the initiation of malignant state. At this stage, if these cells are exposed to further ROS stress, they should normally undergo apoptosis but in some
cases the cells start to proliferate and thus are irreversibly transformed into malignant cells. Thus, according to this model, the malignant cells would innately have high intracellular ROS stress compared to normal cells [213] and maintain an increased level of ROS stress compared to normal cells due to increased metabolism. Also as proposed in the model further ROS stress in these malignant cells could result in cell death either through apoptosis or necrosis and thus the strategy of producing further ROS stress in malignant cells could be successful.
Figure 3.4 Reactive oxygen species (ROS) exposure and cancer cells. Adapted from Fruehauf et al. [213]
3.3.1 Reactive oxygen species (ROS)

ROS can simply be defined as oxygen containing oxidizing agents. It has been reported that up to 1-3% of the pulmonary intake of oxygen by humans is converted into ROS [214]. ROS are generally divided into two subgroups; free radicals such as superoxide radicals (O$_2^-$) and non-radical such as hydrogen peroxide (H$_2$O$_2$). A list of biologically relevant radicals and non-radical ROS is shown in Table 3.4. Both radicals and non-radical ROS share the presence of an oxygen atom, which differentiates them from the reactive nitrogen species (RNS). Free radicals contain one or more unpaired electron and are highly reactive chemical species, with the half life generally ranging in seconds or minutes. Non-radicals on the other hand can exist for longer period of time, but can be converted to radical species by reacting with other radicals.

ROS are products of normal cellular metabolism. It has been reported that in an average adult approximately 10,000-20,000 free radicals attack every cell in the body per day [194]. Although high concentrations of ROS are known to be harmful to the cells, low concentrations of ROS are known to serve a variety of important cellular functions including both the activation and modulation of the signal transduction pathways [191, 215, 216], modulation of the activities of the redox sensitive transcription factors such as NF-κB, AP-1, p53, NFAT, and HIF-1 [215, 217], regulation of apoptosis [218, 219], and the regulation of mitochondrial enzyme activities [215, 220]. A complete list of these functions of ROS is shown in Table 3.5.

The formation of ROS is a consequence of aerobic metabolism [221]. In normal conditions, the balance in the intracellular ROS levels is maintained with the aid of scavenging systems such as glutathione, superoxide dismutase, catalase, and other
antioxidant defense components. However, in certain disorders such as cancer, the balance between the ROS and the antioxidant species is disturbed. The most important source of cellular ROS is the mitochondria, where a continuous production of ROS takes place. This is the result of the electron transport chain located in the mitochondrial membrane which is essential for the energy production inside the cell [20, 222]. Another source of ROS production is in the cytosol, the arachadonic acid cascade involved in the production of prostaglandins and leukotrienes is known to produce ROS when the released lipid is metabolized [223]. Additionally some cytochrome 450 enzymes are also known to produce ROS [224].
Table 3.4 Classification of biologically important ROS. Adapted from Halliwell [17]

<table>
<thead>
<tr>
<th>Reactive Oxygen Species</th>
<th>Free radicals</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superoxide anion (O$_2^-$)</td>
<td>Hydrogen peroxide (H$_2$O$_2$)</td>
</tr>
<tr>
<td></td>
<td>Hydroxyl (‘OH)</td>
<td>Hypobromous acid (HOBr)</td>
</tr>
<tr>
<td></td>
<td>Hydroperoxyl (HO$_2^-$)</td>
<td>Hypochlorous acid (HOCl)</td>
</tr>
<tr>
<td></td>
<td>Peroxy (RO$_2^-$)</td>
<td>Singlet oxygen (O$_2^1$)</td>
</tr>
<tr>
<td></td>
<td>Alkoxy (RO')</td>
<td>Organic peroxides (ROOH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peroxynitrite (ONOO')</td>
</tr>
</tbody>
</table>
Table 3.5 Intracellular functions and targets of ROS. Adapted from Seifried [225]

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Function</th>
<th>Effect of ROS or oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 8</td>
<td>Family of cytosolic aspartate-specific cysteine proteases involved in the initiation and execution of apoptosis</td>
<td>Activated by ROS</td>
<td></td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor of kappa light chain gene enhancer in B cells</td>
<td>Activated by ROS as κB inhibitor and is degraded by oxidation</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor protein</td>
<td>ROS increases the accumulation of the protein but decreases binding to DNA</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Increases the permeability of capillary blood vessels</td>
<td>Decreased by ROS</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
<td>Catalyzes the conversion of superoxide anion to hydrogen peroxide</td>
<td>Accumulation of ROS can damage lipids, proteins and DNA</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
<td>A member of MAPK family, promotes phosphorylation and activation of AP-1, leads to apoptosis or necrosis</td>
<td>ROS leads to activation following dissociation of the Trx-apoptosis signal-regulating kinase 1.</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6 or Interleukin beta</td>
<td>A multifunctional protein that plays important role in host defenses, acute phase reactions, immune responses</td>
<td>ROS decreases activity</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
<td>ASK1 activates</td>
<td>Causes dissociation of the Trx-ASK1 complex leading to</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td><strong>Epidermal Growth Factor Receptor</strong></td>
<td>JNK and P38 and inactive when complexed with Trx</td>
<td>ASK1 activation of JNK and P38</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td></td>
<td>A member of the EGFR family of receptor tyrosine kinases, which activates Raf and MAPKK leading to the activated ERK</td>
<td>Activated by ROS</td>
<td></td>
</tr>
</tbody>
</table>
3.3.1 a Hydrogen peroxide (H$_2$O$_2$)

H$_2$O$_2$ is a pale blue colored liquid, freely miscible with water and most importantly it is known to freely diffuse through cell membranes [226]. H$_2$O$_2$ is less reactive than its radical counterpart such as the superoxide and the hydroxyl radical, but due to the above mentioned ability to diffuse through membranes, H$_2$O$_2$ is a potent cellular oxidant species. The toxicity of H$_2$O$_2$ is largely based on its conversion to hydroxyl radical (·OH) either through ionizing radiation (as shown in reaction $a$ below), by interaction with transition metals such as iron and copper through Fenton chemistry (as shown in reaction $b$ below), or by interaction with superoxide anion radical through Haber-Weiss reaction (as shown in $c$ below) [226]. The superoxide radical, through combination of reaction $d$ and the Fenton reaction, results in the recycling of transition metal between their oxidized and reduced states and results in the formation of hydroxyl radical at an appreciable rate.

$$\begin{align*}
H_2O_2 & \rightarrow 2(\cdot OH) \quad (a) \\
H_2O_2 + Fe^{2+} \text{ or } Cu^+ & \rightarrow Fe^{3+} \text{ or } Cu^{2+} + \cdot OH + \cdot OH \quad (b) \\
O_2^- + H_2O_2 & \rightarrow O_2 + \cdot OH + \cdot OH \quad (c) \\
Fe^{3+} \text{ or } Cu^{2+} + O_2^- & \rightarrow Fe^{2+} \text{ or } Cu^+ + O_2 \quad (d)
\end{align*}$$

Fenton type reactions are one of the most important metal mediated reaction, where the oxidation of a metal (usually transition metals such as iron (II), copper (I), chromium (III), cobalt (II), nickel (II), vanadium (V) by H$_2$O$_2$ leads to the generation of a hydroxyl radical.

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + \cdot OH + \cdot OH$$

The Haber-Weiss reaction is a metal catalyzed reaction which also leads to the formation of hydroxyl radical. However, the metal purely acts as a catalyst in this process.
The hydroxyl radical (•OH) is the most oxidizing radical generated in the human body [227]. The aqueous half life hydroxyl radical is reported to be <1 ns [20]. The redox potential of hydroxyl radical at pH 7.0 has been reported to be approximately +2.31 V [228]. Hydroxyl radical is known to react with DNA and lipid to cause damage and peroxidation, respectively [227].

H$_2$O$_2$ can also be formed by the direct two electron reduction of oxygen, a reaction which is catalyzed by a number of oxidases present in the cell cytoplasm [210]. Antioxidant enzymes are involved in the reduction of H$_2$O$_2$ to water: catalase in peroxisomes and glutathione peroxidase in the mitochondria and in the cytosol [210, 229]. Inspite of the high permeability of H$_2$O$_2$ across cell membranes, the actual gradient of H$_2$O$_2$ acts by the consumption of H$_2$O$_2$ inside the cell by catalase and glutathione reductase (as shown in Figure 3.5) [230]. Therefore, increased activity of catalase and/or glutathione peroxidase leads to a concentration gradient where more H$_2$O$_2$ diffuses inside the cell. Antunes et al. following a H$_2$O$_2$ exposure to Jurkat T-cells, calculated the following gradients: (H$_2$O$_2$)$_{extracellular}/$(H$_2$O$_2$)$_{cytosol} = 7$ and (H$_2$O$_2$)$_{cytosol}/$(H$_2$O$_2$)$_{peroxisome} = 3$ [230], which support the suggestions that H$_2$O$_2$ diffusion from outside the cell would depend on the activity of the antioxidant enzymes involved in H$_2$O$_2$ consumption [230].

H$_2$O$_2$ is considered the key component in the ROS toxicity to cells because it can accumulate in the cell at relatively high concentrations owing to its apparent stability and the likely conversion to radical ROS [229]. H$_2$O$_2$ has been reported to exist in cells at a
concentration of $1 \times 10^{-8}$ M [231]. H$_2$O$_2$ at submicromolar concentrations is known to act as intracellular messenger capable of promoting cell growth responses in molecules such as protein kinases, phosphatases, transcription factors, or transcription factor inhibitors [232]. The concentration of H$_2$O$_2$ shows a biphasic cell effect where low concentrations (50 nM) of H$_2$O$_2$ have been shown to be stimulate *in-vitro* cell growth of some cell lines such as human smooth muscle cells [232], rat fibroblast [233], and hamster fibroblast [234]. However, medium concentrations (1 µM- millimolar) of H$_2$O$_2$ have been known to cause cytotoxicity in cancer cells [190].
Figure 3.5 Kinetics of hydrogen peroxide (H$_2$O$_2$) entry into cells. Adapted from Antunes [230]
3.3.1 b Superoxide radical ($O_2^-$)

Mitochondria is the source of most of the $O_2^-$ present in cells. The intracellular concentration of superoxide radical is reported to be about $1 \times 10^{-11}$ M [201]. Superoxide radical is less reactive compared to hydroxyl radical, but is known to cause considerable damage to the DNA [194]. As mentioned above, superoxide radical reacts with $H_2O_2$ through the Haber-Weiss reaction to produce the toxic hydroxyl radical. Superoxide dismutase (SOD) is a cellular enzyme responsible for the dismutation of superoxide radical into the less reactive $H_2O_2$ through the reaction shown below.

\[
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

Superoxide anion toxicity is seen in the neurodegeneration accompanied with amyotrophic lateral sclerosis (ALS), mainly due to the SOD levels being low [201]. Superoxide radical can also react with glutathione, the major cellular antioxidant to produce damaging glutathionyl radical, as shown below:

\[
O_2^- + GSH + H^+ \rightarrow GS^- + H_2O_2
\]

Jones et al. showed that the rate constant of reaction between superoxide and glutathione is around $200 \text{ M}^{-1}\text{s}^{-1}$. Thus SOD would be able to compete with glutathione for reaction with superoxide radical preventing formation of the glutathionyl radical [235].
3.3.2 Antioxidants

As ROS are normally being produced inside the cells, there has to be a mechanism through which the cells are able to counteract the toxic effects of ROS and able to survive. Antioxidants are chemical compounds present inside the cells which protect them from the oxidative damage. In Table 3.6, antioxidants are classified into four broad categories:

i) ROS scavenging agents

Glutathione is one of the major antioxidant compounds present in the cells and plays vital role for cellular protection against ROS [236-240]. The role of glutathione as an antioxidant is further discussed in section 3.3.2 a.

Thioredoxin (Trx) is a 12 kDa cysteine rich protein containing thiol-disulfide active sites that act as antioxidants by facilitating the reduction of other cellular enzymes by cysteine thiol-disulfide exchange reactions [241]. The reduction of the oxidized thioredoxin is done by the NADPH-dependent flavin adenine dinucleotide containing flavoenzyme, thioredoxin reductase. Thioredoxin/thioredoxin reductase system forms one of the main redox couple in mammalian cells [242, 243 244]. There have been three mammalian thioredoxins shown to be located in the cytoplasm, mitochondria and spermatozoa [243]. The low pKa of the cysteine thiol group leads to its existence as thiolate anion which acts as the attacking group in the reduction of disulfides.

\[
\text{Trx-(SH)}_2 + \text{X-S}_2 \rightarrow \text{Trx-} \text{S}_2 + \text{X(SH)}_2
\]
ii) Dietary antioxidants

Vitamin E: α-Tocopherol is the major constituent of the fat soluble vitamin E. α-Tocopherol is known to inhibit lipid peroxidation by reacting with the peroxyl radical intermediates formed during the chain reaction of lipid peroxidation [22] as shown in the reaction below,

\[ \alpha\text{-Tocopherol} + \text{LOO}^{-} \rightarrow \alpha\text{T}^{-} + \text{LOOH} \]

The resulting tocopherol radical is much less reactive compared to the peroxyl radical, and thus causes the slowing of the lipid peroxidation cascade [245].

Vitamin C: Ascorbate or ascorbic acid is one of the important dietary water soluble radical scavenging antioxidant. The antioxidant activity of ascorbic acid on redox dependent biological function is due to the direct redox role of ascorbic acid and its metabolites as well as due to the generation of the ascorbate free radical [241]. Ascorbic acid is known to regulate transcription factors such as NF-κb, AP-1, GST-pi, MLH1 and is known to regulate apoptosis through IL-1 down regulation and changes in the adhesion molecules [246, 247]. Vitamin C protects the body against oxidative stress and is a cofactor for the biosynthesis of number of important compounds [248]. Several in-vitro and in-vivo studies have been investigated the antioxidant function of ascorbic acid and have been shown to reduce DNA damage [249-251]. A positive relationship between the Vitamin C and E deficiency and lipid peroxidation has also been shown [252].

Selenium in the form of selenocysteine, is an important part of many antioxidant enzymes known as selenoproteins [241, 253]. Glutathione peroxidases and thioredoxin reductase are also well known selenium containing enzymes, which play a critical role in intracellular antioxidant activities [241].
Carotenoids: β and α-carotene are known to protect the cells against oxidative stress caused by radiation and UV exposure. Carotenoids carry out their antioxidant activities through a number of mechanisms [241]. Carotenes are known for their ability to scavenge free radicals such as singlet oxygen (1⁰₂) species [254], alkoxy, and peroxyl radicals [255]. However, carotenoids can also act as electron donors (by transferring electrons) and reducing the free radicals to nonradical species. Thus sub-optimal uptake of carotenoids has been linked to carcinogenesis through increased DNA damage [241, 253].

Antioxidant activity of carotenoids [194]:

\[
\text{ROO}^- + \text{Car} \rightarrow \text{ROO-Car}^-
\]

\[
\text{ROO-Car}^- + \text{ROO}^- \rightarrow \text{ROO-Car-ROO} \text{ (non-radical product)}
\]

Pro-oxidant activity of carotenoids [194]:

\[
\text{Car} + \text{R}^- \rightarrow \text{RH} + \text{Car}^-
\]

\[
\text{Car}^- + \text{O}_2 \rightarrow \text{Car-OO}^-
\]

\[
\text{Car-OO}^- + \text{RH} \rightarrow \text{Car-OOH} + \text{R}^-
\]

where Car represents carotenoids and Car-OO⁻ is the carotenoid-peroxyl radical.

Flavonoids are plant derived polyphenolic antioxidant compounds. There has been extensive research on the *in-vitro* free radical scavenging properties of flavonoids in the last decade [256]. However, their role *in-vivo* as antioxidant has been unclear [257]. The beneficial properties of flavonoids have been largely linked to their antioxidant property but also to their ability to interact with basic cellular mechanism such as modulation of the signal cascade, interaction with membrane receptors, interaction with nucleoproteins, and with nucleic acids [258].

58
iii) *Metal sequestering agents*

These compounds are responsible for sequestering free transition metal ions such as copper and iron from the blood. As these transition metals are known for catalyzing the production of ROS, the metal sequestering agents are very important antioxidants. Ceruloplasmin is the major copper carrying protein in the plasma. As copper is known to be involved in both Fenton type and Haber-Weiss reaction, the availability of free copper in the presence of ROS such as \( \text{H}_2\text{O}_2 \) and superoxide could cause the generation of the highly toxic hydroxyl radical.

Transferrins are an important class of iron binding proteins widely distributed in the human body [259]. The transferrins belong to a family of proteins which includes the serum transferrin, lactoferrin, ovotransferrin and metallotransferrin [259]. Iron is well known to participate in one electron transfer reactions leading to the formation of ROS through either the Fenton or Haber Weiss reaction. Therefore, sequestering of iron by transferrins provides for the safe transport of iron to the cells [260]. Transferrin is known to bind to the transferrin receptor that is then internalized via receptor-mediated endocytosis [259, 261]. Transferrin is known to bind to the transferrin receptor with high affinity \((K_d = 5 \times 10^{-9} \text{ M})\) [259, 261, 262].

Metallothioneins are small sulfhydryl rich peptides [263, 264]. These intracellular peptides are cysteine rich compounds which bind trace metals such as cadmium, mercury, silver, platinum [263]. Due to their binding of the metals they are known to protect cells from potential oxidative stress caused by these metals. The enhanced expression of metallothioneins in cells has been shown to cause anti-apoptotic processes and the lack of
metallothioneins has been reported to increase the susceptibility of cells to apoptosis [263, 265].

iv) *ROS protective enzymes*

Superoxide dismutase (SOD) and catalase work together to protect the cells against superoxide anion and hydrogen peroxide toxicity.

\[
\begin{align*}
O_2^- + O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \quad \text{reaction catalyzed by SOD} \\
2H_2O_2 & \rightarrow 2H_2O + O_2 \quad \text{reaction catalyzed by catalase}
\end{align*}
\]

Superoxide dismutases (SOD) are the only mammalian antioxidant enzymes converting superoxide into $H_2O_2$ [266]. In humans, three different kinds of SOD have been discovered, CuZnSOD is mainly located in the cytoplasm, however it has also been located in cell organelles [267, 268], MnSOD is synthesized in the cytoplasm but transported to the mitochondria [266, 269, 270]. ECSOD is synthesized in the cytoplasm but acts mainly as an extracellular enzyme. Due to its location in the mitochondria, MnSOD has been identified as the SOD which plays an important role in rapidly growing cancer cells [266]. The CuZnSOD and ECSOD contain copper/zinc, while the MnSOD has manganese in their reactive sites [271]. The importance of SOD in normal physiology has been conclusively shown with the recent studies performed in genetically engineered mice. The mice lacking MnSOD died after several days of birth, due to massive oxidative stress [271]. In addition, mice lacking CuZnSOD have been shown to develop hepatic cancer, accelerated muscle loss, and have a reduced lifespan. While the mice lacking ECSOD did not show any signs of oxidative stress and survive to a normal lifespan [272, 273]. SOD has been shown to be overexpressed in malignant cells [266, 274]. The SOD
overexpression *in-vivo* is associated with poor prognosis of some tumors such as breast cancer [275, 276], lung cancer [274, 277], brain tumors [278-280], and leukemia [281].

Catalase (H$_2$O$_2$ oxidoreductase) is present in almost all aerobic cells and is responsible for reducing H$_2$O$_2$ by catalyzing its decomposition without the production of ROS. Catalase exist as dumbbell shaped tetramer of four identical subunits (250-350 kDa), wherein each monomer contains an iron atom at the catalytic center [282]. Catalase mainly exists in the peroxisomes and to some extent is also present in the cytoplasmic fraction [283]. The catalase levels have been shown to be decreased in both the serum and tumor tissue of various malignancies [284].

Glutathione peroxidase (GP) is an enzyme that catalyzes the detoxification of H$_2$O$_2$ by glutathione as shown in the reaction below. The enzyme is responsible for detoxifying H$_2$O$_2$ in addition with catalase. GP is selenium containing tetrameric glycoprotein.

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

Glutathione reductase (GR) catalyzes the reduction of glutathione disulfide to reduced glutathione as shown in the reaction below.

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

1, 3-bis (chloroethyl)-1-nitrosourea (BCNU) is a known specific inhibitor of glutathione reductase.

Thioredoxin reductase (TrxR) is responsible for reducing thioredoxin [283]. Three different thioredoxin reductase proteins in human cells have been described. TrxR1 is present in all compartments in the cells. TrxR2 and TrxR3 are present in the mitochondria. Thioredoxin reductase has selenocysteine in its active site and has been shown to be essential for its activity [243, 283].
Trx-S₂ + NADPH $\rightarrow$ Trx-(SH)_2 + NADP
Table 3.6 Classification of antioxidants in the human body

<table>
<thead>
<tr>
<th>I. ROS scavenging agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
</tr>
<tr>
<td>Thioredoxin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Dietary antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E</td>
</tr>
<tr>
<td>Vitamin C</td>
</tr>
<tr>
<td>Selenium</td>
</tr>
<tr>
<td>Carotenoids (β and α carotene)</td>
</tr>
<tr>
<td>Flavonoids</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>III. Metal sequestering agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>Transferrin</td>
</tr>
<tr>
<td>Metallothioneins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IV. ROS protective enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper/zinc superoxide dismutase (CuZnSOD)</td>
</tr>
<tr>
<td>Extracellular superoxide dismutase (ECSOD)</td>
</tr>
<tr>
<td>Manganese superoxide dismutase (MnSOD)</td>
</tr>
<tr>
<td>Catalase</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
</tr>
</tbody>
</table>
### 3.3.2.a Glutathione

The major cellular antioxidant, glutathione (γ-Glu-CysH-Gly, γ-glutamylcysteinylglycine) is a tripeptide, containing glutamate, cysteine, and glycine amino acids [237]. It is the most widely distributed intracellular thiol and low molecular weight peptide [237]. The γ-peptide bond between glutamate and cysteine in glutathione prevents its hydrolysis by most peptidases [236]. There is a sharp contrast in the concentration of glutathione inside and outside the cells which is important in creating the reductive and oxidative environments inside and outside the cells, respectively. The concentrations of glutathione in mammalian cells are in the millimolar ranges (1-11 mM), whereas only micromolar concentrations are found in the blood [237, 241]. A small proportion of the total cellular glutathione (10-15%) is found in the mitochondria, where it reaches a concentration similar to the cytosol [285]. Glutathione is converted to glutathione disulfide during the reduction of H₂O₂ and other peroxides by the selenium containing glutathione peroxidase [237]. The intracellular concentration of reduced glutathione is maintained by glutathione reductase, a NADPH-dependent enzyme which reduces glutathione disulfide to glutathione [236, 237]. Glutathione is synthesized intracellularly with the sequential action of γ-glutamylcysteine synthetase and glutathione synthetase in a series of six enzyme catalyzed reactions [237]. The physiological role of glutathione is multifaceted and is responsible for multiple cellular functions such as transport of amino acids, synthesis of proteins and nucleic acids, maintenance of enzymes in their active form, and protection against radiation and endotoxin exposure [286]. As the human brain cells are known to utilize about 20% of the oxygen consumed by the body although it only comprises 2% of body weight, the brain generates large quantities...
of ROS during this process. Glutathione acts as the first line of defense against the ROS generated in the brain cells and cells in other parts of the body [286].

The ratio between the reduced glutathione (GSH) and the oxidized glutathione (GSSG) forms the very important redox couple inside the cells and serves as an intracellular redox buffer. In a normal cell, the molar ratio of GSH/GSSG of 30:1 to 100:1 has been measured [287]. This ratio is known to maintain the intracellular reduced conditions and is mainly responsible for the reduction of the oxidative species generated inside the cell. The change in this ratio on the other hand is seen in conditions of oxidative stress such as cancer [286], where the GSH/GSSG ratio could be greatly reduced upto 10 and 1 [286, 288]. Glutathione depletion in humans have been linked to many disorders such as HIV, Wilson’s disease, chronic pancreatitis, chronic pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), neonatal lung damage, and asthma [286]. In addition, GSH depletion has been associated with aging through processes such as reduced responsiveness of T lymphocytes to mitogens, decreased cognitive capacities [289]. Several selective inhibitors of glutathione redox cycle have been developed to study the effect of these components in the glutathione associated antioxidant defense mechanism. For example, the activity of glutathione reductase is inhibited by 1,3-bis (chloroethyl)-1-nitrosourea (BCNU), the activity of glutamylcysteine synthetase is inhibited by buthionine sulfoximine [290], and the activity of glutathione S-transferase inhibited by 1-chloro-2, 4-dinitrobenzene (CDNB) and diethylmaleate (DEM) [291]. Increasing cellular glutathione levels might be beneficial in certain disorders where the levels of glutathione are decreased. Cysteine and N-acetylcysteine have been used to increase the glutathione concentration [236]. Cysteine is usually the limiting amino acid.
in the glutathione biosynthesis and therefore its administration has been shown to increase the glutathione concentration [240, 292]. N-acetylcysteine is deacetylated to cysteine before being used for glutathione biosynthesis [240]. Glutathione itself is not taken up by cells to a significant degree [237, 240]. Moreover, orally administered glutathione is degraded into amino acids extracellularly and the products are then transported inside the cells for subsequent glutathione biosynthesis [236, 238].

Schafer et al. has described the redox potential inside the cell using the intracellular GSH/GSSG couple. As shown in Figure 3.6, the authors have reported that there is a correlation between the redox potential of the GSH/GSSG couple and the biological status of the cells: proliferation: -240 mV; differentiation: -200 mV; or apoptosis: -170 mV [244]. Therefore, the GSH/GSSG provides an overview of the cellular homeostasis.
Figure 3.6 Redox couples in the human body. Adapted from Schafer [244]
3.3.3 ROS and cancer

In a normal cell, there exists a balance between the free radical generation and the antioxidant defense [293]. It has long been documented that cancer cells are under increased and persistent oxidative stress due to elevated levels of intracellular ROS generation. The direct proof of oxidative stress in the cancer cells is shown through the analysis of significantly elevated levels of ROS such as H$_2$O$_2$, superoxide radical, while the indirect proof is shown through the existence of increased levels of lipid oxidation products in the cancer cells compared to surrounding normal cells. The existence of increased ROS induced oxidative stress in cancer cells is also a result of the lower levels of antioxidant enzymes such as SOD, glutathione peroxidase, glutathione reductase, and catalase in these cells.

This increased intrinsic ROS stress in cancer cells has been speculated to be due to a number of factors such as oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction [19]. Cancer is associated with rapid cell growth and differentiation. The rapid cell growth rate requires excessive energy from the mitochondria subsequently resulting in the production of excessive amounts of ROS. This excessive ROS being generated cannot be neutralized by cellular antioxidants and oxidative stress pursues [294]. As a result, cancer cells under sustained ROS stress conditions tend to heavily utilize adaptation mechanisms and may exhaust ROS-buffering capacity while normal cells have low levels of ROS stress and reserve a higher capacity to cope with further oxidative insults [19, 294]. The intracellular redox state which is directly related to the production of intracellular ROS in cancer cells has also been indicated to control the aggressiveness of cancer cells. Chaiswing et al. and colleagues
reported that the difference in the redox status of two prostate cancer cell lines showed remarkable differences in the degree of aggressiveness [295].

Recently, Kondo et al. examined three important questions about oxidative stress in cancer cells, 

1) is the oxidative stress limited to cancer cells and not to other surrounding nontumorous cells,

2) is there a difference between the oxidative stress in benign and malignant tumor cells, and

3) is there a correlation between oxidative stress in-vivo and the level of tumor cell proliferation [296]. The authors observed persistent oxidative stress in human colorectal carcinoma (malignant tumor), which was significantly higher as compared to the corresponding nontumorous epithelial cells. The authors also showed that the level of oxidative stress in cancer cells appears to be maintained high to promote cell proliferation, but concluded that this amount of oxidative stress is insufficient to cause apoptosis or necrosis [296]. Over the past decades numerous studies have shown the existence of higher or significantly higher oxidative stress in various types of malignancies compared to the corresponding normal subjects. The studies supporting the presence of elevated oxidative stress in oral and stomach cancer is shown in Table 3.7 and Table 3.8. We further discuss some studies showing the existence of elevated oxidative stress in various malignancies below.

Senthil et al. reported the evidence of oxidative stress in the circulation of the ovarian cancer patients. The SOD and catalase levels were analyzed to be 0.9 ± 0.1 and 4.4 ± 0.4 (U/mg hemoglobin (Hb)), which were significantly (p<0.001) lower as compared to control individuals levels of 1.9 ± 0.3 and 5.7 ± 0.6 (U/mg Hb), respectively. Also the levels of antioxidants such as Vitamin C and E were 0.4 ± 0.03 and 1.4 ± 0.1 (mg/dL), which were significantly (p<0.001) lower compared to control individual levels
of 1.0 ± 0.1 and 2.8 ± 0.2 (mg/dL), respectively [297]. Additionally, the authors reported that the plasma Thiobarbituric Acid Reactive Substances (TBARS) levels (nmoles malondialdehyde (MDA)/mL of plasma), which is generally employed as an assay for oxidative stress (lipid peroxidation), were significantly higher (p<0.001) in ovarian cancer patients compared to normal individuals (5.6 ± 0.5 compared to 2.1 ± 0.2 in controls) [297].

Aydin et al. investigated the alteration of oxidant/antioxidant status in the circulation of benign and malignant prostrate cancer patients [298]. The authors report a very interesting pattern of the levels of glutathione peroxidase and SOD. The concentrations of both the antioxidant enzymes were significantly lower in malignancy compared to benign disease, and the levels in benign disease levels were significantly lower than in control subjects. The enzyme concentrations were control > benign > malignant. The concentrations of glutathione peroxidase were 9.1 ± 1.8, 8.3 ± 1.4 and 7.1 ± 1.4 (p<0.001 compared to control) (U/mL) in the control, benign and malignant prostrate cancer patients, respectively. While the concentrations of SOD followed the same trend, the concentrations were 168.0 ± 33.3, 144.4 ± 30.1, and 115.2 ± 20.2 (p<0.001 compared to control) (U/mL) in control, benign and malignant prostate cancer patients, respectively [298]. The concentrations of catalase also followed the same trend although there were no significant differences between control, benign and malignant patients. [298].

Ho et al. reported alterations in the antioxidant status in the erythrocytes of patients with Non Small Cell Lung Carcinoma (NSCLC) [299]. The authors reported catalase and SOD concentrations to be lower than in control subjects. The catalase and SOD
concentrations were 7.6 (mU/g Hb) and 13.4 (U/g Hb) in NSCLC patients compared to 20.9 and 48.7 in control subjects, respectively. The glutathione peroxidase concentrations however was shown to be increased in NSCLC patients, 175.2 (mU/g Hb) compared to 49.2 (mU/g Hb) in control subjects [299].

Recently Beevi et al. investigated the antioxidant status in the cervical squamous cell carcinoma patients. SOD, catalase, and the glutathione peroxidase concentrations were shown to be significantly (p<0.001) lower in cervical cancer patients compared to normal subjects [300]. The erythrocyte SOD, catalase, and glutathione peroxidase concentrations were 14.2 ± 2.6, 18.9 ± 2.3, 6.7 ± 1.3 in cervical cancer compared to 18.2 ± 3.8 (U/100 mg protein), 29.3 ± 6.4 (U/mg protein), and 11.3 ± 1.8 (U/mg protein) in control individuals, respectively [300].

Baticioglu et al. recently reported presence of increased oxidative stress in stomach cancer patients [301]. The SOD and catalase concentrations were shown to be significantly (p<0.001) lower compared to control subjects. SOD and catalase concentrations (U/mg protein) were 5.1 ± 1.9 and 6.6 ± 3.2 compared to 8.7 ± 2.6 and 17.6 ± 8.3 in control individuals [301].

Guven et al. investigated the lipid peroxidation and the antioxidant status in patients with Hodgkin’s disease [302]. Selenium and glutathione peroxidase concentrations were shown to be significantly (p<0.001) lower in the erythrocytes of cancer patients compared to controls. While the SOD levels were shown to be higher in cancer patients [302]. The marker for lipid peroxidation (nmol MDA/ g Hb) was significantly increased in the erythrocytes of cancer patients [302].
Navarro et al. examined the changes in glutathione status and the antioxidant system in blood and in cancer cells associated with tumor growth in-vivo [303]. The GSH/GSSG ratio was reduced, which was mainly due to increase in the GSSG levels. The authors explained the higher GSSG levels were due to the increase in the H$_2$O$_2$ production by tumor as well as to the changes in the activity of the glutathione related antioxidant enzymes [303].
Table 3.7 Lipid peroxidation and antioxidant system status in plasma of oral cancer patients vs. control [304]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=16)</th>
<th>Oral Cancer Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stage II (n=16)</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/L)</td>
<td>223.8 ± 17.7</td>
<td>204.1 ± 13.7**</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>4.2 ± 0.3</td>
<td>3.6 ± 0.7**</td>
</tr>
<tr>
<td>Catalase (U/L)</td>
<td>0.5 ± 0.03</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>50.6 ± 5.2</td>
<td>44.1 ± 5.6**</td>
</tr>
<tr>
<td>Vit.E (mg/dL)</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.10**</td>
</tr>
</tbody>
</table>

** p<0.01 compared to controls, *** p<0.001 compared to controls
Figure 3.8 Lipid peroxidation and antioxidant system status in tissue of stomach cancer patients vs. control [301].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=18)</th>
<th>Stomach cancer (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/mg protein)</td>
<td>8.7 ± 2.6</td>
<td>5.1 ± 1.9***</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>17.6 ± 8.3</td>
<td>6.6 ± 3.2***</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/mg protein)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>MDA (µmol/g tissue)</td>
<td>13.5 ± 4.7</td>
<td>21.8 ± 5.7***</td>
</tr>
</tbody>
</table>

***p<0.001 compared to controls
3.3.3.a Leukemia and oxidative stress

All forms of leukemia including Acute Lymphocytic Leukemia (ALL), Acute Nonlymphocytic Leukemia (ANLL), Chronic Myeloid Leukemia (CML) have been reported to have significantly increased levels of various ROS such as superoxide radical, H$_2$O$_2$, and alteration in the levels of enzymes such as SOD, glutathione peroxidase, glutathione reductase, and catalase compared to healthy individuals [70, 293, 305-308]. Additionally there have been studies showing that trace metals such as copper levels are significantly higher in leukemia patients as compared to healthy individuals [70].

Devi et al. reported significant increase in the superoxide anion, SOD, glutathione peroxidase in leukemia patients compared to that of controls [293]. The superoxide anion levels were shown to be significantly increased (p<0.01) at 8.0 ± 3.4 in leukemia patients compared to 5.1 ± 2.8 (nmoles/10$^6$ cells/30 min) for controls, while the SOD levels were also reported to significantly elevated (p<0.001), 3464.1 ± 1999.4 in leukemia patients compared to 1413 ± 243 (U/g Hb) in control individuals. Additionally, the glutathione peroxidase levels were shown to be significantly elevated (p<0.001) in leukemia patients, 231.6 ± 203.3 compared to 62.1 ± 17.5 (U/g Hb) in control individuals [293]. The authors also reported on increase in the H$_2$O$_2$ generation in leukemia cells: however, the levels were not significantly different compared to controls [293]. The authors concluded that the increased free radical generation by leukemia leukocytes, increased antioxidant enzyme activities, and normal MDA levels indicated the existence of transient oxidative stress *in-vivo* in leukemia patients [293]. Other studies supporting the presence of oxidative stress in leukemia patients are shown in Table 3.9 and Table 3.10.
Table 3.9 Antioxidant levels in leukemia patients compared to controls [70].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=20)</th>
<th>Acute Leukemia (n=41)</th>
<th>Acute nonlymphoid leukemia (n=34)</th>
<th>Acute lymphoid leukemia (n=7)</th>
<th>Chronic myelogeneous leukemia (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-Zn SOD (U/mL)</td>
<td>59.8 ± 8.8</td>
<td>67.9 ± 9.5*</td>
<td>67.6 ± 10.4*</td>
<td>68.3 ± 8.7*</td>
<td>65.1 ± 14.7</td>
</tr>
<tr>
<td>T-SOD (U/mL)</td>
<td>76.7 ± 9.8</td>
<td>86.4 ± 11.2**</td>
<td>85.5 ± 11.9**</td>
<td>83.1 ± 10.2*</td>
<td>85.5 ± 14.3</td>
</tr>
<tr>
<td>Mn-SOD (U/mL)</td>
<td>16.9 ± 1.8</td>
<td>17.5 ± 1.9</td>
<td>17.9 ± 2.0</td>
<td>17.2 ± 1.9</td>
<td>17.9 ± 2.1</td>
</tr>
<tr>
<td>LPO (nmol/L)</td>
<td>1.9 ± 0.6</td>
<td>2.5 ± 0.8</td>
<td>2.4 ± 0.6</td>
<td>2.4 ± 0.7</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>GPx (U/g Hb)</td>
<td>11.8 ± 2.6</td>
<td>27.8 ± 9.9**</td>
<td>28.1 ± 10.5**</td>
<td>27.1 ± 9.9*</td>
<td>17.6 ± 5.4</td>
</tr>
</tbody>
</table>

*p<0.05 and ** p<0.01 compared to controls
Table 3.10 Oxidants and antioxidants in leukemia patients compared to controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=10)</th>
<th>Chronic Myelogeneous Leukemia (n=11)</th>
<th>Chronic Lymphocytic Leukemia (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH (mM)</strong></td>
<td>5.4 ± 0.4</td>
<td>9.1 ± 0.9*</td>
<td>8.2 ± 0.6*</td>
</tr>
<tr>
<td><strong>Ascorbic acid (mg/dL)</strong></td>
<td>9.5 ± 0.6</td>
<td>6.5 ± 0.7*</td>
<td>5.93 ± 0.5*</td>
</tr>
<tr>
<td><strong>H₂O₂ (µM)</strong></td>
<td>51.5 ± 4.9</td>
<td>75.1 ± 7.0*</td>
<td>79.8 ± 7.2*</td>
</tr>
<tr>
<td><strong>MDA (mmol/mL.h)</strong></td>
<td>157.7 ± 13.8</td>
<td>401.3 ± 40.1*</td>
<td>368.9 ± 40.1*</td>
</tr>
<tr>
<td><strong>Total Antioxidant capacity (mM)</strong></td>
<td>2.57 ± 0.25</td>
<td>4.02 ± 0.41*</td>
<td>3.7 ± 0.5*</td>
</tr>
</tbody>
</table>

*p<0.05 compared to control*
3.3.3.b Breast cancer and oxidative stress

Analogous to leukemia, there has been accumulating evidence in support of significantly higher oxidative stress in breast cancer patients [284, 309-314]. The excessive oxidative stress in breast cancer has been correlated to the severity of the disease e.g. Stage IV patients have been shown to have higher levels of oxidative stress compared to III, who have been shown to have higher levels of ROS stress compared to Stage II patients and so on for Stage I and control subjects [309, 310, 313, 314]. Table 3.11 and 3.12 shows changes in the glutathione and antioxidant status in breast cancer patients.

Khanzode et al. investigated the levels of antioxidant enzymes and lipid peroxidation in different stages of breast cancer patients [309]. The serum SOD and MDA concentrations were show to be significantly increased (p<0.001), in the various stages IV > III > II > I > control. The plasma ascorbic acid concentrations were significantly lowered (p<0.001), stage IV < II < II < I breast cancer patients compared to control individuals [309].

Kumaraguruparan et al. examined the antioxidant profile in the circulation of patients with fibroadenoma and adenocarcinoma of the breast [310]. The concentration of plasma glutathione (mg/dL), vitamin C and E (mg/dL) were significantly lower (p<0.05) in both fibroadenoma and adenocarcinoma patients compared to their age matched control subjects [310]. The concentrations of antioxidant enzymes such as SOD, catalase, glutathione peroxidase, glutathione S-transferase in the erythrocyte lysate of fibroadenoma and adenocarcinoma patients were also found to be significantly lower (p<0.05) compared to age-matched control subjects [310].
Sener et al. explored the lipid peroxidation and total antioxidant status in breast cancer patients [312]. The authors reported significantly lower (p<0.05) total antioxidant capacity in breast cancer patients compared to control subjects, while the serum MDA levels (indicator of oxidative stress) were significantly higher (p<0.05) in cancer patients [312]. However, the author did not find any differences between the lipid hydroperoxide levels in breast cancer patients [312].

Polat et al. compared the oxidant/antioxidant status in the blood of patients with malignant tumor and benign breast disease [311]. The MDA levels were significantly higher (p<0.05) is both benign and malignant patients. The levels of SOD, catalase, glutathione peroxidase were found to be significantly lowered (p<0.05) in malignant cancer patients compared to both the benign disease and control individuals [311].

Ray et al. examined the lipid peroxidation, free radical production and the antioxidant status in breast cancer patients [284]. The authors reported that the rate of production of ROS such as superoxide and H₂O₂ in the plasma were significantly increased (p<0.001) in all stages of breast cancer, while the concentration of antioxidant such as SOD, glutathione peroxidase were significantly lower (p<0.001) in all stages of breast cancer patients, while the concentrations of catalase was decreased in all stages of breast cancer [284].

It should be noted that these and other documented studies suggesting the existence of oxidative stress in cancer patients have been performed: \textit{i}) in different geographical locations, \textit{ii}) with completely different patients and control populations, and \textit{iii}) the studies have been reported over the last three to four decades. Therefore, these well documented differences in the levels of oxidative stress in cancer cells and lower
concentrations of the antioxidant enzymes provide us with the unique opportunity to selectively target cancer cells by designing therapies which employ these differences as a mechanism to kill tumor cells.
Table 3.11 Glutathione status in breast cancer patients [314]

<table>
<thead>
<tr>
<th></th>
<th>Stage I (n=19)</th>
<th>Stage II (n=86)</th>
<th>Stage III (n=112)</th>
<th>Total (n=112)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor tissue</td>
<td>Adjacent tissue</td>
<td>Tumor tissue</td>
<td>Adjacent tissue</td>
</tr>
<tr>
<td>GSH (µM/g)</td>
<td>498 ± 862</td>
<td>164 ± 476</td>
<td>902 ± 2084*</td>
<td>82.9± 236</td>
</tr>
<tr>
<td></td>
<td>93.9 ± 284</td>
<td>792 ± 186</td>
<td>1236 ± 2644*</td>
<td>196 ± 385</td>
</tr>
<tr>
<td></td>
<td>1096 ± 2367*</td>
<td>202 ± 455</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG (µM/g)</td>
<td>109 ± 108*</td>
<td>52.2 ± 143</td>
<td>167 ± 320*</td>
<td>56.4 ± 97.8</td>
</tr>
<tr>
<td></td>
<td>152 ± 286*</td>
<td>54.1 ± 104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Glutathione (µM/g)</td>
<td>716 ± 1010*</td>
<td>269 ± 747</td>
<td>1236 ± 2644*</td>
<td>196 ± 385</td>
</tr>
<tr>
<td>GSH/Total Glutathione (%)</td>
<td>36.4</td>
<td>22.2</td>
<td>49.1*</td>
<td>36.1</td>
</tr>
</tbody>
</table>

*p<0.05 compared to adjacent tissues
Table 3.12 Antioxidant status in breast cancer patients [313]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=117)</th>
<th>Breast cancer patients (n=117)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion (counts/10 s × 10^3 WBC)</td>
<td>434 ± 209</td>
<td>664 ± 563*</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>2.7 ± 0.9</td>
<td>3.3 ± 1.5*</td>
</tr>
<tr>
<td>GSH (µmol/L)</td>
<td>1289 ± 317</td>
<td>506 ± 222*</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>103 ± 51.1</td>
<td>81 ± 60*</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>16.3 ± 11.5</td>
<td>9.2 ± 7.0*</td>
</tr>
<tr>
<td>SOD (U/g Hb)</td>
<td>806 ± 149</td>
<td>895 ± 350*</td>
</tr>
<tr>
<td>GPX (U/g Hb)</td>
<td>46.8 ± 13.0</td>
<td>53.5 ± 21.4*</td>
</tr>
<tr>
<td>GRX (U/g Hb)</td>
<td>7 ± 3</td>
<td>9.78 ± 2.9*</td>
</tr>
<tr>
<td>Vit C (mg/L)</td>
<td>7.8 ± 2.9</td>
<td>6.5 ± 2.9*</td>
</tr>
<tr>
<td>Vit A (µg/dL)</td>
<td>245 ± 86</td>
<td>240 ± 86.5</td>
</tr>
<tr>
<td>Vit E (µg/mL)</td>
<td>15.5 ± 9.8</td>
<td>12.7 ± 4.6</td>
</tr>
</tbody>
</table>

*p<0.05 compared to controls
3.3.4 ROS in cancer treatment

In the last two decades ROS has been shown to be involved in each step of cancer development namely, initiation, promotion and progression [219, 315]. Additionally, the levels of antioxidant enzyme such as catalase and SOD have been shown to be altered in tumor cells [316]. It has been clearly documented that tumor cells are under persistent oxidative stress and the antioxidant system in the cancer cells are under constant attack, as they have to cope up with this excessive ROS being produced. Thus, there have been suggestions for exploiting this condition in cancer cells as a potential treatment strategy for cancer [317-321]. Two main strategies have been proposed:

i) Inhibition of the antioxidant enzymes and the antioxidant system in general in cancer cells

ii) Production of ROS (H$_2$O$_2$ and/or superoxide) in cancer cells resulting in apoptosis and/or necrosis.

Additionally, the higher ROS stress in cancer cells compared to normal cells also provides conditions for selectively targeting the cancer cells with agents that can generate further ROS and result in apoptosis and/or necrosis, while the normal cells should possess enough antioxidant enzymes to combat the extra ROS production. The above strategy is also schematically shown in Figure 3.7. The use of ROS in potential treatment of cancer was investigated as early as the 1950’s. Green et al. [322] and Sugiura et al. [323] in 1958 reported the use of H$_2$O$_2$ for treating tumors in animal tumor models. H$_2$O$_2$ has been the ROS of choice for anti-cancer therapy due to its apparent stability compared to other radical species such as hydroxyl and superoxide radical. However, due to the presence of high concentrations of catalase in the blood and the ability of H$_2$O$_2$ to diffuse through
membranes, it is extremely difficult to achieve any biological effect *in-vivo* with the use of H$_2$O$_2$ alone [219, 322-325]. The first use of an agent which would act as a precursor to ROS generation *in-vivo* was reported by Nathan et al. in 1981. The authors employed glucose oxidase as a H$_2$O$_2$ generating agent and this resulted in significant anti-tumor activity in an animal tumor model [326]. Since then several H$_2$O$_2$ and superoxide radical generating agents have been investigated as potential anti-cancer agents [327-329]. Also, many chemotherapeutic agents such as doxorubicin, vinblastine, vincristine, camptothecins have been shown to exhibit their anticancer activity through the generation of either H$_2$O$_2$ itself or through the activation of H$_2$O$_2$ dependent apoptotic pathways inside the cells [330]. Alexandre et al. reported that the accumulation of H$_2$O$_2$ is an early and crucial step for paclitaxel-induced cancer death both *in-vitro* and *in-vivo* [331]. The authors showed that H$_2$O$_2$ was in fact generated both *in-vitro* in A549, human lung cancer cells and in the LLC1 lung cancer cells implanted *in-vivo* in mice [331]. Studies over the last decade both *in-vitro* and *in-vivo* with a number of agents which produce ROS such as H$_2$O$_2$ or superoxide radical have shown that their administration leads to anti-cancer effects and the cytotoxic effect is limited to the cancer cells and there was limited or no toxicity in the surrounding normal tissue [332].

Procarbazine, Buthionine sulfoximine, and Motexafin gadolinium are some of the anti-cancer agents whose anti-cancer activity is known to be dependent on production of ROS or their interaction with ROS scavenging enzymes.

Berneis et al. proposed that procarbazine as a new class of tumor inhibiting compound based on its generation of ROS (H$_2$O$_2$), as an intermediate during its anti-tumor activity [333]. The oxidation of procarbazine in aqueous solution was shown to
generate $\text{H}_2\text{O}_2$ [316, 333]. Procarbazine has been approved for the treatment of Hodgkin’s lymphoma, non-Hodgkin’s lymphoma and primary brain tumors to enhance the effect of radiation [316].

Buthionine sulfoximine is a specific inhibitor of glutamyl synthetase and thus can inhibit glutathione synthesis. Several *in-vitro* and *in-vivo* studies have shown that the depletion of glutathione with the aid of BSO increases the sensitivity to cancer cells to the chemotherapeutic agents [334-336]. In addition, with the aid of BSO the resistance towards cytotoxic drugs such as paclitaxel was shown to be directly correlated to total antioxidant capacity in the cells [337]. A phase I study has shown that BSO lowered glutathione to 10% of its pretreatment values in tumor samples and blood lymphocytes [338, 339]. Maeda et al. and colleagues recently reported that the combination of BSO with arsenic trioxide resulted in the effective treatment of advanced solid tumor [340]. The cytotoxicity of arsenic trioxide has been reported to be due to the generation of ROS [341], and has been shown to cause clinical remission in patients with acute promyelocytic leukemia [342]. The combination of arsenic trioxide with a glutathione synthesis inhibitor, BSO resulted in inhibition of *in-vitro* growth in cell lines of breast, prostate, lung, colon, cervix, bladder, and kidney cancer [340, 343].

Xcytrin® (Motexafin gadolinium) selectively accumulates in the tumor due to the increased rates of metabolism of the tumor [344, 345]. Motexafin gadolinium is currently being investigated in several phase II and III clinical trials in the treatment of brain metastases, and non-small cell lung cancer [346, 347]. Once inside the cells, Motexafin gadolinium induces apoptosis by disrupting the redox dependent pathways [345]. The drug has been suggested to have multiple mechanisms of action which leads to the
disruption of the intracellular redox pathways such as the potential to inhibition of the enzyme thioredoxin reductase, generate ROS, deplete reducing compounds such as protein thiols, thioredoxin, ascorbate and glutathione [316, 348, 349].

Toshikazu et al. have demonstrated that the production of oxidative radicals with the aid of a hypoxanthine and xanthine oxidase reaction resulted in anticancer effect in an experimental rabbit model wherein VX2 carcinomas were implanted [332]. The authors reported that the parenteral administration of xanthine oxidase and hypoxanthine resulted in increased production of superoxide radical in the VX2 carcinoma tissue compared to the muscle tissue surrounding the tumor region [332]. Recently, Fang et al. employed polyethylene glycol (PEG) to conjugate superoxide and H₂O₂ generating enzymes such as xanthine oxidase (XO) and D-amino acid oxidase (DAO) to deliver these enzymes to potentially increase their intra-tumor accumulation [350].

We have recently proposed the use of D-penicillamine (D-pen), a potent copper chelator, as an anti-cancer agent based on its production of ROS (H₂O₂) during the copper chelation [187]. We proposed this strategy because it potentially exploits two crucial differences between cancer cells and normal cells:

i) Copper levels have been documented to be significantly higher in a variety of malignancies including breast, leukemia, stomach, colon cancers. We have shown that D-pen co-incubated with cupric sulfate resulted in cytotoxicity in human breast cancer and leukemia cells [190]. We propose that D-pen administered in-vivo would interact with the excess copper in tumor tissue and results in anti-cancer effect.
Cancer cells in comparison to the normal cells are under elevated levels of persistent oxidative stress and therefore they may have exhausted or have limited antioxidant defense mechanisms against any further ROS production. We have recently shown that the *in-vitro* copper pre-treatment of human leukemia cells in culture resulted in D-pen cytotoxicity, while D-pen treatment of copper un-treated (naïve) human leukemia cells did not result in any toxic effects [351].
Figure 3.7 Mechanism of ROS based anti-cancer therapies. Adapted from Nicco [321] and Lopez-Lazaro [319]
3.4 Intra-tumor delivery of hydrophilic anticancer drugs

Chemotherapeutic agents belong to various classes of chemical compounds and thus they have varied physicochemical properties. The majority of the anti-cancer drugs are lipophilic molecules, which mean they generally have very good permeability through biological membranes, but they exhibit poor to limited aqueous solubility. Several novel delivery forms such as nanoparticles, liposomes and microparticles have been developed to mask the lower aqueous solubility of these agents and improve their biological activity.

There are however, some examples of anti-cancer drugs which belong to the other side of the physicochemical spectrum, the biological activity of these molecules limited by their low lipophilicity. Over the years, several novel strategies have been proposed to effectively deliver these agents to the tumor site. Novel delivery systems such as nanoparticles, microspheres, liposomes and polymer-drug conjugates have all been employed to mask the physicochemical properties of hydrophilic drugs in order to increase their intracellular concentration.
3.4.1 Polymer drug conjugates

The polymer-drug conjugates have been classified by Vincent and Duncan to be in the umbrella of polymer therapeutics, which also includes polymer-protein conjugates, polymer micelles, multicomponent polyplexes [352, 353]. Polymer-drug conjugates are generally composed of two components: a bioactive agent that is attached to a water soluble polymer. The main goal of this delivery system is to transport high amounts of drugs such as cytotoxic agents to the tumor site and into tumor cells [353, 354]. There are some critical properties which have to be considered when selecting water soluble polymers. The ideal polymer is biocompatible, biodegradable, non-immunogenic, and has a high capacity of loading active agent. Based on the above properties polymers such as poly(ethylene glycol) (PEG) [355, 356], N-(2-hydroxypropylmethacrylamide) (HPMA) copolymer [357-359], polyglutamic acid (PGA) [360-362], gelatin [363, 364], chitosan [365, 366] have been investigated as potential polymer candidates for the synthesis of polymer-drug conjugates.

Several polymer-drug conjugates are in phase I, II, and III clinical trials as listed in Table 3.13 [352, 354]. The polymer conjugates in clinical trials mostly include polymer linked to well established chemotherapeutic agents such as paclitaxel [359], doxorubicin [367] and camptothecins [355, 356]. These conjugates have been shown to increase the amount of drug reaching the tumor site mainly through passive targeting by the Enhanced Permeability and Retention (EPR) effect. The EPR effect has been cited as the mechanism of passive tumor targeting of the polymer drug conjugates [352, 354]. One of the unique features of the tumor vasculature is their leakiness as a result of the discontinuity of the endothelium [368]. The pore size of these microvessels has been
reported to be in the range of 100 to 750 nm depending largely on the tumor location [369, 370]. The leaky vasculature of the tumor allows extravasations of the circulating molecules resulting in the accumulation of these delivery systems. Once in the tumor vasculature, the conjugates can either enter the cancer cells or release the cytotoxic agent outside the cell [370].

Other than the selection of the suitable polymer, another important property which needs investigation is the potential bond formed between the drug and the polymer. For bioconjugates, the nature of the linker between the active moiety and the polymer has been described to dictate the successful delivery [371]. Both covalent and non-covalent interactions have been used for polymer-drug conjugate synthesis. The non-covalent interactions include high affinity ligand-receptor interactions and the electrostatic complexation [371, 372], while the covalent interactions include amide, ester, disulfide, malonate, and carbamate [352, 354]. The choice of the bond between the polymer and the active moiety is largely dependent on two important properties: biological stability and reversibility. When the release of active moiety from the polymer conjugate is necessary, the disulfide bond is the ideal choice due to its potential reversibility in the cell [371]. The relative stability in the plasma and the reversibility of the disulfide bond is based on the difference between glutathione concentration in the plasma (10 µM) versus in the cell (1-11 mM) [149, 240]. Saito et al. have described this high redox potential difference between the oxidizing extracellular space and the reducing intracellular space makes the disulfide bond most desirable as a potential delivery tool [371].

However, there are certain restrictions in the successful delivery of drugs with the aid of disulfide conjugates, as polymer conjugates have low diffusivity due to their size
and higher aqueous and therefore low lipid solubility. The cell membrane restricts the entry of these conjugates in the cytosol [372]. The process by which the polymer-drug conjugate is taken up by the cell is generally thought to be through endocytosis. The pH of the early endosomes and the lysosomes have been described as acidic and oxidative in nature compared to the cytosol, and therefore, the reduction of the disulfide conjugates is slow and limited process [371-373]. In addition, the reduction in acidic environment is known to be inefficient as it requires the deprotonation of thiols [373]. Cysteine has been known to be the major reducing agent in lysosomes [374, 375], but the presence of other cysteine and glutathione containing enzyme has been recently discovered. Gamma-interferon-inducible lysosomal thiol reductase (GILT) could also aid in the disulfide reduction [371, 376, 377]. GILT is a 30 kDa soluble glycoprotein which is expressed in the lysosomes and endosomes of antigen presenting cells in humans [377, 378]. GILT has a pH activity optimum at 4.0-5.5, compared to thioredoxin whose activity is optimal at pH 7.3 [377, 379].

The synthesis of disulfide conjugates depend on the presence of thiol moieties on both the polymer and the active agent. Most of the polymers described above do not have endogenous thiol moiety. Therefore, the addition of thiol moiety to these compounds (either polymer of active agent or both) has to be accomplished. The modification of primary amines has been a popular choice for introducing thiol moieties [371]. Heterobifunctional cross-linkers, which have amino reactive group on one end and a thiol reactive group on the other end, have been commonly employed for modification of the amine compounds. Heterobifunctional cross-linkers such as N-succinimidyl-3-(2-pyridylthio) propionate (SPDP), N-succinimidyl S-acetyltioacetate (SATA), 4-
succinimidyl oxy-carbonyl-2-pyridylthio toluene (SMPT) have been used in the synthesis of disulfide conjugates [380-382].

Mylotarg® (anti-CD33, antibody-S-S-calicheamicin) is a chemotherapeutic agent composed of recombinant humanized antibody conjugated to the cytotoxic agent, calicheamicin with the aid of a disulfide bond. Mylotarg is one of the first disulfide conjugates recently approved by the FDA for the treatment of acute leukemia [371, 383].
Table 3.13 Polymer-drug conjugates in clinical trials. Adapted from Vincent [352]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Commercial Name</th>
<th>Status</th>
<th>Polymer characteristics</th>
<th>Linker</th>
<th>Drug (loading)</th>
<th>Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglutamate-paclitaxel</td>
<td>XYOTAX</td>
<td>Phase II/III</td>
<td>PGA</td>
<td>Ester</td>
<td>Paclitaxel (37% w/w)</td>
<td>Esterase</td>
</tr>
<tr>
<td>Polyglutamate Camptothecin</td>
<td>CT2106</td>
<td>Phase I</td>
<td>PGA</td>
<td>Ester</td>
<td>Camptothecin (33-35% w/w)</td>
<td>Esterases</td>
</tr>
<tr>
<td>HPMA copolymer-doxorubicin</td>
<td>PK1</td>
<td>Phase I</td>
<td>HPMA</td>
<td>Amide</td>
<td>Doxorubicin (8.5% w/w)</td>
<td>Cathepsin B</td>
</tr>
<tr>
<td>HPMA copolymer-doxorubicin-galactosamine</td>
<td>PK2</td>
<td>Phase I/II</td>
<td>HPMA</td>
<td>Amide</td>
<td>Doxorubicin (7.5% w/w)</td>
<td>Cathepsin B</td>
</tr>
<tr>
<td>HPMA copolymer-carboplatin palatinate</td>
<td>AP5280</td>
<td>Phase I/II</td>
<td>HPMA</td>
<td>Malonate</td>
<td>Platinum (7% w/w)</td>
<td>Hydrolysis</td>
</tr>
<tr>
<td>HPMA copolymer-DACH-platinate</td>
<td>AP5346</td>
<td>Phase I/II</td>
<td>HPMA</td>
<td>Malonate -DACH</td>
<td>Platinum (7% w/w)</td>
<td>Hydrolysis</td>
</tr>
<tr>
<td>PEG-Camptothecin</td>
<td>PROTHE CAN</td>
<td>Phase I</td>
<td>PEG</td>
<td>Ester</td>
<td>Camptothecin (1.7% w/w)</td>
<td>Esterases</td>
</tr>
</tbody>
</table>
3.4.1.a Gelatin conjugates

Gelatin is a commonly used natural polymer which is derived from collagen [384]. Two kinds of gelatin can be produced based on the extraction process from collagen. Gelatin A and B are the acid and base derived forms of gelatin, respectively. In the pharmaceutical industry, gelatin is used as a suspending agent, encapsulating agent, and tablet binder [384, 385]. Gelatin is considered as Generally Regarded As Safe (GRAS) by the FDA. The characteristic properties of gelatin which make it an ideal polymer candidate are low level of immunogenicity and cytotoxicity, biodegradability, and the capacity for modification (mainly due to the presence of large number of amino groups which could be used for conjugation) [384-387]. Gelatin has been successfully investigated as a vehicle for gene delivery [388, 389], as a vehicle for the release of bioactive molecules [390-392], and in the generation of scaffolds for tissue engineering [393, 394]. Novel delivery formulations employing gelatin have been previously described such as conjugates [390, 392, 395], nanoparticles [363, 388, 389, 396, 397], microparticles [363, 398, 399], and tissue scaffolds [400, 401]. Gelatin conjugates with cytotoxic agents have been shown to be successful in delivering these agents to cancer cells. Ofter et al. recently described the synthesis of a gelatin-methotrexate conjugate for the increased intracellular delivery of methotrexate in cancer cells [390]. Chung et al. have earlier described the enzymatic synthesis and antioxidant property of gelatin-catechin conjugates [395]. The conjugate was shown to be a good scavenging activity against superoxide radical. Additionally, the conjugate was also shown an amplified effect on human low density lipoprotein oxidation [395].
Chapter 4

An investigation into copper catalyzed D-penicillamine oxidation and subsequent hydrogen peroxide generation

4.1 Summary

D-penicillamine (D-pen) is a potent copper (Cu) chelating agent. D-pen reduces Cu(II) to Cu(I) in the process of chelation while at the same time being oxidized to D-pen disulfide (D-pen-S-S-D-pen). It has been proposed that hydrogen peroxide (H$_2$O$_2$) is generated during this process. However, definitive experimental proof that H$_2$O$_2$ is generated remains lacking. Thus, the major aims of these studies were to confirm and quantitatively assess the in-vitro production of H$_2$O$_2$ during copper catalyzed D-pen oxidation, and to determine the reaction kinetics. The biological effect of the proposed cytotoxic H$_2$O$_2$ generation was also investigated in-vitro against MCF-7 human breast cancer cells. Cell cytotoxicity resulting from the incubation of D-pen with copper was compared to that of D-pen, copper and H$_2$O$_2$. The mechanism of copper catalyzed D-pen oxidation and simultaneous H$_2$O$_2$ production was investigated as a function of time, concentration of cupric sulfate or ferric chloride, temperature, pH, anaerobic condition and chelators such as EDTA and bathocuproinedisulfonic acid (BCS). A simple, sensitive and rapid HPLC assay was developed to simultaneously detect D-pen, its major oxidation product D-pen disulfide, and H$_2$O$_2$ in a single run. H$_2$O$_2$ was shown to be generated in a concentration dependent fashion as a result of D-pen oxidation in the presence of cupric sulfate. Chelators such as EDTA and BCS were able to inhibit D-pen oxidation. The
incubation of MCF-7 human breast cancer cells with D-pen plus cupric sulfate resulted in the production of reactive oxygen species (ROS) within the cell and cytotoxicity that was comparable to free H₂O₂.
4.2 Introduction

Thiol containing compounds such as glutathione and cysteine play an important role in protecting biological systems against oxidative stress [154, 402, 403]. However, it is recognized that these endogenous thiols can also be oxidized to disulfides in the presence of transition metals such as copper and iron [404-406]. This oxidation process is known to generate hydrogen peroxide (H$_2$O$_2$) which leads to oxidative stress [152, 407, 408]. The interaction of copper and endogenous thiols has received considerable focus since the ratio between the oxidized and the reduced thiol could greatly affect the cellular redox status and modulate the activity of several thiol dependent enzymes [204, 409].

D-penicillamine (D-pen) is an aminothiol and a potent copper chelating agent. It is currently approved by the FDA (CUPRIMINE®, Merck) for the treatment of Wilson’s disease and rheumatoid arthritis. D-pen is known to remove excess copper accumulated in Wilson’s disease patients [39, 131]. The efficacy of D-pen in rheumatoid arthritis is more complicated as a variety of therapeutic mechanisms have been reported [24, 115, 142]. D-pen binds Cu(II) and reduces it in the process of chelation to Cu(I) [23, 410]. D-pen then binds to Cu(I) with a stability constant of 18.8 [47]. It has been proposed that the interaction between D-pen and copper results in the formation of a multivalent D-pen-copper complex (Cu$_8^{II}$Cu$_6^{II}$D-pen$_{12}$Cl$_5^{-}$), although its in-vivo presence is yet to be conclusively proven [143, 144]. Kato et al. demonstrated using in-vitro $^1$H-NMR studies that D-pen incubation with copper in the presence of glutathione resulted in the formation of a multivalent D-pen-glutathione-copper complex [144]. It has been proposed that in the D-pen copper complex, Cu(II) is in equilibrium with the surrounding aqueous medium while Cu(I) is removed from equilibrium [144]. Thus, the continued presence of
Cu(II) leads to the potential of Cu(II) being available for catalyzing thiol oxidation. Therefore, the process of D-pen chelation with Cu(II) and subsequent reduction of Cu(II) to Cu(I) may catalyze oxidation that leads to the generation of reactive oxygen species (ROS) such as superoxide radicals, H$_2$O$_2$, and hydroxyl radicals (Figure 4.1). These ROS can induce damage to proteins and DNA and cause membrane peroxidation as previously described [211, 212, 411].

Although D-pen can generate H$_2$O$_2$, it can also react with the generated H$_2$O$_2$. The pKa of thiol group of D-pen is known to play a critical role in its reaction with H$_2$O$_2$ [154, 412]. The thiol group of D-pen has a pKa of is 7.9 compared to that of 8.3 and 8.75 for cysteine and glutathione, respectively. Since the rate of thiol reaction with H$_2$O$_2$ increases with decreasing pKa [154, 412], there is more competition with D-pen between the production of H$_2$O$_2$ and reaction with H$_2$O$_2$ in the biological environment.

Previous studies in the literature investigating the metal catalyzed thiol oxidation have employed a wide variety of techniques to detect or quantify H$_2$O$_2$ including horseradish peroxidase plus scopoletin [413], the use of an oxygen electrode [414], or by its inhibition with catalase [415]. The loss of thiol is commonly measured with Ellman’s reagent while the final product of oxidation, the respective disulfide, is generally not measured. These methods employed to quantify D-pen and H$_2$O$_2$ are relatively insensitive and non-specific. Newer techniques that can simultaneously quantify the disappearance of D-pen and the appearance of D-pen disulfide and H$_2$O$_2$ would greatly enhance the understanding of copper catalyzed D-pen oxidation mechanisms. One of the major difficulties in D-pen assay development has been to maintain D-pen in its reduced state and prevent its spontaneous oxidation in air to D-pen disulfide. It is notable that D-pen
disulfide is highly resistant to reduction even in the presence of DTT, TCEP and other commonly used reducing agents [185, 186].

The aim of the present studies was to provide experimental evidence that concentration dependent \( \text{H}_2\text{O}_2 \) is generated \textit{in-vitro} as a result of copper catalyzed D-pen oxidation. A simple, sensitive and rapid HPLC assay was developed that simultaneously detected and quantified D-pen, D-pen disulfide and \( \text{H}_2\text{O}_2 \) in a single run. The mechanism of copper catalyzed D-pen oxidation and simultaneous \( \text{H}_2\text{O}_2 \) production was investigated as a function of time, concentration of cupric sulfate or ferric chloride, temperature, pH, anaerobic condition and chelators such as EDTA and bathocuproinedisulfonic acid (BCS). The biological implications of cytotoxic \( \text{H}_2\text{O}_2 \) generation as a result of chelation of D-pen with copper was examined against MCF-7 human breast cancer cells and compared to the cytotoxic effects of D-pen, cupric sulfate and \( \text{H}_2\text{O}_2 \).
4.3 Materials and methods

Reagents

D-penicillamine, D-penicillamine disulfide, cupric sulfate (CuSO₄), ferric chloride (FeCl₃), hydrogen peroxide (H₂O₂) solution 30% w/w, bathocuproinedisulfonic acid (BCS) disodium salt, catalase (bovine liver), ethylenediaminetetraacetic acid (EDTA) disodium salt, tris (2-carboxyethyl) phosphine (TCEP), dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and o-phosphoric acid (85%) were HPLC grade and purchased from Fisher Scientific Co. (Pittsburg, PA). MCF-7 cell line, fetal bovine serum (FBS), penicillin-streptomycin antibiotic solution, trypsin-EDTA solution and MTT assay kit were purchased from the American Type Culture Collection (Rockville, MD). RPMI-1640 media was purchased from Gibco (Carlsbad, CA). 2’-7’-dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Molecular Probes (Eugene, OR).

High Performance Liquid Chromatography (HPLC)

The HPLC system (Spectra System® (ThermoSeparation Products)) consisted of the following: a pump (model Spectra System® P4000; ThermoSeparation Products) with a flow rate of 1 mL/min; an autosampler (model Spectra System® AS3000; ThermoSeparation Products), an UV detector (model Spectra system® UV6000; ThermoSeparation Products) set at 214 nm (rise time 2 s) and a data acquisition system (Chromoquest® software Version. 2.5); an analytical column (YMC ProPack C₁₈ with 5-μm beads; 150 mm x 4.6 mm; Waters Corp. Milford, MA). The mobile phase (50% - 50% v/v mixture of solvent A (50 mM phosphoric acid) and solvent B (50 mM
phosphoric acid + 5% acetonitrile)), the pH of both solvents was adjusted to pH 2.5 and were pumped at a flow rate of 1 mL/min, which led to a typical backpressure of 1600 psi. 

H₂O₂, D-pen disulfide and D-pen were detected by UV absorption at 214 nm with retention times of 1.69, 2.8 and 3.07 min, respectively. Sample concentrations (µM) were obtained from the regression line of peak area versus standard concentrations (µM). These were calculated using a ten-point calibration curve of H₂O₂, D-pen disulfide and D-pen standards. Water used in the mobile phase was deionized and further purified with Milli-Q® Synthesis A10 Ultra Pure Water System, Millipore Ltd. (Billerica, MA).

**Evidence of hydrogen peroxide generation as a result of D-pen oxidation**

D-pen oxidation was investigated in deionized water as follows: either I) D-pen (50, 100 and 500 µM) was incubated alone, or II) D-pen (50, 100 and 500 µM) was incubated with cupric sulfate (4 µM), or III) D-pen (50, 100 and 500 µM) was incubated with cupric sulfate (4 µM) and catalase (100 U/mL) for 15 min at 25 °C. The percentage of D-pen oxidized was calculated as: D-pen oxidized (%) = (D-pen disulfide (µM) / D-pen (µM)) × 100.

**Effect of incubation time and cupric sulfate concentration on copper catalyzed D-pen oxidation**

To investigate the effect of incubation time on copper catalyzed D-pen oxidation, solutions containing D-pen (100 µM) and cupric sulfate (4 µM) were incubated at 25°C. The oxidation reaction was stopped with the addition of EDTA (1 mM) and samples were analyzed at predetermined time points between 0 and 20 min. These studies were
performed in deionized water. To compare the copper catalyzed D-pen oxidation profile with that of another transition metal, the above studies were repeated under similar experimental conditions with ferric chloride (4 µM).

To investigate the effect of increasing cupric sulfate concentration on copper catalyzed D-pen oxidation, D-pen (100 µM) was incubated with a range of cupric sulfate concentrations of 0.1 to 20 µM for 20 min followed by addition of EDTA (1 mM). Samples were analyzed for the disappearance of D-pen and the appearance of D-pen disulfide and H₂O₂ using the developed HPLC assay.

**Effect of chelators, pH, and temperature on copper catalyzed D-pen oxidation**

EDTA and a Cu(I) specific chelator (BCS) were used to investigate the effect of other chelating agents on copper catalyzed D-pen oxidation. A solution containing D-pen and cupric sulfate (4 µM) was incubated with varying concentrations (0-50 mM) of either EDTA or BCS for 1 h.

The effect of pH was investigated in the presence of cupric sulfate (3 µM), ferric chloride (3 µM), cupric sulfate (3 µM) + ferric chloride (12 µM), cupric sulfate (3 µM) + ferric chloride (3 µM) and cupric sulfate (12 µM) + ferric chloride (3 µM). These studies were performed in phosphate buffer (0.1 M) at pH 6.0 and 7.0.

The effect of temperature was investigated by incubating D-pen (850 µM) with cupric sulfate (4 µM) at 4, 25 and 37°C at pH 7.4 in phosphate buffer (0.1 M). As a negative control, D-pen was also incubated without cupric sulfate at 25°C at pH 7.4 in phosphate buffer (0.1 M). Concentration (µM) versus time curves for the disappearance of D-pen and the appearance of D-pen disulfide at 4, 25 and 37°C was fitted to a first-
order model using non-linear least squares regression analysis (SCIENTIST®, Micromath Inc., St Louis, MO).

**Cell cytotoxicity studies with breast cancer cells (MCF-7)**

MCF-7 cells were cultured in RPMI media supplemented with 10% FBS at 37°C in 5% CO₂. Cells were transferred to a 96-well plate and seeded at 1×10⁵ cells/well and allowed to attach for 24 h before the commencement of the experiment. Cell cytotoxicity was determined using an MTT assay kit and reported as the % viable cells compared to control cells after 48 h. Cells were incubated with D-pen alone (10, 100 and 1000 µM), cupric sulfate alone (10 µM), H₂O₂ alone (50, 250, 500 and 1000 µM), and D-pen (100, 500 and 1000 µM) + cupric sulfate (10 µM), D-pen (1000 µM) + cupric sulfate (10 µM) + catalase (1000 U/mL).

**Determination of intracellular reactive oxygen species (ROS) with 2'-7’ dichlorodihydrofluorescein diacetate (H₂DCFDA)**

MCF-7 cells (4×10⁵ cells/well) were incubated with 10 µM 2'-7’ dichlorodihydrofluorescein diacetate (H₂DCFDA) in PBS (pH 7.4) for 40 min at 37°C to load the cells with the intracellular reactive oxygen species marker. This was followed by washing the cells twice with fresh PBS to remove excess H₂DCFDA. Complete RPMI media containing cupric sulfate (10 µM), D-pen (1000 µM), and D-pen (1000 µM) + cupric sulfate (10 µM) was added and cells were incubated for 2, 4 and 6 h, and the cell-associated fluorescence was measured using a Biotek FL600 Spectrophotometer.
(Winooski, VT) (excitation 485 nm, emission 530 nm) and compared to that of untreated but H₂DCFDA loaded MCF-7 cells.

**Statistical analysis**

Statistical analysis on Figure 4.4 was performed with one-way analysis of variances (ANOVA) followed by Dunnett’s multiple comparison post-test. Statistical analysis on Figures 4.5 and 4.7 was performed using a two-way ANOVA followed by Bonferroni post test. Statistical analysis on Figure 4.6 was performed using a one-way ANOVA with Newman-Keuls multiple comparison post-test with GraphPad Prism® 4 software (San Diego, CA).
4.4 Results and discussion

Determination of D-pen, D-pen disulfide and hydrogen peroxide

In earlier published reports investigating thiol oxidation [23, 413], the disappearance of thiol was solely analyzed by Ellman’s reagent (DTNB) which reacts with free sulfhydryl (-SH) groups to form a yellow colored compound. The appearance of H$_2$O$_2$ was analyzed by non-specific or insensitive methods [413-415] while the concentration of D-pen disulfide was not analyzed. Although HPLC methods to separate and quantify thiols and their disulfides have been published earlier by a few groups [416, 417], we were not aware of a method that could separate and quantify D-pen and D-pen disulfide. The majority of the earlier published HPLC methods for detecting D-pen mostly involved an initial step of derivatization of D-pen [165, 171] mainly through Michael’s addition with the thiol group. This derivatization led to efficient and sensitive detection of D-pen, but it also meant that D-pen could be detected only in its reduced form. An HPLC assay was sought that could detect D-pen, D-pen disulfide and H$_2$O$_2$ simultaneously in the same run. Luo et al. [418] previously described a simple HPLC method that detected cysteine, its respective disulfide, and H$_2$O$_2$ in a single run. Due to apparent structural similarities between cysteine and D-pen, we decided to modify the method of Luo et al. to achieve the separation, detection and quantification of D-pen, D-pen disulfide and H$_2$O$_2$. A representative chromatogram of simultaneous H$_2$O$_2$, D-pen disulfide and D-pen elution is shown in Figure 4.2. The retention times for H$_2$O$_2$, D-pen disulfide and D-pen were 1.69 ± 0.01 min, 2.8 ± 0.01 min and 3.07 ± 0.01 min, respectively. Although D-pen disulfide and D-pen eluted close to one another, there was excellent resolution to separate and quantify both compounds. D-pen disulfide and H$_2$O$_2$
were stable if stored at 4°C; however, D-pen had to be analyzed immediately due to its oxidation to form D-pen disulfide. The limit of quantification (LOQ) for D-pen, D-pen disulfide and H$_2$O$_2$ was 5 µM. The coefficient of variation (C.V.) for intra-run and inter-run for H$_2$O$_2$, D-pen disulfide and D-pen ranged between 1-3%, respectively. A linear concentration range of 5 to 1000 µM for H$_2$O$_2$, D-pen disulfide and D-pen was established. The C.V. for response factors in the linear range between 5 to 1000 µM was 8-9%, 4-4.5% and 3–3.4% for H$_2$O$_2$, D-pen disulfide and D-pen, respectively.

Although this assay for D-pen is not as sensitive as other fluorescence or electrochemical HPLC methods [417], it has the advantages of 

1) being easier than the previous assay requiring derivatization of D-pen and, 

2) being able to simultaneously detect H$_2$O$_2$, D-pen disulfide and D-pen.

Evidence of hydrogen peroxide generation as a result of D-pen oxidation

D-pen oxidation was preliminarily investigated in deionized water at 25°C for 15 min. D-pen (50, 100 and 500 µM) was incubated alone, with cupric sulfate (4 µM), or D-pen was incubated with cupric sulfate (4 µM) and catalase (100 U/mL). When D-pen was incubated alone at concentrations of 50, 100 and 500 µM, D-pen was fully recovered giving, 50.1 ± 1.3, 97.5 ± 7.0 and 500 ± 15.9 µM D-pen. However, when cupric sulfate (4 µM) was added to D-pen at concentrations of 50, 100 and 500 µM, D-pen was completely oxidized to D-pen disulfide resulting in detectable D-pen disulfide concentrations at 15 min of 25.7 ± 0.4 µM, 51.4 ± 1.0 µM and 252.4 ± 6.3 µM, respectively, with the simultaneous production of H$_2$O$_2$ at concentrations of 21.1 ± 0.9 µM, 43.4 ± 1.4 µM and 116.5 ± 8.6 µM, respectively. To confirm that H$_2$O$_2$ was being
generated, the same experiment was repeated in the presence of catalase (100 U/mL). D-pen at concentrations of 50, 100 and 500 µM, was completely oxidized to D-pen disulfide to produce final concentration of D-pen disulfide of 24.8 ± 0.1 µM, 57.4 ± 0.6 µM and 254 ± 0.7 µM, respectively, with no H2O2 being detected. It was also noted that the molar ratio of H2O2 generated to D-pen oxidized was approximately 1:2 mol/mol at lower concentration of D-pen (50 and 100 µM) which is in agreement with the mechanism proposed by Starkebaum et al. [23] for copper catalyzed D-pen oxidation and subsequent H2O2 generation (Figure 4.1). However, at higher D-pen concentration (500 µM), only 116.5 ± 8.6 µM of H2O2 was detected. This was presumably due to the loss of H2O2 as well as further oxidation and loss of D-pen due to reaction of D-pen with H2O2.

Effect of time of incubation and concentration of cupric sulfate on the copper catalyzed D-pen oxidation

As shown in Figure 4.3a, when D-pen (100 µM) was incubated with cupric sulfate (4 µM), D-pen oxidation was complete after 10 min resulting in the generation of 45.7 ± 1.7 µM H2O2, which is in agreement with the 1:2 molar ratio of H2O2 to D-pen mentioned above. After D-pen oxidation was complete, the H2O2 generated remained constant until the end of the experiment demonstrating that H2O2 generation was directly related to the availability of D-pen.

D-pen (100 µM) was then incubated with a range of cupric sulfate concentration (0.1-20 µM) at 25°C for 20 min. This was followed by the addition of EDTA (1 mM) to stop further D-pen oxidation. Samples were analyzed to determine the D-pen disappearance and the appearance of H2O2 and D-pen disulfide. As shown in Figure 4.3b,
a maximum of 42 ± 4.3 µM H₂O₂ was generated using 4 µM cupric sulfate. It should be noted that almost all copper *in-vivo* is bound to the copper carrying protein ceruloplasmin and/or albumin and that the concentration of free copper is very low. Each ceruloplasmin binds about 6-7 copper atoms although it has been suggested that at least one of the copper atoms is loosely bound and therefore could be available for catalyzing D-pen oxidation [419, 420]. The binding of albumin to copper is much weaker compared to ceruloplasmin; thus, the ability of D-pen to remove excess copper in Wilson’s disease could be due to its ability to interact with albumin and remove albumin bound copper [420-422]. In any case, it can be seen from the present experiments that ≤1000 nM cupric sulfate was able to oxidize D-pen and generate H₂O₂ which then suggests that even nanomolar concentrations of copper is enough for significant H₂O₂ generation.

When increasing concentrations of ferric chloride (0.1-20 µM) was incubated with D-pen (100 µM) under similar experimental conditions, it resulted in only ~6-8% oxidation of D-pen confirming earlier published reports [423-425] that iron catalyzed oxidation rates for thiols are significantly less than that of copper catalyzed (data not shown).

**Effect of ETDA and BCS on copper catalyzed D-pen oxidation**

When D-pen was incubated with cupric sulfate (4 µM) in the presence of EDTA and BCS at concentrations from 0.5-50 mM, D-pen was completely protected from oxidation due to the chelation of copper (data not shown). The possible explanation would be that EDTA is known to chelate Cu (II) compared to D-pen which reduces Cu(II) first to Cu(I) before chelation. Therefore, EDTA was able to protect D-pen from
oxidation in the presence of Cu(II). The mechanism proposed by Starkebaum et al. (Figure 4.1) of D-pen oxidation suggests that the first step involves the reduction of Cu (II) to Cu(I) by D-pen. As confirmed in these studies, the reduction of Cu(II) to Cu(I) is the initial step in H₂O₂ production, and thus a Cu(I) specific chelator is able to block any D-pen oxidation and subsequent generation of H₂O₂.

**Effect of copper and iron concentration on D-pen oxidation at pH 6 and 7**

The effect of pH and the presence of either cupric sulfate or ferric chloride alone or in combination on D-pen oxidation was investigated. The experiments were performed in phosphate buffer (0.1 M) at either pH 6 or 7 at 25°C for 2 h. When D-pen (450 µM) was incubated with cupric sulfate (3 µM) alone, complete oxidation (p<0.01) was observed at both pH 6 and 7. When ferric chloride (3 µM) was employed, only ~25% and ~20% (p<0.01) oxidation was observed at pH 6 and 7, respectively (Figure 4.4). When a combination of ferric chloride and cupric sulfate (12 µM + 3 µM, 3 µM + 3 µM and 3 µM + 12 µM) were employed complete D-pen oxidation was observed (p<0.01) at both pH 6 and 7. This suggested that the rate of D-pen oxidation is dominated by the presence of copper. Finally, when D-pen was incubated with cupric sulfate (3 µM) in the absence of oxygen, only ≤10% D-pen was oxidized at both pH 6 and 7 (p<0.01) suggesting that oxygen is essential for the oxidation of D-pen.

**Effect of additional D-pen on copper catalyzed D-pen oxidation**

As shown in Table 4.1, D-pen (90 µM) was initially incubated with cupric sulfate (4 µM) in phosphate buffer (0.1 M) at either pH 6.2 and 7.4 for 1 h at 25°C. Samples were
analyzed and complete D-pen oxidation was confirmed with the formation of 38.2 ± 4.2 µM and 38.7 ± 2.3 µM of D-pen disulfide at pH 6.2 and 7.4, respectively. The state of copper after it has completely oxidized D-pen was investigated. Specifically, additional D-pen (90 µM) was added and samples were again incubated for 1 h followed by analysis. It was found that D-pen oxidation was complete as 81.7 ± 4.3 µM and 86.9 ± 6.7 µM of cumulative D-pen-disulfide was formed at pH 6.2 and 7.4, respectively. Additional D-pen (90 µM) was added and samples were incubated for 2 h followed by analysis. Complete D-pen oxidation resulted in the cumulative formation of 126 ± 1.5 µM and 134.7 ± 5.4 µM of D-pen disulfide at pH 6 and 7, respectively. Finally, D-pen (90 µM) was added one additional time and incubated for 10 h followed by analysis. All of the available D-pen was oxidized resulting in the formation of 181.9 ± 3.5 µM and 186.4 ± 2.7 µM of D-pen disulfide at pH 6 and 7, respectively. Thus, after each addition of D-pen and subsequent incubation there was a consistent formation of D-pen disulfide indicating that copper had retained its catalytic activity after initial interaction with D-pen. These data strongly suggests that the formed D-pen copper complex is still able to oxidize available D-pen.

**Effect of temperature on copper catalyzed D-pen oxidation**

Copper catalyzed D-pen oxidation was strongly influenced by temperature as shown in Figure 4.5. After 5 h incubation of D-pen (850 µM) with cupric sulfate (4 µM), 49% D-pen was oxidized at 4°C (p<0.01), 78% D-pen was oxidized at 25°C (p<0.001) and 99% D-pen was oxidized at 37°C (p<0.001). In the absence of cupric sulfate, only 18% D-pen was oxidized after 5 h at 25°C. The first-order D-pen oxidation rate constants
in the presence of cupric sulfate (4 µM) at 4°C, 25°C and 37°C were 0.131 ± 0.001 h⁻¹, 0.273 ± 0.002 h⁻¹, and 0.459 ± 0.012 h⁻¹, respectively. In contrast, in the absence of cupric sulfate, the oxidation rate constant at 25°C was 0.036 ± 0.001 h⁻¹. The rate constants generated through curve fitting at 4, 25 and 37°C were well described by the Arrhenius Equation as a plot of (1/K x 10³) (x-axis) versus $k$ (y-axis) yielded an $r^2$ value of 0.9921 with an activation energy (Ea) of 20.57 kJ/mol (data not shown).

**Cytotoxicity studies with breast cancer cells (MCF-7)**

The *in-vitro* cell cytotoxicity of D-pen supplemented with cupric sulfate was investigated using MCF-7 human breast cancer cells to ascertain the potential biological effect of H₂O₂ generation. Cell viability was investigated at 48 h using the MTT assay. Cell incubation with D-pen alone resulted in no significant cytotoxicity, with 94.1 ± 5.5%, 91.2 ± 5.6% and 97.9 ± 4.6% cells remaining viable after 48 h incubation with D-pen at concentrations of 10, 100 and 1000 µM, respectively. Likewise, cupric sulfate incubation at a concentration of 10 µM did not result in significant cytotoxicity, with 98 ± 2.1% cells surviving after 48 h incubation. Cell viability was 92 ± 0.6%, 98 ± 4 %, 97 ± 1.6% and 77.3 ± 6.2% after incubation with H₂O₂ concentrations of 50, 250, 500 and 1000 µM for 48 h, respectively. However when MCF-7 cells were incubated with a combination of D-pen (100, 500 and 1000 µM) + cupric sulfate (10 µM), the cell viability was 84 ± 2.8%, 86.7 ± 2.4% and 83.9 ± 5.6%, respectively, after 48 h incubation. The reason for a lack of dose-dependent response is currently under investigation. However, the current hypothesis is that since the membrane permeability of Cu, D-pen, and the D-pen/Cu complex are very low, a sufficiently high
extracellular concentration of these species is required by Fick’s law to drive the diffusion of these species into the cell.

As shown in Figure 4.6, the cytotoxicity resulting from incubation MCF-7 cells with a combination of D-pen (1000 µM) + cupric sulfate (10 µM) was significantly lower than either D-pen (1000 µM) and cupric sulfate (10 µM) alone (p<0.05). It is to be noted that there was no significant difference between the cytotoxicity resulting from the incubation of the combination of D-pen (1000 µM) + cupric sulfate (10 µM) and H$_2$O$_2$ (1000 µM) (p>0.05). To confirm that the cytotoxicity resulting from the incubation of D-pen + cupric sulfate was directly related to H$_2$O$_2$ generation, catalase (1000 U/mL) was added to D-pen (1000 µM) plus cupric sulfate (10 µM) and the above experiment was repeated, a complete recovery of cell viability was observed (Figure 4.6) compared to D-pen (1000 µM) + cupric sulfate (10 µM) (p<0.01) confirming the direct involvement of H$_2$O$_2$ in cell cytotoxicity.

H$_2$O$_2$ has been shown to be generated in-vitro in cell cytotoxicity studies when D-pen plus copper were incubated with ductal carcinoma cell line [426], murine plasmocytoma cells [427], endothelial cells [6, 23], T-lymphocytes [141, 428] and human fibroblast [142]. Samoszuk et al. [427] demonstrated that a combination of D-pen plus copper exhibited a significant antiproliferative effect on tumor cells in-vitro and the fact that this effect was inhibited by the presence of catalase clearly demonstrated that H$_2$O$_2$ was responsible for the cytotoxicity.
Generation of intracellular oxidant species as determined with H$_2$DCFDA

H$_2$DCFDA readily enters the cell and is hydrolyzed by esterases to the non-fluorescent 2'-7' dichlorodihydrofluorescein (H$_2$DCF). H$_2$DCF undergoes subsequent conversion to the fluorescent 2'-7' dichlorofluorescein (DCF) upon reaction with oxidant species [429-431]. To further provide proof that H$_2$O$_2$ and other reactive oxygen species were in fact being generated as a result of D-pen oxidation, D-pen (1000 µM) + cupric sulfate (10 µM) were incubated with H$_2$DCFDA loaded MCF-7 cells and cell-associated fluorescence was measured at 2, 4, and 6 h. As shown in Figure 4.7, while D-pen (1000 µM) and cupric sulfate (10 µM) alone did not show statistically significant cell-associated fluorescence compared to control at 2, 4 and 6 h, D-pen and cupric sulfate incubated together at the concentrations above caused a 2 fold increase in the cell-associated fluorescence at 2, 4 and 6 h. This confirmed that copper catalyzed D-pen oxidation leads to the generation of reactive oxygen species within the cells. These data strongly suggest that the production of hydrogen peroxide led to the formation of intracellular reactive oxygen species that caused cell cytotoxicity. However, additional studies are on-going to provide conclusive evidence.

D-pen is currently being investigated as an anti-angiogenic agent [4, 8, 86], mainly due to its copper chelating efficacy. However, the present work suggests that D-pen in the presence of copper is also capable of generating cytotoxic concentrations of H$_2$O$_2$. It has been reported that the serum and tumor levels of copper and ceruloplasmin are higher in diverse malignant conditions such as breast [59, 60], leukemia [432], lung [72], and gynecological carcinomas [71]. Additionally, the copper and ceruloplasmin levels have been shown to relate to the stage and kind of malignancy, with maximum rise in copper
levels in poorly differentiated variety [59]. Thus, these reports combined with the results obtained in this study warrant further examination of the possibility of employing D-pen as dual (anti-angiogenic and cytotoxic) anti-cancer agent.

**Conclusions**

The current work describes an experimental evidence of H$_2$O$_2$ generation as a result of copper catalyzed D-pen oxidation. A simple, sensitive and rapid HPLC assay was developed to simultaneously detect D-pen, its major oxidation product D-pen disulfide and H$_2$O$_2$ in a single run. The HPLC assay was employed to study effect of several factors on the copper catalyzed D-pen oxidation. It was shown that D-pen supplemented with copper resulted in the formation of reactive oxygen species within breast cancer cells and *in-vitro* cytotoxicity.

**Table of abbreviations**

D-penicillamine (D-pen)
D-pen disulfide (D-pen-S-S-D-pen)
Hydrogen peroxide (H$_2$O$_2$)
Bathocuproinedisulfonic acid (BCS)
Reactive oxygen species (ROS)
Dithiothreitol (DTT)
Tris (2-carboxyethyl)phosphine (TCEP)
Cupric sulfate (CuSO$_4$)
Ferric chloride (FeCl$_3$)
Ethlenediaminetetraacetic acid (EDTA)

2’-7’-dichlorodihydrofluorescein diacetate (H₂DCFDA)

2’-7’-dichlorodihydrofluorescein (H₂DCF)

2’-7’-dichlorofluorescein (DCF)

5,5’-Dithio-bis(2-nitro-benzoic acid) (DTNB)

*The content of this chapter were published in *Journal of Inorganic Biochemistry*, Gupte, A., Mumper, R. J., An investigation into copper catalyzed D-penicillamine oxidation and subsequent hydrogen peroxide generation, 2007;101:594-602. Copyright 2008 with permission from Elsevier.
Table 4.1 Effect of D-pen added in succession on the copper catalyzed D-pen oxidation at pH 6.2 and 7.4.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Sample</th>
<th>pH 6.2 D-pen disulfide (μM)</th>
<th>pH 7.4 D-pen disulfide (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-pen (90 μM) + CuSO₄ (4 μM)</td>
<td>38.2 ± 4.2</td>
<td>38.7 ± 2.3</td>
</tr>
<tr>
<td>2</td>
<td>+ D-pen (90 μM)</td>
<td>81.7 ± 4.3</td>
<td>86.9 ± 6.7</td>
</tr>
<tr>
<td>4</td>
<td>+ D-pen (90 μM)</td>
<td>126 ± 1.5</td>
<td>134.7 ± 5.4</td>
</tr>
<tr>
<td>14</td>
<td>+ D-pen (90 μM)</td>
<td>181.9 ± 3.5</td>
<td>186.4 ± 2.7</td>
</tr>
<tr>
<td>Reaction</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2\text{PSH} + 2\text{Cu}^{2+} \rightarrow 2\text{PS}^\cdot + 2\text{Cu}^{+} + 2\text{H}^+$</td>
<td>Initial reaction: formation of thiyl radical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{Cu}^{+} + \text{O}_2 \rightarrow \text{Cu}^{2+} + \text{O}_2^-$</td>
<td>Maintenance reaction: Regeneration of Cu$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{Cu}^{+} + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2$</td>
<td>Maintenance of radical chain reaction and generation of hydrogen peroxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{PS}^\cdot + \text{PS}^\cdot \rightarrow \text{PSSP}$</td>
<td>Chain termination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2\text{PSH} + \text{O}_2 \rightarrow \text{PSSP} + \text{H}_2\text{O}_2$</td>
<td>Overall reaction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.1 Mechanism of copper catalyzed D-pen oxidation.** The mechanism was initially proposed by Starkebaum [23]. PSH = D-penicillamine, PSSP = D-penicillamine disulfide, $\text{H}_2\text{O}_2$ = hydrogen peroxide, $\text{PS}^\cdot$ = D-penicillamine (thiyl) radical, $\text{PS}^-$ = D-penicillamine anion, $\text{O}_2^-$ = oxygen radical.
Figure 4.2 HPLC chromatogram showing elution of H$_2$O$_2$, D-pen disulfide and D-pen. H$_2$O$_2$ (1.69 min), D-pen disulfide (2.80 min) and D-pen (3.07 min).
Figure 4.3 Copper catalyzed D-pen oxidation to D-pen disulfide and the generation of H$_2$O$_2$ at 25°C. a) Effect of incubation time: CuSO$_4$ (4 µM) was incubated with D-pen (100 µM) for 0-20 min. b) Effect of cupric sulfate concentration: CuSO$_4$ (0.1-20 µM) was incubated with D-pen (100 µM) for 20 min. Data represents the mean ± SD (n = 3).
Figure 4.4 Effect of pH and copper and iron concentration on D-pen oxidation to D-pen disulfide. D-pen (450 µM) incubated in phosphate buffer (0.1 M) at pH 6.0 or 7.0 in the presence of cupric sulfate (3 µM), ferric chloride (3 µM) and cupric sulfate + ferric chloride (4:1, 1:1, and 1:4) for 2 h at 25°C. Data represents the mean ± SD (n = 3).

**p<0.01 compared to the control group of 0.1 M phosphate buffer.
Figure 4.5 Effect of temperature on copper catalyzed D-pen oxidation. D-pen (850 μM) was incubated alone at 25°C and with cupric sulfate (4 μM) in phosphate buffer (0.1 M) at 4°C, 25°C or 37°C. Data represents mean ± SD (n = 3). *p<0.05, **p<0.01, ***p<0.001 compared to the control group of no cupric sulfate at 25°C. The first-order rate constant, k, for each condition is shown above each plot.
**Figure 4.6 Cytotoxicity of the copper catalyzed D-pen oxidation.** The cytotoxicity of D-pen and cupric sulfate was compared to H$_2$O$_2$ after 48 h. Free D-pen and free cupric sulfate were used as controls. In addition, an additional sample contained catalase (1000 U/mL) to quench generated H$_2$O$_2$. Data represents the mean ± SD (n = 3). *group c was statistically different compared to groups a and b (p<0.01) and group e (p<0.001). **group d was statistically different compared to groups a and b (p<0.05) and group e (p<0.01). There was no statistical difference between groups c and d.
Figure 4.7 Copper catalyzed D-pen oxidation results in the generation of intracellular reactive oxygen species. The intracellular ROS was measured using 2′-7′-dichlorodihydrofluorescin diacetate (H$_2$DCFDA) loaded MCF-7 cells. D-pen (1000 µM), CuSO$_4$ (10 µM), and D-pen (1000 µM) + CuSO$_4$ (10 µM) were added to cells and the cell-associated fluorescence was measured after 2, 4, and 6 h. *** p<0.001 for D-pen (1000 µM) + CuSO$_4$ (10 µM) compared to D-pen (1000 µM) and CuSO$_4$ (10 µM) alone.
Copper chelation by D-penicillamine generates reactive oxygen species that are cytotoxic to human leukemia and breast cancer cells

5.1 Summary

Serum and tumor copper levels are significantly elevated in a variety of malignancies including breast, ovarian, gastric, lung cancer and leukemia. D-penicillamine (D-pen), a copper chelating agent, at low concentrations in the presence of copper generates concentration dependent cytotoxic hydrogen peroxide (H₂O₂). The purpose of these studies was to investigate the in-vitro cytotoxicity, intracellular reactive oxygen species (ROS) generation, and the reduction in intracellular thiol levels due to H₂O₂ and other ROS generated from copper catalyzed D-pen oxidation in human breast cancer cells (BT474, MCF-7) and human leukemia cells (HL-60, HL-60/VCR, HL-60/ADR). D-pen (≤ 400 µM) in the presence of cupric sulfate (10 µM) resulted in concentration dependent cytotoxicity. Catalase was able to completely protect the cells, substantiating the involvement of H₂O₂ in cancer cell cytotoxicity. A linear correlation between the D-pen concentration and the intracellular ROS generated was shown in both breast cancer and leukemia cells. D-pen in the presence of copper also resulted in a reduction in intracellular reduced thiol levels. The H₂O₂-mediated cytotoxicity was greater in leukemia cells compared to breast cancer cells. These results support the hypothesis that D-pen can be employed as a cytotoxic copper chelating agent based on its ROS generating ability.
5.2 Introduction

D-penicillamine (D-pen) is an aminothiol and a potent copper chelating agent [39, 117]. D-pen is currently approved for the treatment of Wilson’s disease and rheumatoid arthritis. Based on its ability to effectively chelate and remove copper, it has also been investigated as an anti-angiogenic agent [4, 8, 52, 117].

Copper has been established as a key co-factor required by a number of pro-angiogenic molecules including fibroblast growth factor (FGF) [433], vascular endothelial growth factor (VEGF) [105], and interleukin-1 [105]. Several in-vitro studies have shown that high copper concentrations facilitate the proliferation of cancer cells [52, 90, 434]. Serum and tumor copper levels have been shown to be significantly elevated in breast cancer [60, 83, 435], lung cancer [69, 72], leukemia [70], and gynecological cancer [71]. Thus, anti-copper therapy has been investigated as an anti-angiogenic strategy for cancer treatment [4, 8] and include copper chelating agents, tetrathiomolybdate [105, 433], clioquinol [102, 436], and D-pen [4, 8, 52].

In the process of chelating copper, D-pen reduces Cu(II) to Cu(I) leading to the generation of hydrogen peroxide (H₂O₂) and other ROS [6, 23, 427]. D-pen has been shown to inhibit human endothelial cell proliferation in-vitro and neovascularization in-vivo [6], and suppress human fibroblast proliferation [142] in the presence of copper. Auto-oxidation of other thiols (cysteamine, homocysteine) in the presence of copper has also been shown to generate H₂O₂ and cause cytotoxicity [425, 437].

Thiol containing compounds are known to be cytotoxic at moderate concentrations but not at low and high concentrations [438, 439]. At low thiol concentration, very small levels of ROS are produced and the cells possess sufficient anti-oxidant capacity to
defend themselves against this ROS stress [152]. At high concentrations, thiols react with
the generated H$_2$O$_2$ and other ROS and thus act as anti-oxidants [152]. It has been shown
that the rate of thiol reaction with H$_2$O$_2$ and superoxide is inversely related to its pKa
[154]. Therefore, thiol toxicity depends on the interplay between the rate of transition
metal (copper, iron) catalyzed thiol oxidation and the rate of thiol reaction with H$_2$O$_2$ and
other ROS generated during thiol oxidation [439]. Thiolate ion plays a critical role in
metal catalyzed thiol oxidation [23]. The pKa of thiol group of D-pen is 7.9, therefore,
the degree of ionization for D-pen at physiological pH would be higher than that
compared to cysteamine (pKa 8.6), and homocysteine (pKa 8.9). In addition, D-pen has
been shown to be oxidized at higher rates in the presence of copper compared to other
thiols (e.g. cysteine, n-acetylcysteine, glutathione) [439]. Thus, we hypothesized that D-
pen at low concentration would generate H$_2$O$_2$ and ROS in the presence of copper and
cause cytotoxicity against cancer cells, in contrast to high D-pen concentrations, wherein
D-pen is more likely to act as an anti-oxidant against excess ROS.

Cancer cells differ from typical cells as they exhibit increased intrinsic ROS stress
due to a number of factors including the oncogenic stimulation, increased metabolic
activity, and mitochondrial malfunction [19, 316, 440]. As a result, cancer cells under
sustained ROS stress conditions tend to heavily utilize adaptation mechanisms and may
exhaust ROS-buffering capacity while normal cells have low levels of ROS stress and
reserve a higher capacity to cope with further oxidative insults [316, 440]. Therefore, the
generation of ROS can be exploited therapeutically in the treatment of cancer [316].
Several anticancer agents currently employed in cancer treatment including
anthracyclines, bleomycin, and cisplatin are known to either generate cellular ROS or to
impair the cellular redox buffering [19, 244].

We have recently investigated the mechanism of copper catalyzed D-pen oxidation
and simultaneous H$_2$O$_2$ production as a function of time, concentration of cupric sulfate
or ferric chloride, temperature, pH, anaerobic conditions, and in the presence of chelators
such as EDTA and bathocuproinedisulfonic acid (BCS) [187]. It was demonstrated that
H$_2$O$_2$ was generated in a concentration dependent manner as a result of D-pen oxidation
in the presence of cupric sulfate. Chelators such as EDTA and BCS were able to inhibit
D-pen oxidation [187]. Additionally, it was shown that the in-vitro copper catalyzed D-
pen oxidation generates H$_2$O$_2$ in a 2:1 mole ratio at low D-pen concentrations (<500 µM)
[187]. Therefore, the purpose of these studies was to, 1) examine the cytotoxicity due to
the ROS generating ability of D-pen, and 2) if the cytotoxicity of D-pen in the presence
of copper correlated to the in-vitro non-cell based molar ratio of D-pen and H$_2$O$_2$. Breast
cancer cell lines differing in HER2 expression (MCF-7 (HER2 negative) and BT474
(HER2 positive)), and leukemia cell lines differing based on their anthracycline
sensitivity (HL-60 (wild type), HL-60/VCR (P-gp) and HL-60/ADR (MRP-1)) were used
in these studies to ascertain differences in H$_2$O$_2$ and ROS cytotoxic effects.

The specific aim of the current study was to assess whether D-pen at low
concentration ($\leq$ 400 µM) in the presence of copper would cause intracellular generation
of ROS and result in cytotoxicity in cancer cells. Indeed, in the current studies, it is
demonstrated that D-pen generates concentration dependent ROS only in the presence of
copper and exhibits concentration dependent cytotoxicity in cancer cells.
5.3 Materials and methods

Cell lines and culture conditions

The human breast cancer cell lines BT474 (HER2 positive and ER+), and MCF-7 (HER2 negative and ER+) and human leukemia cell line, HL-60, were purchased from American Type Cell Culture Collection (ATCC, Rockville, MD). Resistant leukemia cell lines, HL-60/VCR (P-gp) and HL-60/ADR (MRP-1) were kindly provided by Dr Baer (Roswell Park Cancer Institute, Buffalo, NY). Cells were routinely cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% Fetal Bovine Serum (FBS) (ATCC, Rockville, MD) and maintained at 37°C in a humidified 5% CO₂ incubator. Plasmocin (5 µg/mL) (InvivoGen, San Diego, CA) was added to the cell culture media as a prophylactic measure to prevent mycoplasma contamination. Cell viability was regularly determined by trypan blue exclusion test.

Reagents

D-penicillamine (D-pen), hydrogen peroxide (H₂O₂) 30% w/w and cupric sulfate (CuSO₄), catalase (2860 U/mg), glutathione, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), EDTA, were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Coomassie Plus Protein Assay kit was purchased from Pierce Biotech Inc. (Rockford, IL). Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburg, PA). Cell cytotoxicity was assessed with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) assay, phosphate buffer saline (PBS), pH 7.4 cell culture grade was
purchased from ATCC (Rockville, MD), 2’-7’-dichlorodihydrofluorescein diacetate (H₂DCFDA), and hydroethidine were purchased from Invitrogen Inc (Carlsbad, CA).

**Assessment of cell viability**

Cell viability was measured using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyloxazolium bromide (MTT) assay. The adherent breast cancer cells (MCF-7 and BT474) were seeded at an initial concentration of 3×10⁴ cells/well 24 h prior to the commencement of the experiments to allow them to attach, while the suspension leukemia cells, HL-60, HL-60/VCR and HL-60/ADR were seeded at 3×10⁴ cells/well on the same day of the experiment in a 96 well plate. Cells were then treated with H₂O₂ (1-200 µM), D-pen (1-400 µM), cupric sulfate (1-50 µM), D-pen (1-400 µM) + cupric sulfate (10 µM), and D-pen (1-400 µM) + cupric sulfate (10 µM) + catalase (500 U/mL). The MTT assay was performed at 48 h and absorbance was measured with the microplate reader (Biotek ELx 800, Biotek Instruments, Winooski, VT) at a wavelength of 595 nm. Data is reported as cell viability (% control) and corresponds to the percent viable cells compared to untreated cells.

**Determination of intracellular reactive oxygen species (ROS)**

2’-7’-dichlorodihydrofluorescein diacetate (H₂DCFDA) was used as an indicator of intracellular ROS generation. H₂DCFDA is a cell permeable probe, it enters the cell and is deacetylated to a non-fluorescent product, 2’-7’-dichlorodihydrofluorescein (H₂DCF) by cellular esterases and is oxidized by ROS to a fluorescent product, 2’-7’-dichlorofluorescein (DCF). MCF-7 and HL-60 cells were loaded with H₂DCFDA and
were seeded in a 96 well plate at a concentration of $3 \times 10^4$ cells/well. Briefly, cells were incubated with PBS buffer containing 5 µM H$_2$DCFDA (dissolved in DMSO) for 30 min at 37ºC. H$_2$DCFDA was then removed and cells were washed twice with fresh PBS to remove excess H$_2$DCFDA. Cells were then incubated with either drug in media or media alone (positive control). Non-H$_2$DCFDA loaded cells were used as negative control. The fluorescence of control and sample wells was recorded at excitation 485 ± 20 nm, emission 530 ± 25 nm with Biotek FL600 (Biotek Instruments, Winooski, VT) at 30, 60, and 90 min. Then data is reported as DCF fluorescence (fold increase vs. control) corresponding to the increase of fluorescence associated with the sample cells compared to that of untreated cells.

**Intracellular levels of thiols (-SH)**

The intracellular reduced glutathione levels were measured with 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB). Briefly, HL-60 cells ($5 \times 10^6$) were seeded in a 12 well plate and incubated for 4 and 48 h with or without D-pen (50 and 200 µM) plus cupric sulfate (10 µM). Cells were collected, washed with ice-cold PBS buffer and then suspended in pH 7.4 lysis buffer comprised of 25 mM Tris-HCl + 1 mM EDTA + 0.5% Triton X-405. Cells were then lysed in the buffer with a homogenizer (Tissue Tearor, Model 985370 Variable Speed, Biospec Products Inc, Bartlesville, OK). 25 µL of standard (glutathione) or samples (cell lysate) + 25 µL DTNB (0.4 mg/mL) + 150 µL of 50 mM HEPES buffer, pH 7.4 with 5 mM EDTA were incubated for 10 min at room temperature and read at 405 nm on a microplate reader.
Protein assay

The total protein content of the cell lysate was analyzed with Coomassie Plus® Bradford Protein Assay using Glutathione as a standard.

Statistical analysis

Data are represented as the mean ± standard error (SE). Each experimental group consisted of n = 6 and experiments were repeated two times. Statistical analysis was performed with two-way analysis of variance (ANOVA) followed by Bonferroni post test where significance was set at p<0.05 with GraphPad Prism® 4 Software (GraphPad software Inc. San Diego, CA). The IC_{50} was calculated with GraphPad Prism® non-linear regression program.
5.4 Results

In-vitro cytotoxicity of D-pen plus cupric sulfate and H2O2

Leukemia cells

HL-60, HL-60/VCR and HL-60/ADR cells were treated with H2O2 (1, 10, 25, 50, 100 and 200 µM) and D-pen (1, 50, 100, 150, 200 and 400 µM) plus cupric sulfate (10 µM). Cells were also treated with cupric sulfate alone as control (0.1, 1, 10, 25 and 50 µM). The concentrations of H2O2 and D-pen were chosen based on our previous in-vitro non-cell based studies [187], where it was determined that at low D-pen concentrations (< 500 µM) copper catalyzed D-pen oxidation resulted in H2O2 generation in the molar ratio of 2:1 (D-pen: H2O2). Therefore, we wanted to compare the cytotoxicity in these present studies using H2O2 alone and versus H2O2 generated from D-pen plus cupric sulfate wherein the theoretical H2O2 generated corresponded to a 2:1 molar ratio of D-pen to H2O2.

Figure 5.1 shows the concentration dependent cytotoxicity of H2O2 alone (Figure 5.1a) and D-pen plus cupric sulfate (Figure 5.1b), respectively, in leukemia cells (HL-60, HL-60/VCR and HL-60/ADR). HL-60 and HL-60/VCR cells were highly sensitive to H2O2 with IC50 of 20 ± 1.0 µM and 31.5 ± 1.1 µM, respectively. The IC50 of D-pen plus cupric sulfate in these two leukemia cell lines was 102.1 ± 1.0 µM and 123.7 ± 1.0 µM, which was approximately 5-fold and 4-fold more than corresponding IC50 of H2O2 alone in these two cell lines. The HL-60/ADR cells were shown to be less sensitive to the cytotoxic effects of both H2O2 and D-pen plus cupric sulfate with IC50 of H2O2 of 162.5 ± 1.1 µM, while the IC50 of D-pen plus cupric sulfate was beyond the concentration range of D-pen used in the present studies. Cupric sulfate alone (0.1-50 µM) did not result in
any appreciable loss in cell viability (data not shown). The purpose of employing the resistant leukemia cells, HL-60/VCR (p-gp) and HL-60/ADR (MRP-1), was to compare the effect of H₂O₂ and D-pen plus cupric sulfate cytotoxicity on these cells versus the HL-60 cells. The order of sensitivity of leukemia cells to both H₂O₂ and D-pen plus cupric sulfate cytotoxicity was HL-60 > HL-60/VCR > HL-60/ADR and correlated with the sensitivity of these cells to anti-cancer agents. These results support the relationship between ROS and drug resistance [19].

**Breast cancer cells**

The cytotoxicity of H₂O₂ and D-pen plus cupric sulfate in breast cancer cells is shown in Figure 5.2. The IC₅₀ of H₂O₂ was 115.5 ± 1.6 µM and 96.1 ± 1.1 µM for MCF-7 and BT474 breast cancer cells (Figure 5.2a). In comparison, as shown in Figure 5.2b, the IC₅₀ of D-pen plus cupric sulfate was 246.1 ± 1.1 µM and 287.4 ± 1.1 µM for MCF-7 and BT474 cells, respectively. The IC₅₀ of H₂O₂ was approximately 2-fold and 3-fold lower than the IC₅₀ of D-pen plus cupric sulfate in MCF-7 and BT474 cells, respectively.

**Catalase protects MCF-7 and HL-60 cells from D-pen plus cupric sulfate cytotoxicity**

Figure 5.3 demonstrates that catalase (500 U/mL) was able to completely protect both MCF-7 (Figure 5.3a) and HL-60 cells (Figure 5.3b) from D-pen plus cupric sulfate cytotoxicity.
Intracellular reactive oxygen species (ROS) generation in MCF-7 and HL-60 cells

Figure 5.4 shows that intracellular ROS was produced in both MCF-7 (Figure 5.4a) and HL-60 cells (Figure 5.4b) in the presence of D-pen (200 µM) plus cupric sulfate (10 µM). D-pen alone (200 µM) or cupric sulfate alone (10 µM) failed to produce any ROS, supporting the fact that copper interaction with D-pen is essential and is responsible for the generation of ROS. Further, a 4-fold increase in ROS production was shown in D-pen plus cupric sulfate treated HL-60 cells vs. H2DCFDA loaded untreated HL-60 control cells compared to a 2-fold increase in ROS production for D-pen plus cupric sulfate treatment of MCF-7 vs. H2DCFDA loaded untreated MCF-7 control cells. In addition, the presence of catalase (500 U/mL) with D-pen plus cupric sulfate completely inhibited ROS generation, demonstrating that H2O2 was the major ROS generated. As shown in Figure 5.4 (A and B), when H2O2 alone (100 µM) was incubated with HL-60 and MCF-7 cells, 5-fold greater ROS was generated in each cell line (compared to D-pen plus cupric sulfate) which agrees very well with the 5-fold reduced IC50 calculated for H2O2 compared to D-pen plus cupric sulfate in HL-60 cells.

In separate cell studies, hydroethidine was used as a quantitative marker for intracellular superoxide anion production. Hydroethidine is freely permeable into cells and can be directly oxidized to a fluorescent compound by intracellular superoxide anion. These studies showed that there was no statistical difference in the intracellular superoxide anion at up to 90 min post-incubation between all treatment and control groups (data not shown), suggesting that the observed cytotoxicity was not caused by superoxide anion but by hydrogen peroxide.
Figure 5.5 shows the linear relationship between D-pen (50, 100 and 200 µM) in the presence of cupric sulfate and the increase in the DCF fluorescence (indicator of intracellular ROS) in both MCF-7 (Figure 5.5a) and HL-60 (Figure 5.5b). The correlation between D-pen concentration and DCF fluorescence was shown to increase over time, as indicated by the $r^2$ values becoming higher over time and closer to $r^2 = 1$.

**Intracellular reduced thiols**

Figure 5.6 shows the levels of intracellular thiols (mainly glutathione) in HL-60 cells after incubation with D-pen (50 and 200 µM) plus cupric sulfate (10 µM) for 4 and 48 h. The levels of reduced thiols was shown to be significantly decreased ($p<0.05$) after incubation with D-pen (200 µM) plus cupric sulfate compared to control at 4 h. After 48 h incubation, intracellular thiol levels were significantly lower ($p<0.01$) after D-pen plus cupric sulfate incubation compared to control.
5.5 Discussion

Therapeutic selectivity (cancer cells versus normal cells) and drug resistance are two important factors that greatly affect the chances for success of cancer therapy. Therefore, it is important to explore key biochemical differences between cancer cells and normal cells and develop strategies that could potentially use these differences to improve therapeutic selectivity and/or overcome drug resistance [19]. Oxidative stress occurs when the production of ROS exceeds their removal with anti-oxidant compounds or enzymes [440]. Cellular defenses against ROS include anti-oxidants scavengers such as glutathione, ascorbate, thioredoxin and enzymes like catalase, superoxide dismutase, and glutathione peroxidase [19]. Strong evidence from studies in literature suggests that cancer cells differ from normal cells in being under persistent ROS stress [208, 441-443]. Additionally, it has been suggested that the ability of a cell to defend itself against ROS could be associated with resistance against chemotherapy [203, 316]. Several chemically diverse compounds have been shown to generate ROS and exhibited anti-cancer activity alone or in combination with other chemotherapeutic agents [340, 444, 445].

D-pen is a potent copper chelating agent that has been investigated in the recent years as a potential anti-angiogenic agent based on its efficient copper chelating and removing abilities [8, 86]. However, D-pen interaction with copper during chelation has been shown to generate H$_2$O$_2$ and other ROS [23, 187, 427]. A few reports have also shown that D-pen in the presence of copper causes the inhibition of human fibroblasts proliferation [142], and inhibition of human endothelial cells proliferation *in-vitro* and their *in-vivo* neovascularization [6].
Starkebaum et al. [23] proposed a free radical mechanism of copper catalyzed D-pen oxidation to D-pen disulfide and the subsequent generation of H$_2$O$_2$. The mechanism involves an initial reduction of Cu(II) to Cu(I) by D-pen. This is followed by the generation of superoxide anion and finally of H$_2$O$_2$ during the spontaneous oxidation of Cu(I) to Cu(II). We have recently further investigated the mechanism proposed by Starkebaum et al. as a function of time, concentration of cupric sulfate or ferric chloride, temperature, pH, anaerobic conditions and in the presence of chelators [187]. We have shown with a novel HPLC assay that H$_2$O$_2$ was indeed generated in a concentration dependent fashion as a result of D-pen oxidation in the presence of cupric sulfate.

In the present studies, it was hypothesized that D-pen at low concentrations (≤ 400 µM) in the presence of cupric sulfate would generate cellular H$_2$O$_2$ and ROS and result in cytotoxicity. The anthracycline sensitive HL-60 and the mildly resistance HL-60/VCR leukemia cells were found to be highly susceptible to D-pen plus cupric sulfate cytotoxicity. The IC$_{50}$ of H$_2$O$_2$ alone was approximately 5-fold and 4-fold lower compared to D-pen plus cupric sulfate in HL-60 and HL-60/VCR cells, respectively. The ROS assay showed that approximately 5-fold higher cellular ROS was generated in HL-60 cells due to incubation with H$_2$O$_2$ alone compared to D-pen plus cupric sulfate, which was remarkably similar to the measured 5-fold higher cytotoxicity with H$_2$O$_2$ alone compared to D-pen. H$_2$O$_2$ was also established to be the major ROS species, as the presence of catalase completely inhibited D-pen plus cupric sulfate cytotoxicity. Breast cancer cells (MCF-7 and BT474) were less sensitive to both H$_2$O$_2$ and D-pen cytotoxicity, with 5-fold higher IC$_{50}$ compared to leukemia cells. The IC$_{50}$ of H$_2$O$_2$ was
approximately 2-fold and 3-fold lower compared to D-pen plus cupric sulfate in MCF-7 and BT474 cells, respectively.

In conclusion, these studies demonstrated that low concentration of D-pen (≤ 400 µM) in the presence copper resulted in a concentration dependent H\textsubscript{2}O\textsubscript{2}-mediated cytotoxicity in both breast cancer and leukemia cells. D-pen in the presence of copper also was shown to generate concentration dependent cellular ROS and to decrease cellular reduced thiol content in cancer cells. Further, it was shown that leukemia cells were highly sensitive to D-pen plus cupric sulfate cytotoxicity. A four-fold increase in ROS generation was shown in leukemia cells after D-pen plus cupric sulfate treatment compared to untreated control. It was demonstrated that D-pen has effective ROS generating ability in the presence of copper. Since copper levels are significantly elevated in the serum and tumor tissue in a variety of malignancies, these findings provides a novel and exciting opportunity to exploit D-pen as an anti-cancer agent having both anti-angiogenic and cytotoxic mechanism of action.

**Abbreviations:**

D-pen: D-penicillamine  
CuSO\textsubscript{4}: cupric sulfate  
ROS: reactive oxygen species  
H\textsubscript{2}O\textsubscript{2}: Hydrogen peroxide  
H\textsubscript{2}DCFDA: 2′-7′-dichlorodihydrofluorescein diacetate  
DTNB: 5,5′-dithiobis (2-nitrobenzoic acid)  
PBS: phosphate buffer saline
GSH: reduced glutathione

P-gp: P-glycoprotein

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a) HL-60: 20.0 ± 1.0 µM  
HL-60/VCR: 31.5 ± 1.1 µM  
HL-60/ADR: 162.5 ± 1.1 µM

b) HL-60: 102.1 ± 1.0 µM  
HL-60/VCR: 123.7 ± 1.0 µM  
HL-60/ADR: ND

H2O2 (µM) vs. Cell viability (% control)
Figure 5.1 The concentration dependent *in-vitro* cytotoxicity of H$_2$O$_2$ alone compared to D-pen plus cupric sulfate in HL-60, HL-60/VCR and HL-60/ADR leukemia cells. a) H$_2$O$_2$ alone and b) D-pen plus cupric sulfate.

Cells ($3 \times 10^4$ cells/well) were treated with increasing concentrations of H$_2$O$_2$ alone and D-pen plus cupric sulfate (10 µM) for 48 h in media. Cell viability was determined with the MTT assay. Absorbance was measured at 595 nm and adjusted to the absorbance of untreated cells. Data represent mean ± SE; n = 12 (two independent experiments performed in n = 6). Inhibitory concentrations (IC$_{50}$) were reported as the mean ± SE in the figure legend.
a) MCF-7: 115.5 ± 1.6 µM
BT474: 96.1 ± 1.1 µM

b) MCF-7: 246.1 ± 1.1 µM
BT474: 287.4 ± 1.1 µM
Figure 5.2 The concentration dependent *in-vitro* cytotoxicity of H$_2$O$_2$ alone compared to D-pen plus cupric sulfate in MCF-7 and BT474 breast cancer cells.

a) H$_2$O$_2$ alone. b) D-pen plus cupric sulfate.

Cells ($3 \times 10^4$ cells/well) were treated with increasing concentration of H$_2$O$_2$ and D-pen plus cupric sulfate for 48 h in media. Cell viability was determined with MTT assay. Absorbance was measured at 595 nm and adjusted to the absorbance of untreated cells. Data represent mean ± SE; n = 12 (two independent experiments performed in n = 6). Inhibitory concentration (IC$_{50}$) was reported as mean ± SE.
a)

![Graph a)

b)

![Graph b]}
Figure 5.3 Catalase inhibits the D-pen plus cupric sulfate cytotoxicity in breast cancer and leukemia cells.

a) MCF-7 cells b) HL-60 cells.

Cells (3×10^4/well) were treated with D-pen, D-pen plus cupric sulfate (10 µM) and D-pen plus cupric sulfate (10 µM) plus catalase (500 U/mL) for 48 h in media. Cell viability was determined with MTT assay. Absorbance was measured at 595 nm and adjusted to the absorbance of untreated cells. Data represent the mean ± SE; n = 12 (two independent experiments performed in n = 6). *p<0.05 and ***p<0.001 for D-pen plus cupric sulfate compared to D-pen alone and D-pen plus cupric sulfate plus catalase.
Figure 5.4 D-pen in the presence of cupric sulfate generates intracellular ROS in breast cancer and leukemia cells.

a) MCF-7 cells (3×10⁴/well) were loaded with ROS probe H₂DCFDA (5 µM). *p<0.05 for D-pen plus cupric sulfate (10 µM) compared to D-pen alone. ***p<0.001 for H₂O₂ compared to D-pen alone.
b) HL-60 cells (3×10⁴/well) were loaded with ROS probe H₂DCFDA (5 µM). **p<0.01 for D-pen plus cupric sulfate compared to D-pen alone. ***p<0.001 for H₂O₂ compared to D-pen alone. Cells were incubated without (positive control) or with H₂O₂, H₂O₂ plus catalase, cupric sulfate, D-pen, D-pen plus cupric sulfate, D-pen plus cupric sulfate plus catalase in media. Unloaded cells were used as negative control. The cell associated fluorescence was measured at 30, 60, 90 min at excitation and emission of 485 ± 20 nm and 530 ± 25 nm, respectively. Data are reported as the DCF fluorescence (fold increase versus control). Data represent mean ± SE; n = 12 (two independent experiments performed in n = 6).
Figure 5.5 The correlation between D-pen concentration and the intracellular ROS generation in breast cancer and leukemia cells. a) MCF-7 cells (3×10⁴/well) were loaded with ROS probe H₂DCFDA (5 µM). b) HL-60 cells (3×10⁴/well) were loaded with ROS probe H₂DCFDA (5 µM).
Cells were incubated without (positive control) or with 50, 100 and 200 µM D-pen plus cupric sulfate (10µM) in media. Unloaded cells were used as negative control. The cell associated fluorescence was measured at 30, 60, 90 and 120 min at excitation and emission of 485 ± 20 nm and 530 ± 25 nm, respectively. Data are reported as Fluorescence Units or the raw fluorescence of DCF. Data represent the mean ± SE; n = 12 (two independent experiments performed in n = 6).
Figure 5.6 D-pen in the presence of cupric sulfate causes the reduction in intracellular thiol levels in leukemia cells. HL-60 cells (5×10^6/well) were plated in a 12 well plate and treated with D-pen plus cupric sulfate (10 µM) for 4 and 48 h. Cells were counted with trypan blue assay, washed with PBS buffer and homogenized in lysis buffer. Cells were centrifuged and the supernatant was used for total intracellular protein and thiol assay. Data represent the mean ± SE; n = 6 (two independent experiments performed in triplicate). *p<0.05 and **p<0.01 for D-pen (200 µM) plus cupric sulfate (10 µM) compared to control.
Supplemental Study (not included in the manuscript published in Free Radical Biology and Medicine)

Intracellular superoxide radical generation

Hydroethidine from Invitrogen (Carlsbad, CA) was used as a quantitative marker for superoxide anion. Hydroethidine is freely permeable into cells and can be directly oxidized by superoxide anion produced inside the cells [446]. It has been reported that hydrogen peroxide, hydroxyl radical, nitric oxide derived oxidants do not react with hydroethidine [447]. Therefore, the oxidation of hydroethidine was employed as the intracellular superoxide anion marker. Briefly, HL-60 cells were incubated with hydroethidine (10 µM) for 30 min at 37ºC. Cells were plated at 30,000/well in a 96 well plate. Cells were incubated with media alone (control) or D-pen (200 µM), CuSO₄ (10 µM), D-pen (200 µM) + CuSO₄ (10 µM), D-pen (200 µM) + CuSO₄ (10 µM) + SOD (10 U), and H₂O₂ (100 µM). Cells not treated with hydroethidine were used as negative control. Fluorescence was read at 30, 60 and 90 min at 485 ± 10 nm and 610 ± 20 nm. Superoxide anion being a charged species cannot enter the cell. Therefore, the intracellular oxidation of Hydroethidine would exclusively detect the intracellular generated superoxide anion. As shown in the Figure below there was no statistical difference after incubation of D-pen + cupric sulfate compared to control cells at any time points indicating that superoxide anion did not play a role in the of the intracellular ROS being produced from D-pen + cupric sulfate incubation with breast cancer and leukemia cells. Xanthine and Xanthine oxidase was used as a positive control for the superoxide production (not shown in the figure).
Figure 5.7 Intracellular superoxide anion generations in HL-60 cells. Cells were incubated in media alone (control), cupric sulfate, D-pen, D-pen + cupric sulfate, D-pen + cupric sulfate + superoxide dismutase, and hydrogen peroxide.
Chapter 6

Enhanced intracellular delivery of the reactive oxygen species (ROS)-generating copper chelator D-penicillamine via a novel gelatin-D-penicillamine conjugate

6.1 Summary

D-penicillamine (D-pen) is an established copper chelator. We have recently shown that the copper catalyzed D-pen oxidation generates concentration dependent hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Additionally, D-pen co-incubated with cupric sulfate resulted in cytotoxicity in human leukemia and breast cancer cells due to the extracellular generation of reactive oxygen species (ROS). The inherent physicochemical properties of D-pen such as its short \textit{in-vivo} half life, low partition coefficient and rapid metal catalyzed oxidation limit its intracellular uptake and the potential utility as an anti-cancer agent \textit{in-vivo}. Therefore, to enhance the intracellular delivery and to protect the thiol moiety of D-pen, we designed, synthesized, and evaluated a novel gelatin-D-pen conjugate. D-pen was covalently coupled to gelatin with a biologically reversible disulfide bond with the aid of a heterobifunctional crosslinker, (N-succinimidyl-3-(2-pyridyldithio)-propionate) (SPDP). Additionally, fluorescein labeled gelatin-D-pen conjugate was synthesized for cell uptake studies. D-pen alone was shown not to enter leukemia cells. In contrast, the qualitative intracellular uptake of the conjugate in human leukemia cells (HL-60) was shown with confocal microscopy. The conjugate exhibited slow cell uptake (over the period of 48 to 72 h). The gelatin amino groups were modified with SPDP and conjugated with D-pen. A novel HPLC assay was developed to simultaneously quantify both D-pen and glutathione in a single run. The conjugate was shown to completely
release D-pen in the presence of glutathione (1 mM) in approximately 3 h in PBS buffer, pH 7.4. The gelatin-D-pen conjugate resulted in significantly greater cytotoxicity compared to free D-pen, gelatin alone and a physical mixture of gelatin and D-pen in human leukemia cells. Further studies are warranted to assess the potential of D-pen conjugate in the delivery of D-pen as a ROS generating anti-cancer agent.
6.2 Introduction

D-penicillamine (D-pen) is a copper chelating agent that is currently approved by the FDA for the treatment of Wilson’s disease and rheumatoid arthritis. In recent years there has been immense interest in the use of copper chelators including D-pen as anti-angiogenic agents [4, 8]. This is due to the now established role of copper in angiogenesis. Copper is known to act as a co-factor for a variety of important endogenous pro-angiogenic compounds such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [4]. Thus, it has been proposed that the copper chelation by D-pen could lead to an anti-angiogenic effect. It is also known that the interaction of D-pen with copper during chelation leads to the generation of hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS) [8, 23, 24]. We have recently both qualitatively and quantitatively confirmed that the copper catalyzed D-pen oxidation generated concentration dependent H₂O₂ [187]. In addition, we have shown that D-pen co-incubated with cupric sulfate resulted in concentration dependent cytotoxicity in both human breast cancer (MCF-7, BT474) and leukemia cells (HL-60, HL-60/VCR) in culture [190].

As copper levels have been reported to be significantly elevated in the serum [60, 70] and the tumor tissue [9, 59, 84] of cancer patients, we believe that our studies in the investigation of D-pen cytotoxicity in the presence of copper have significant potential. Therefore, we hypothesized that novel formulation strategies that could increase the intracellular delivery of D-pen would potentially lead to the use of D-pen as a dual anti-cancer agent via, 1) cytotoxicity, through the intracellular generation of H₂O₂ and other ROS, 2) anti-angiogenesis, through the well known copper chelation process. The
physicochemical properties of D-pen would limit the anti-cancer efficacy of D-pen \textit{in-vivo}. These limiting properties include, \textit{i}) extreme hydrophilicity (Log P: -0.39) \cite{448} thus limiting its intracellular uptake in cancer cells \cite{114, 123} \textit{ii}) rapid elimination from the blood exhibiting biphasic kinetics \cite{116, 449}, and \textit{iii}) sensitivity to oxidation. The thiol group of D-pen is critical to both copper chelation and H$_2$O$_2$ generation; however, D-pen is prone to oxidation resulting in inactive D-pen disulfide or mixed disulfides. Therefore, the efficacy of D-pen as an anti-cancer agent would depend on its successful delivery to cancer cells in its reduced form or a modified form which could then be reversibly converted intracellularly to bioactive D-pen.

We have previously reported on a novel D-pen-nanoparticle formulation for metal chelation therapy \cite{450}. Also, Chvapil et al. recently reported on the synthesis of a hexyl-D-pen-ester that converted D-pen to a more lipophilic form (Log P: 1.61), but resulted in very slow D-pen release \cite{448, 451}. The ester prodrug strategy for the D-pen delivery would potentially have other problems such as \textit{i}) the lack of protection of the critical thiol group, and \textit{ii}) the slow rate of D-pen release from the lipophilic ester prodrug. This could result in less than effective free D-pen concentrations \textit{in-vivo}. To overcome these potential problems, we designed a novel conjugate of D-pen wherein D-pen was linked to a macromolecular polymer via a biologically reversible disulfide bond. In the recent years, the covalent conjugation of low molecular weight drugs with soluble macromolecular carriers (peptides, protein and polymers) has been the focus of considerable research \cite{390, 452}.

Gelatin is a partially hydrolyzed form of collagen \cite{452}. Gelatin has been used in oral delivery technologies such as hard and soft capsules and is recently being examined
as a carrier in novel drug delivery [384]. Gelatin has been reported to be both biocompatible and biodegradable [384, 389]. These and other desirable physiochemical properties of gelatin make it a suitable carrier for the delivery of genes [453-455], chemotherapeutic agents formulated as: conjugates [390, 392, 395, 452], as nanoparticles [384, 388, 396, 456, 457], as complex coacervates [454], and as microspheres [384, 455]. Both the carboxyl and the amino groups of gelatin have been modified to directly conjugate drugs [389, 392].

In the present studies, we describe the synthesis of a soluble gelatin-D-pen conjugate through the modification of gelatin with the aid of a heterobifunctional cross-linker, sulfosuccinimidyl 6-(3´(2-pyridyldithio)-propionamido) hexanoate (sulfo-LC-SPDP) (water soluble derivative of SPDP). Gelatin was coupled to D-pen with the aid of SPDP to form a potentially biologically reversible disulfide bond. The disulfide bond was an attractive strategy for D-pen delivery due to, i) enhanced protection of the thiol group of D-pen from oxidation before it reaches the site of action, ii) its potential for intracellular reversibility (due to the presence of ~1-11 mM of glutathione inside the cells) [371, 458, 459] and iii) its reported relative stability in plasma [460].

The overall objectives of the present studies were to: 1) synthesize and characterize a novel disulfide gelatin-D-pen conjugate, 2) evaluate the \textit{in-vitro} release of D-pen from the gelatin-D-pen conjugate as a function of glutathione concentration, time of incubation with glutathione and pH, 3) evaluate the qualitative intracellular uptake of fluorescein labeled gelatin-D-pen conjugate in human leukemia cells (HL-60), and 4) investigate the \textit{in-vitro} cytotoxicity of gelatin-D-pen conjugate in HL-60 cells compared to free D-pen, gelatin alone and the physical mixture of gelatin with D-pen.
6.3 Materials and methods

Materials

Type B gelatin (75 bloomstrength) with 100-115 mmol of carboxylic acid per 100 g of protein, an isoelectric point of 4.7-5.2, and an average molecular weight of 20,000-25,000 Da was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-penicillamine (D-pen), D-penicillamine disulfide, Glutathione, Glutathione disulfide, Dithiothreitol (DTT) was also purchased from Sigma-Aldrich. Sulfosuccinimidyl 6-(3’(2-pyridyldithio)-propionamido) hexanoate (Sulfo-LC-SPDP), 2,4,6-trinitrobenzene sulfonic acid (TNBS) reagent, N-hydroxysuccinimide-Fluorescein (NHS-Fluorescein), M-PER mammalian cell extraction reagent were purchased from Pierce Biotech Inc. (Rockford, IL). Acetonitrile, o-phosphoric acid (85%) were purchased from Fisher Sci. (Pittsburg, PA). Microcon® (YM-10, MWCO: 10 kDa) centrifugal filter devices were purchased from Millipore (Billerica, MA). All aqueous solutions were prepared in deionized distilled water (MilliQ®, Millipore Inc.).

Cell lines and culture conditions

The human leukemia cell line (HL-60) was purchased from American Type Cell Culture Collection (ATCC, Rockville, MD). Cells were routinely cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% Fetal Bovine Serum (FBS) (ATCC, Rockville, MD) and maintained at 37°C in a humidified 5% CO₂ incubator. Plasmocin (5 µg/mL) (InvivoGen, San Diego, CA) was added to the cell culture media as a prophylactic measure to prevent
mycoplasma contamination. Cell viability was regularly determined by trypan blue dye (0.4% in phosphate buffered saline) (ATCC, Rockville, MD).

**Synthesis and purification of the gelatin-D-pen conjugate**

A heterobifunctional cross-linker (sulfo-LC-SPDP) was employed to conjugate D-pen with gelatin. Sulfo-LC-SPDP has an amine reactive N-hydroxysuccinimide (NHS) and a thiol reactive 2-pyridylthio group. The conjugate synthesis was performed in two steps. Briefly, gelatin (2 mg/mL) was incubated with SPDP for 2 h at room temperature in PBS, pH 7.4. After incubation, excess SPDP was separated from gelatin with microcon centrifugation devices (MWCO: 10 kDa). As described below, the pyridine-2-thione and the TNBS assays were performed to determine the degree of SPDP modification of gelatin. The SPDP-modified gelatin was then incubated with excess D-pen. The gelatin-D-pen conjugate was separated from the unconjugated D-pen with microcon centrifugal devices. The HPLC assay as described below was performed on the filtrate and retentate to determine the amount of D-pen conjugated to gelatin.

The complete and efficient separation of gelatin from unreacted SPDP or D-pen was performed with microcon centrifugation filter devices (MWCO: 10 kDa). Briefly, 500 µL of reaction mixture was added to the microcon, the mixture was centrifuged at 14,000 g for 30 min at room temperature. The filtrate was then collected and the retentate was washed twice with 400 µL of fresh PBS buffer. The retentate was finally collected by centrifuging at 3,000 g for 5 min. Gelatin and D-pen concentration both in the filtrate and the retentate were quantified.
Synthesis and purification of the fluorescein labeled gelatin-D-pen conjugate

The fluorescent labeling of the conjugate was performed with the aid of an amine reactive fluorescent probe, NHS-fluorescein. Briefly, NHS-fluorescein was added to gelatin in a 1:1 mole ratio and the mixture was incubated with constant shaking for 1 h at room temperature. The unreacted free fluorescein was separated from fluorescein-gelatin using the microcon centrifugal filter devices. The fluorescein-gelatin was washed three times with PBS, pH 7.4 until the free fluorescein was completely removed. The fluorescein-gelatin was then used to synthesize the conjugate as described above.

Characterization of the gelatin-D-pen conjugate

Amino content determination

2, 4, 6-trinitrobenezene sulfonylic acid (TNBS) is a rapid and sensitive assay reagent employed for the determination of free amino groups. Briefly, 50 µL of 0.01% (w/v) TNBS was added to 100 µL of either gelatin or SPDP-modified gelatin in PBS buffer, pH 7.4 and was incubated for 2 h at 37°C. Twenty five (25) µL of 1 N HCl was added to stop the reaction. The resulting absorbance was recorded at 335 nm with the Synergy™ 2 Multi-Detection Microplate Reader (Biotek, Winooski, VT). A standard curve for amino groups was generated using known concentrations of D-pen. The primary amino group was expressed as the amount of TNBS reactive amino groups in 1 g of gelatin. The % amino groups conjugated were calculated as follows: % amino groups conjugated = (amino group of SPDP modified gelatin / amino groups in gelatin alone) × 100%. 
**Gelatin determination**

The gelatin concentration was analyzed using the Coomassie Plus assay. Briefly, 10 µL of standard or sample was added to 150 µL of Coomassie Plus Protein Assay Reagent (Pierce Biotech, Rockford, IL), mixed and incubated for 10 min at room temperature and the absorbance was read at 595 nm with the Synergy™ 2.

**Determination of the level of SPDP modification**

The degree of SPDP modification of gelatin was determined by quantifying the release of pyridine-2-thione group after exposure of the SPDP-modified gelatin with DTT. Briefly, 100 µL purified SPDP-modified gelatin was diluted to 1 mL with PBS buffer, pH 7.4. Ten (10) µL DTT (15 mg/mL) was added and samples were incubated for exactly 15 min and the absorbance was recorded at 343 nm with the Synergy™ 2. The molar ratio of SPDP to gelatin modification as follows = (ΔA × 8080) × (Average M.W. of gelatin × mg/mL of gelatin), where the value 8080 reflects the extinction coefficient (8.08 x 10³ M⁻¹ cm⁻¹) for pyridine-2-thione at 343 nm.

**HPLC determination of D-pen and glutathione**

D-pen and glutathione were simultaneously analyzed with a modification of our previously reported HPLC method [187]. Briefly, a HPLC system (Finnigan™ Surveyor System, Thermo Electron Corp., San Jose, CA) was used and the data was analyzed with the ChromQuest™ software version 4.2. The mobile phase employed was a 50:50% v/v mixture of solvent A (50 mM phosphoric acid) and solvent B (50 mM phosphoric acid + 5% acetonitrile), both adjusted to pH 2.5, and pumped at a flow rate of 1 mL/min. Both
D-pen and glutathione were detected by UV absorption at 214 nm with retention time of 3.1 ± 0.01 and 2.79 ± 0.01 min, respectively. Sample concentrations (µM) were obtained from the regression line of peak area versus standard sample concentration (µM). These were calculated using a ten-point calibration curve of D-pen and glutathione dissolved in PBS buffer, pH 7.4.

**D-pen release from the conjugate under reducing conditions**

To evaluate the amount of D-pen released from the conjugate under reducing conditions, the gelatin-D-pen conjugate was incubated in PBS buffer, pH 6.2 and 7.4 with increasing concentrations of glutathione (0, 0.1, 1 and 10 mM) for 2 h at 37°C. Additionally, D-pen release from the conjugate at various time was also determined in PBS at pH 6.2 and 7.4 after incubation in the presence of 1 mM of glutathione at 37°C.

**Cancer cell uptake studies**

**Quantitative cell uptake of free D-pen**

The quantitative cell uptake of free D-pen was investigated in HL-60 cells. Briefly, D-pen (100 µM) was incubated with HL-60 (1×10^6) cells in PBS, pH 7.4 at 37°C in a 5% CO₂ incubator. Cells were incubated for 1-4 h. At pre-determined time the cells were separated by centrifugation and the supernatant was analyzed for the remaining concentration of D-pen as present as either free D-pen or D-pen disulfide using the HPLC assay previously described [187].
Intracellular uptake of the conjugate

The uptake of the fluorescein-gelatin-D-pen conjugate was determined qualitatively using confocal microscopy. HL-60 cells (1×10^5) were suspended in a 24 well plate in the medium alone (control), or containing either fluorescein-gelatin (fluorescein labeled gelatin) (control) or fluorescein-gelatin-D-pen conjugate. At 4, 24, 48 72 h post-treatment, cells were transferred to a centrifuge tube, washed twice with PBS and resuspended in 200 µL fresh media. Cells were transferred onto a slide for visualizing using Zeiss 510 Meta Laser Scanning Confocal Microscope (Carl Zeiss, Thornwood, NY). Differential Interference Contrast (DIC) images, fluorescence images and the overlapped images taken from the microscope were visualized using the Zeiss AIM Viewer (Carl Zeiss, Thornwood, NY).

Additionally to support the qualitative cell uptake of the conjugate we measured the lysate fluorescence of the fluorescence-gelatin-D-pen conjugate treated HL-60 cells. Briefly, after treatment and washing with PBS as described above the HL-60 cells were lysed with M-PER mammalian cell extraction reagent. Fluorescence of the cell lysate was measured at 485 ± 20 nm (excitation) and 520 ± 25 nm (emission).

Gelatin-D-pen conjugate cytotoxicity

HL-60 cells were plated in 48 well plates at a concentration of 20,000 cells/well. The cells were treated with the gelatin-D-pen conjugate (1 mg/mL), D-pen (100 µM), gelatin (1 mg/mL), a physical mixture of gelatin (1 mg/mL) plus D-pen (100 µM). The medium was replaced every two days, and no further dose of any treatment group was
added. Cytotoxicity was determined over a period of 10 days (on the 2, 4, 6, 8, and 10 day) using the MTT assay.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism® 4 Software (GraphPad software Inc. San Diego, CA). Figure 6.7 was analyzed with two-way analysis of variance (ANOVA) followed by Berfontini’s post test, where significance was set at p<0.05.
6.4 Results and discussion

Synthesis of gelatin-D-pen conjugate

The gelatin-D-pen conjugate was prepared by a simple two step reaction as shown in Figure 6.1: *i*) gelatin was modified with the heterobifunctional cross-linker (sulfo-LC-SPDP); *ii*) D-pen was conjugated to the modified gelatin (SPDP-gelatin) with simple thiol exchange. The choice of the amine-thiol reactive heterobifunctional cross-linker (sulfo-LC-SPDP) was based on two important properties: *i*) availability of amino groups in gelatin for chemical modification, *ii*) the protection of the thiol group of D-pen, through a reversible bond between polymer (gelatin) and the drug (D-pen). A disulfide bond compared to the irreversible (maleimide) bond provides potential biological reversibility.

As shown in Figure 6.2 the amino group content of gelatin was reduced to approximately 50% of the original gelatin as the ratio of SPDP added to gelatin increased to 0.23% w/w. To confirm and quantify the amount of SPDP modification of gelatin in the process of loss of amino groups, the SPDP modified gelatin was incubated with DTT to release the pyridine-2-thione group. Figure 6.2 shows that approximately 3 moles of SPDP were conjugated per mole of gelatin as SPDP added to gelatin increased to 0.23% w/w. This corresponded to $20.5 \pm 0.5$ to $102.4 \pm 1.4 \mu$mol D-pen/g gelatin or $3.1 \pm 0.07$ to $15.2 \pm 0.2$ mg D-pen/g gelatin as the ratio of SPDP to gelatin was increased from 0.02-0.23% w/w (shown in Table 6.1).
Synthesis of fluorescein labeled gelatin-D-pen conjugate

A fluorescein-gelatin-D-pen conjugate was synthesized for cell uptake studies. The degree of labeling of fluorescein on gelatin was 0.1 mole of fluorescein per mole of gelatin. Gelatin was labeled with fluorescein through the reaction of the NHS-Fluorescein with gelatin to form fluorescein labeled gelatin. The fluorescein-gelatin was then conjugated to D-pen as described above to form the fluorescein-gelatin-D-pen conjugate. Fluorescein-gelatin alone was used as a control for cell uptake studies.

D-pen release in the presence of glutathione

The stability of the disulfide bond between D-pen and gelatin was probed using glutathione, an endogenous reducing molecule. The intracellular glutathione concentration has been reported to range from 1-11 mM [459]. Therefore, in-vitro release studies were performed in the presence of both low (1 mM) and high (10 mM) concentrations of glutathione. The pH 7.4 and 6.2 were based on the reported pH of the early endosomal and cytosolic conditions. In the presence of 1 mM glutathione in pH 7.4, D-pen was completely released in 4 h, while only ~50% D-pen was released at pH 6.2 (Figure 6.3a). As shown in Figure 6.3b, the 2 h incubation of the conjugate with glutathione (0.1-10 mM) at pH 7.4 resulted in the release of approximately 30%, 80% and 100% of D-pen in presence of 0.1, 1, and 10 mM glutathione, respectively. In contrast, D-pen release at pH 6.2 was approximately 5%, 20% in the presence of 0.1, 1, and 10 mM glutathione, respectively. D-pen and glutathione were simultaneously quantitated as shown in Figure 6.4. These studies underline the significance of interaction
of the conjugate at favorable pH and local concentration of glutathione for successful D-pen release and the subsequent cytotoxicity.

**Intracellular uptake of free D-pen**

Lodemann et al. reported the inability of D-pen to cross cell membrane of mammalian cells [126]. The inability to be transported is likely explained by the three highly ionizable functional groups of D-pen. The novel HPLC assay developed provides accurate determination of both D-pen and D-pen disulfide. As shown in Figure 6.5 when the supernatant was analyzed for remaining D-pen in the supernatant at 1, 2, 3 and 4 h post-incubation, approximately 100% D-pen was recovered. These results support the previously reported studies by Lodermann et al. [126] and signify the need for developing a novel delivery system for D-pen to enhance intracellular delivery.

**Gelatin-D-pen conjugate uptake**

The intracellular uptake of the gelatin-D-pen conjugate was studied using confocal laser scanning microscopy in HL-60 cells. The fluorescein-gelatin-D-pen conjugate uptake into HL-60 cells was a slow process with maximum uptake seen at the 72 h (Figure 6.6a) compared to 4 h (Figure 6.6b). The conjugate was shown to accumulate in the cytoplasm and the distribution of the conjugate was uniform throughout the cytoplasm. We have confirmed the cell uptake of the conjugate by measuring the cell lysate fluorescence of the conjugate treated HL-60 cells (data not shown). Ofner et al. previously showed a similar cellular distribution and slow uptake (over 96 h) of fluorescein-labeled gelatin in HL-60 cells [390].
Conjugate cytotoxicity in HL-60 cells

We have reported that D-pen alone in the presence of cupric sulfate was more cytotoxic in leukemia cells compared to breast cancer cells [190]. The data suggest that this may be due to the higher innate copper levels in the cultured leukemia cells compared to breast cancer cells (unpublished data). Therefore, the cytotoxicity of the conjugate was evaluated in human leukemia cells (HL-60). The cells were treated with gelatin and D-pen alone, a physical mixture of gelatin plus D-pen and the gelatin-D-pen conjugate. As the conjugate was shown to enter the cells, the differences in cytotoxicity of the conjugate was compared with the free gelatin and D-pen and the physical mixture of gelatin plus D-pen in the presence of innate levels of intracellular copper in HL-60 cells. During the studies, media was replaced every two days. As shown in Figure 6.7, the conjugate exhibited significantly increased cytotoxicity compared to all the controls (p<0.001 on day 4 and 10 and p<0.01 on day 6 and 8, respectively). As we have reported earlier, the mechanism of D-pen cytotoxicity is due to the generation of H_2O_2 and other ROS in presence of copper [187, 190]. Thus, the intracellular efficacy of D-pen is dependent upon two important processes: i) the interaction of the conjugate with glutathione and the subsequent release of D-pen and ii) the interaction of the released D-pen with the intracellular innate copper present in leukemia cells to produce its cytotoxic effect. We are presently investigating the intracellular D-pen release from the conjugate and the localization of intracellular copper to improve the conjugate anti-cancer effect. Other conjugates are being designed and synthesized that may increase the rate and extent of uptake and subsequent D-pen release.
6.5 Conclusions

A novel method for the synthesis and characterization of a novel gelatin-D-pen conjugate is described. The disulfide bond between gelatin and D-pen in the conjugate provides protection of D-pen from oxidation. Additionally, the disulfide bond in the conjugate was shown to be biologically reversible through the complete release of D-pen only in the presence of biologically relevant concentration of glutathione. It was shown that free D-pen does not enter cells. The delivery of D-pen as a novel gelatin-D-pen conjugate was shown to increase the intracellular accumulation of conjugate in cancer cells over time, and this directly led to enhanced cytotoxicity in human leukemia cells. The cytotoxicity was of the conjugate in leukemia cells was sustained and significant compared to the physical mixture of gelatin and D-pen indicating to the importance of disulfide bond between gelatin and D-pen.

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Table 6.1

The effect of SPDP/gelatin concentration on the amount of D-pen conjugated to gelatin

<table>
<thead>
<tr>
<th>SPDP/gelatin (% w/w)</th>
<th>D-pen (µmol)/gelatin (g) ± S.D</th>
<th>D-pen (mg)/gelatin (g) ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>20.5 ± 0.5</td>
<td>3.1 ± 0.07</td>
</tr>
<tr>
<td>0.06</td>
<td>52.8 ± 2.0</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>58.8 ± 0.9</td>
<td>8.7 ± 0.14</td>
</tr>
<tr>
<td>0.18</td>
<td>84.3 ± 9.1</td>
<td>12.6 ± 1.3</td>
</tr>
<tr>
<td>0.23</td>
<td>102.4 ± 1.4</td>
<td>15.3 ± 0.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD (n = 3).
Figure 6.1
Figure 6.1 Schematic illustration of gelatin-D-pen conjugate synthesis.

The amino group of gelatin was modified with the amine reactive N-hydroxysuccinimide (NHS) portion of sulfo-SPDP and D-pen was conjugated to the SPDP modified gelatin by reacting with sulphhydryl reactive 2-pyridothione
Figure 6.2 Effect of increasing SPDP concentration on the modification of gelatin.

The amine groups and moles of SPDP conjugated to gelatin. The pyridine-2-thione assay was used to determine the SPDP modification of gelatin. The TNBS assay was used to determine the level of SPDP modification of gelatin. Each value represents mean ± SD (n = 3).
Figure 6.3

a) 

![Graph showing % D-pen release over time for PBS buffer, pH 6.2 and pH 7.4.]

b) 

![Graph showing % D-pen release at different Glutathione concentrations for PBS buffer, pH 6.2 and pH 7.4.]

175
Figure 6.3 Release of D-pen from the gelatin-D-pen conjugate under reducing conditions.

a) The gelatin-D-pen conjugate was incubated with glutathione (1 mM) at 37°C in phosphate buffer saline (PBS, pH 7.4 and pH 6.2) and the release of D-pen was analyzed with HPLC. Each value represents mean ± SD. (n = 3).

b) The gelatin-D-pen conjugate was incubated with increasing concentration of glutathione (0, 0.1, 1, 10 mM) at 37°C for 2 h. D-pen release was analyzed with HPLC. Each value represents mean ± SD (n = 3).
Figure 6.4

Figure 6.4 HPLC chromatogram showing glutathione and D-pen elution.
Figure 6.5 Cell uptake of D-pen. D-pen (100 µM) was added to HL-60 (1×10^6) cells in PBS (pH 7.4) in a 24-well plate at 37°C in 5% CO₂ incubator. Cells were separated by centrifugation and the supernatant was analyzed by HPLC assay for D-pen concentration. Data represents mean ± SD (n = 4).
Figure 6.6

a)

b)
Figure 6.6 Intracellular uptake of gelatin-D-pen conjugate. HL-60 cells were incubated with the Fluorescein labeled gelatin-D-pen conjugate at 37°C for a) 4 and b) 72 h. Cells were washed twice with PBS and cells then suspended in phenol red free RPMI media. Cells were imaged by Confocal Laser Scanning Microscopy. Fluorescence (left), Differential Contrast (DIC) (right) and overlapped Images (bottom).
Figure 6.7 Cytotoxicity of the gelatin-D-pen conjugate in HL-60 cells. Cells (2×10⁴) were incubated with gelatin alone (1 mg/mL), D-pen alone (100 µM), gelatin (1 mg/mL) plus D-pen (100 µM), and gelatin-D-pen conjugate (1 mg/mL) After every 2 day interval media was replaced with fresh media. MTT assay was performed on day 2, 4, 6, 8 and 10. Data represents mean ± SD (n = 6). **p<0.01 and ***p<0.001 compared to gelatin plus D-pen.
Chapter 7

Copper pre-treatment of human leukemia cells augments the cytotoxicity resulting from D-penicillamine chelation of copper

7.1 Summary

The aim of present research was to investigate the effect of cupric sulfate (CuSO$_4$) pre-treatment on D-penicillamine (D-pen) and gelatin-D-pen conjugate cytotoxicity in human leukemia cells (HL-60). The CuSO$_4$ pretreatment resulted in reduced thiol level and significantly increased cellular copper content compared to untreated cells. Whereas both free D-pen and gelatin-D-pen conjugate lacked cytotoxicity in un-treated cells, they both caused concentration dependent cytotoxicity in CuSO$_4$ pre-treated leukemia cells. D-pen was more cytotoxic compared to the conjugate under the conditions tested. These findings warrant further investigation into the role of copper (amounts and intracellular distribution) on D-pen mediated cytotoxicity.
7.2 Introduction

Copper is an essential trace metal needed for the normal physiological functions in the body [2]. Copper acts as a co-factor for many important enzymes such as cytochrome c oxidase, lysyl oxidase, and superoxide dismutase [2, 3]. The human body regulates the copper homeostasis through a complex system of uptake and excretory pathways [36]. Copper influx into cells is known to be accomplished by the human copper transporter (hCTR1) although the exact mechanism remains to be determined [30]. hCTR1 is present in the plasma membrane and its ability to import copper has been reported to depend on temperature, pH and potassium ion concentration [461]. Once inside the cell copper binds to a number of pathway specific copper metallochaperones such as ATOX1, COX17 and CCS [30]. The metallochaperones deliver copper to various sub-cellular sites [30, 462]. The copper efflux from the cells is mediated by P-type ATPases, ATP7A and ATP7B [30]. Wilson’s disease and Menke’s disease are genetic disorders associated with abnormal copper homeostasis [2]. Copper has also been postulated to play a role in the etiology of Alzheimer’s disease, Parkinson’s disease and other neurodegenerative disorders [48].

The role of copper in cancer has been extensively investigated in the past two to three decades [86, 87]. Copper is now known to act as a co-factor for a number of important endogenous angiogenic compounds such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [86, 87]. As the importance of copper in angiogenesis was shown, subsequent strategies have been developed for copper targeting. The copper chelating agents such as D-penicillamine (D-pen) [8, 188],
tetrathiomolybdate [86, 87], and clioquinol [86] are at the forefront as investigative anti-
angiogenic drugs for cancer therapy.

The serum and tumor copper levels have been shown to be significantly elevated in
various malignancies including in leukemia patients [70, 76, 78]. Copper levels in the
serum of patients with acute leukemia, acute lymphoid leukemia (ALL), acute
nonlymphoid leukemia (ANLL) and chronic myelogeneous leukemia (CML) have been
reported to be 1.3 ± 0.5 µM (p<0.01), 1.3 ± 0.5 µM (p<0.01), 1.4 ± 0.5 µM (p<0.001),
and 1.1 ± 0.5 µM (p<0.05), respectively compared to 0.9 ± 0.2 µM in healthy individuals
[70]. Additionally the levels of ceruloplasmin, the major copper carrying protein, have
also been shown to be elevated in some malignancies [65]. These elevated copper levels
have also been shown to correlate to progression and severity of malignancy [91].

However, cancer cells in culture do not accumulate the same levels of copper as that
of cells in-vivo [463]. Therefore, to simulate increased copper levels in cultured leukemia
cells, HL-60 cells were pre-treated with a copper salt, cupric sulfate. Daniel and Dou
have previously described similar cupric sulfate pre-treatment with breast cancer cells to
study the effect of copper chelator clioquinol as an apoptosis inducer [436, 463, 464].

Cancer cells are known to be under persistent oxidative stress [204, 294]. An
interactive relationship between leukemia and oxidative stress has been shown [293,
305]. Significantly increased levels of ROS such as superoxide radical and H$_2$O$_2$
production has been measured in both the serum and the leukemic cells of leukemia
patients. Significant alterations in the levels of antioxidant enzymes such as SOD,
 glutathione peroxidase, glutathione reductase, and catalase have also been reported [293,
305]. Additionally, the increased oxidative stress has been shown to be limited to cancer
cells and not present in the surrounding normal cells [296]. Even though the presence of elevated copper levels and the increased oxidative stress in leukemia cells is well known, their exact interplay in the etiology of the disease still remains largely unclear. However these apparent differences in oxidative stress in cancer compared to normal cells may be exploited for selective anti-cancer therapy.

D-pen is an extremely hydrophilic (log P: -0.39) copper chelating agent [451]. The intracellular uptake of D-pen is proposed to be limited due to the presence of three ionizable functional groups, the amino (pKa: 10.8), the carboxyl (pKa: 1.8) and the thiol (pKa: 7.9) [116, 123]. At physiological pH of 7.4, D-pen is expected to exist as a highly polar zwitterion, which would limit its intracellular uptake. We [187] have recently shown with the aid of a novel HPLC assay that D-pen produces hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS) through a copper catalyzed oxidation to D-pen disulfide. Subsequently, we also showed that the ROS produced during the copper catalyzed oxidation of D-pen can cause in-vitro cell cytotoxicity in human breast cancer and leukemia cells [190]. A direct correlation between ROS and D-pen cytotoxicity was also shown [190]. In these previous D-pen cytotoxicity studies [190], cupric sulfate was externally added with D-pen to the breast cancer and leukemia cells to examine D-pen cytotoxicity in presence of copper. This does not simulate the in-vivo condition of copper being already present either intracellularly or in the extracellular tumor microenvironment at elevated levels in leukemia cells. Thus, in the present studies, HL-60 cells were pre-treated with cupric sulfate in order to simulate the elevated copper concentrations in-vivo and provide an improved model for examining the cytotoxic effect of free D-pen and of gelatin-D-pen conjugate. The novel disulfide gelatin-D-pen
conjugate cannot bind copper before being reduced to release D-pen. The conjugate was designed in order to increase the intracellular D-pen concentration and to compare the cytotoxicity to that of free D-pen.

Therefore, the aims of these studies were: i) pre-treat human leukemia cells (HL-60) for pre-determined time with cupric sulfate, ii) assess the increase in cell number, change in the intracellular thiol status and cellular copper concentration after the cupric sulfate pre-treatment, iii) assess the in-vitro cytotoxicity of free D-pen and the novel disulfide gelatin-D-pen conjugate in naïve cells compared to the cupric sulfate pretreated human leukemia cells.
7.3 Materials and methods

Materials

D-penicillamine, cupric sulfate, glutathione, Type B gelatin (75 bloom strength with 100-115 mmol of carboxylic acid per 100 g of protein, an isoelectric point of 4.7-5.2, and an average molecular weight of 20,000-25,000 Da) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sulfosuccinimidyl 6-(3’(2-pyridyldithio)-propionamido) hexanoate (Sulfo-LC-SPDP), Coomassie Plus® Bradford Protein Assay reagent, M-PER mammalian cell extraction reagent, Ellman’s reagent was purchased from Pierce Biotech Inc. (Rockford, IL). A copper standard (1000 µg/mL) for inductively coupled plasma atomic absorption spectroscopy (ICP-AAS) was purchased from Fisher Scientific Co. (Pittsburg, PA). Phosphate buffer saline (PBS), pH 7.4 cell culture grade was purchased from ATCC (Rockville, MD). All aqueous solutions were prepared in deionized distilled water (MilliQ®, Millipore Inc.).

Cell line and culture conditions

The human leukemia cell line (HL-60) was purchased from American Type Cell Culture Collection (ATCC, Rockville, MD). Cells were routinely cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% Fetal Bovine Serum (FBS) (ATCC, Rockville, MD) and maintained at 37°C in a humidified 5% CO₂ incubator. Plasmocin (5 µg/mL) (InvivoGen, San Diego, CA) was added to the cell culture media as a prophylactic measure to prevent mycoplasma contamination.
Synthesis and purification of a gelatin-D-pen conjugate

A Gelatin-D-pen conjugate was designed and synthesized to increase the intracellular concentrations of D-pen. The synthesis was performed in two steps. Briefly, gelatin (10 mg/mL) was incubated with increasing amounts of Sulfo-LC-SPDP for 2 h at room temperature in PBS, pH 7.4. Excess SPDP was separated from gelatin with microcon centrifugation devices (MWCO: 10 kDa). The SPDP-modified gelatin was then incubated with D-pen. The gelatin-D-pen conjugate was separated from the unconjugated D-pen with microcon centrifugal devices.

Pre-treatment of human leukemia cells with cupric sulfate

HL-60 cells were seeded at a density of $3.75 \times 10^5$ cells/mL in 10 mL (3.75 million cells) in triplicate in a 6-well plate. Cells were either incubated in media alone (untreated) or media containing various concentrations of cupric sulfate (10, 25, 50, and 100 µM) for 96 h. After 96 h incubation, both naïve and the cupric sulfate treated cells were collected, washed twice with PBS, and then resuspended in fresh copper free media.

Cellular copper determination

The amount of cell associated copper was analyzed as described below. Briefly, cells were collected, centrifuged to remove the copper containing media, and then washed twice with PBS, pH 7.4. The cell suspension was then suspended in 5% HNO$_3$ and heated at 80°C for 48 h under continuous stirring. The cell suspension at the end of the extraction process turned clear indicating that cell lysate has been completely digested. Copper concentration was determined by inductively coupled plasma-atomic absorption
spectroscopy (ICP-AAS, Varion Inc.). Yttrium was used as an internal standard. Copper and yttrium were analyzed at a wavelength of 327.39 and 371.03 nm, respectively. An eight point (10-250 ng/mL) copper standard curve was used to quantify copper concentration in cancer cells. Water used in the mobile phase was deionized and further purified with Milli-Q® Synthesis A10 Ultra Pure Water System, Millipore Ltd. (Billerica, MA). The 5% HNO₃ dilution vehicle for the cells was used as blank in the copper analysis. Additionally, several known amounts of copper were spiked into leukemia cell lysate and copper analysis was performed to validate the extraction process. Data represents mean ± SD (n = 3).

**Cell number determination**

The number of cells was determined with trypan blue exclusion assay. Cells were diluted with trypan blue exclusion dye introduced into the hemacytometer for counting.

**Intracellular levels of thiols (-SH)**

The intracellular reduced glutathione levels were measured with 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB). Briefly, cupric sulfate pre-treated or naïve cells were collected, washed twice with ice-cold PBS buffer, and then suspended in the M-PER mammalian cell extraction reagent. Twenty five (25) µL of standard (glutathione) or samples (cell lysate) + 25 µL DTNB (0.4 mg/mL) + 150 µL of 50 mM HEPES buffer, pH 7.4 were incubated for 10 min at room temperature and absorbance was measured at wavelength of 412 nm with the Synergy™ 2 multi-detection microplate reader (Biotek, Winooski, VT).
Protein assay

The total protein content of the cell lysate was analyzed with Coomassie Plus® Bradford Protein Assay using glutathione as a standard.

Cell viability determination

Cell viability was measured using the 3-(4,5-dimethyl-2-yl)-2,5-diphenylterazolium bromide (MTT) assay. Un-treated (naïve) or cupric sulfate pre-treated HL-60 cells were plated at concentration of $3 \times 10^4$ cells/well ($n = 3$) in a 96 well plate. Cells were treated with free D-pen, gelatin, gelatin plus D-pen, and gelatin-D-pen conjugate for 48 h. The MTT assay was performed at 48 h and absorbance was measured at a wavelength of 570 nm with the Synergy™ 2 Multi-Detection Microplate Reader (Biotek, Winooski, VT). Data are reported as cell viability (% control) and corresponds to i) the percent viable cells after 48 h compared to the untreated (naïve) cells or ii) the percent viable cells after 48 h compared to the cupric sulfate pre-treated cells.

Statistical analysis

Data represents mean ± standard deviation (SD) ($n = 3$). Statistical analysis was performed with two-way analysis of variance (ANOVA) followed by Bonferroni post test where significance was set at $p<0.05$ with GraphPad Prism® 4 Software (GraphPad software Inc. San Diego, CA).
7.4 Results

The HL-60 cells were pre-treated with increasing concentrations of cupric sulfate (10, 25, 50 and 100 µM) for 96 h. The cupric sulfate pre-treatment groups (50 and 100 µM) showed significantly lower (p<0.01) cell number compared to the untreated (naïve) HL-60 cells. As shown in Table 7.1, the fold increase in cell number was significantly lower (p<0.01) in 50 and 100 µM pre-treated cells (2.3 ± 0.2 and 2.2 ± 0.1) compared to the naïve cells (3.3 ± 0.3). The total cellular thiol content showed significant reduction (p<0.01) on all the cupric sulfate pre-treatment groups (Figure 7.1). The total cellular thiol concentration after 96 h was 103 ± 4.2 nmol/mg protein in naïve cells compared to 85.9 ± 5.6, 84.9 ± 3.7, 88.3 ± 9.0, and 77.4 ± 10.1 nmol/mg protein for the 10, 25, 50 and 100 µM cupric sulfate pre-treatment group, respectively. Whereas the total cellular copper content significantly increased from the baseline levels after 96 h of 45.6 ± 12.1 ng in naïve cells to 131.9 ± 58.8 ng (p<0.01), 92.7 ± 12.6 ng, 220.5 ± 61.2 ng (p<0.001), 335.1 ± 28.0 ng (p<0.001) in the 10, 25, 50 and 100 µM cupric sulfate pre-treatment groups, respectively. After 96 h pre-treatment, when the ratio of the amount of cellular copper was normalized against the total number of both naïve and pre-treated cells as shown in Figure 7.1, the copper (ng) per million cells in 50 and 100 µM pre-treatment groups were shown to have significantly higher (p<0.01) copper levels compared to the naïve cells. It should be noted that the studies with all the pre-treatment groups including the naïve cells were performed after washing the cells twice with PBS to remove any external copper associated with cells and suspending the cells in fresh copper free media.

The naïve and the copper pre-treated leukemia cells were then incubated with increasing concentrations of either free D-pen (Table 7.2a), D-pen plus cupric sulfate
(Figure 7.2), the gelatin-D-pen conjugate (Table 7.3a) and a physical mixture of gelatin plus D-pen (Table 7.3b). Cytotoxicity assay was performed after 48 h. The free D-pen did not cause any significant toxicity in naïve HL-60 cells (Table 7.2a). This was most likely due to the inability of D-pen to enter the cancer cells, and thus failure to bind any endogenous intracellular copper [126]. It should be noted that endogenous copper was analyzed in the culture media used in these studies and was found to be undetectable (below the detection limit of copper with atomic absorption spectroscopy of 10 ng/mL).

In contrast, when increasing concentrations of free D-pen was incubated with cupric sulfate pre-treated cells, concentration dependent cytotoxicity was observed in all pre-treated groups (Table 7.2a). The IC$_{50}$ for free D-pen in cupric sulfate (10 µM) pre-treated HL-60 cells was $131.8 \pm 3.0$ µM. These results were contrary to expectations as free D-pen would still not be able to interact with elevated level of intracellular copper. However, the results could be explained with the copper efflux phenomena to the copper free media once the copper pre-treatment media is substituted with the copper free media. When free D-pen is added to the cells, it interacts with this effluxed copper and results in the extracellular $\text{H}_2\text{O}_2$ generation which causes the observed cytotoxicity. The copper efflux phenomenon from cancer cells has been verified once they are removed from copper containing media (data not shown). To confirm that D-pen would be cytotoxic in presence of cupric sulfate present in culture media, cupric sulfate (10 µM) was simultaneously added with increasing concentrations of D-pen to naïve HL-60 cells. As shown in Figure 7.2, this resulted in concentration dependent cytotoxicity in naïve HL-60 cells with D-pen IC$_{50}$ of $102.1 \pm 1.0$ µM. As copper acts as a catalyst in the process of ROS generation from D-pen oxidation, the D-pen cytotoxicity can be proposed to be
independent of copper concentration. The cytotoxicity would be dependent on two factors: D-pen concentration and the availability of copper to catalyze the oxidation. This was experimentally supported by the comparable D-pen IC\textsubscript{50} in cupric sulfate pre-treated cells of 131.8 \pm 3.0 \mu M and 102.1 \pm 1.0 \mu M in the cells when cupric sulfate was simultaneously added with D-pen.

To improve the intracellular uptake of D-pen, a Gelatin-D-pen conjugate was successfully synthesized and characterized for D-pen release in the presence of glutathione [465]. The conjugate has a disulfide bond between gelatin and D-pen. Due to the lack of appreciable levels of glutathione (major biological reducing agent) in the culture media, the reduction of the conjugate in media was not expected. The conjugate has been shown to enter the cells slowly over a period of 48 to 72 h time period [465]. In the present studies the effect of treatment of gelatin-D-pen conjugate, gelatin alone and the physical mixture of gelatin plus D-pen (which contains gelatin and D-pen concentrations equivalent to the conjugate) in naïve and cupric sulfate pre-treated HL-60 cells was examined. As shown in the Table 7.3a and b, free D-pen, gelatin-D-pen conjugate and gelatin plus D-pen showed no cytotoxicity in the naïve HL-60 cells. While gelatin alone had no cytotoxic effect in either the naïve and the cupric sulfate pre-treated group (data not shown), the conjugate exhibited modest cytotoxicity of \(~\)55% at the highest gelatin concentrations (2000 \mu g/mL) for almost all the pre-treated groups (except 100 \mu M cupric sulfate pre-treated cells) (Table 3a), while the gelatin plus D-pen treatment resulted in the considerable cytotoxicity in all pre-treated groups (Table 3b) which was comparable to that seen with free D-pen. These data suggest that gelatin alone is not cytotoxic under the conditions tested.
7.5 Discussion

Leukemia patients have been reported to have significantly elevated copper levels compared to normal individuals [70, 76]. Additionally, the leukemia cells from patients have been shown to have increased level of H$_2$O$_2$, superoxide anion and enhanced levels of antioxidants such as superoxide dismutase and glutathione peroxidase which are hallmarks of cells being under increased oxidative stress [293]. This cellular imbalance between the oxidant-antioxidant species in the favor of oxidant environment in cancer cells supports the current emerging interest in the use of reactive oxygen species (ROS) in selective treatment in cancer [19, 316].

We have previously shown that copper chelator D-pen in the presence of cupric sulfate caused a concentration dependent cytotoxicity in leukemia cells [190]. We have also shown that leukemia cells were more sensitive to the D-pen plus cupric sulfate cytotoxicity compared to breast cancer cells (MCF-7, BT474) [190]. Copper was externally added in the above studies because: 1) D-pen cannot cross cell membranes and thus cannot interact with intracellular copper, and 2) cancer cells in culture are known not to accumulate to similar levels of copper as observed in-vivo [436, 463]. Therefore, in these present studies we pre-treated the HL-60 cells with cupric sulfate in order to simulate the in-vivo condition of elevated copper levels. The effect cupric sulfate pre-treatment on the sensitivity of HL-60 to D-pen induced ROS mediated cytotoxicity was investigated. The cupric sulfate pre-treatment of cancer cells has been previously reported to simulate in-vivo levels [19]. Additionally, it has been reported that when cells in culture are exposed to copper salts they effectively take up copper; however, the cells were also shown to efflux certain fraction of copper when copper salts were removed.
Katano et al. showed that copper was effluxed from ovarian cancer cell lines in culture after they were removed from copper containing media [467]. This process of copper efflux from cultured cells has not been extensively studied. However, it has been proposed to depend on two important factors: the copper concentration employed during the pre-treatment studies and the cell type. The process of copper efflux could explain the cytotoxicity of free D-pen in the present studies. This would allow free D-pen to quickly chelate copper and cause the H₂O₂ mediated cytotoxicity, as we have elucidated previously [190]. Copper acts as a catalyst in the mechanism of ROS generation which leads to the D-pen cytotoxicity [187, 190]. Therefore, D-pen cytotoxicity should be solely dependent on D-pen concentration and copper availability for catalysis although independent of the copper concentration. This was clearly supported by the comparable IC₅₀ of D-pen in HL-60 cells when D-pen was added with cupric sulfate and D-pen added to the cupric sulfate pre-treated HL-60 cells. However, the copper concentration in cupric sulfate pre-treated cells was more than 100-fold less compared to when cupric sulfate was added with D-pen.

The moderate cytotoxicity of the disulfide gelatin-D-pen conjugate may be explained as follows: i) slow uptake of the conjugate into the leukemia cells (data not shown), and ii) an additional step of disulfide bond reduction to release D-pen for biological activity, iii) the inability of the gelatin-D-pen conjugate to bind copper [465]. Thus, the moderate conjugate cytotoxicity seen against the HL-60 cells is perhaps an indication of a slower process compared to free D-pen. In order to support these differences in the cytotoxicity of free D-pen versus the conjugate we are currently
investigating the intracellular accumulation, cellular localization and the intracellular D-
pen release from the conjugate.

In conclusion, these results show that cellular copper levels in the cultured leukemia
cells could be elevated with pre-treatment with copper salts. The copper levels in cultured
leukemia cells were shown to be significantly lower than the cupric sulfate pre-treated
cells. In the present study under the conditions of cupric sulfate pre-treatment the copper
chelator D-pen was shown to be cytotoxic selectively in the cupric sulfate pre-treated but
not in the naïve leukemia cells indicating the need for copper availability for its
cytotoxicity. The current copper pre-treatment method needs optimization so that more
biologically relevant in-vitro model of copper uptake could be developed.

Acknowledgement

The authors would like to thank Tricia Coakley at the Environmental Training Labs
(ERTL) at the University of Kentucky for copper analysis with Inductively Coupled
Plasma- Atomic Absorption Spectroscopy (ICP-AAS).

*The contents of this chapter were submitted to Cancer Lett. Gupte, A., Mumper, R. J.,
Copper pre-treatment of human leukemia cells augments the cytotoxicity resulting from
D-penicillamine chelation of copper.
Table 7.1 The effect of cupric sulfate pretreatment on HL-60 cells. The changes in cell number, total cellular thiol status, total copper levels are compared between the untreated (naïve) and the cupric sulfate pre-treated HL-60 after 96 h incubation. Data represents mean ± SD (n = 3). **p<0.01 and ***p<0.001 compared to untreated (naïve) cells.

<table>
<thead>
<tr>
<th>CuSO4 (µM)</th>
<th>Fold increase in cell number (millions)</th>
<th>Cellular thiol (nmol/mg cellular protein)</th>
<th>Cellular Copper (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.25 ± 0.34</td>
<td>103.4 ± 4.16</td>
<td>45.6 ± 12.1</td>
</tr>
<tr>
<td>10</td>
<td>2.7 ± 0.42</td>
<td>85.9 ± 5.6**</td>
<td>131.9 ± 58.8**</td>
</tr>
<tr>
<td>25</td>
<td>2.6 ± 0.49</td>
<td>84.9 ± 3.7**</td>
<td>92.7 ± 12.6</td>
</tr>
<tr>
<td>50</td>
<td>2.33 ± 0.17**</td>
<td>88.3 ± 9.05**</td>
<td>220.5 ± 61.2***</td>
</tr>
<tr>
<td>100</td>
<td>2.15 ± 0.13**</td>
<td>77.44 ± 10.1**</td>
<td>335.1 ± 28.0***</td>
</tr>
</tbody>
</table>
Table 7.2 In-vitro cytotoxicity of D-pen in HL-60 cells. Un-treated (naïve) and cupric sulfate (10, 25, 50, 100 µM) pre-treated cells were plated at concentration of $3 \times 10^4$ cells/well. Cells were treated with D-pen (2-200 µM) for 48 h. Data represents mean ± SD (n = 3) *p<0.05, **p<0.01, ***p<0.001 compared to un-treated cells for the corresponding D-pen concentration.

<table>
<thead>
<tr>
<th>D-pen (µM)</th>
<th>Un-treated (naïve)</th>
<th>CuSO₄ (10 µM) Pre-treated</th>
<th>CuSO₄ (25 µM) pre-treated</th>
<th>CuSO₄ (50 µM) pre-treated</th>
<th>CuSO₄ (100 µM) Pre-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>111.2 ± 29</td>
<td>86.2 ± 18.2</td>
<td>85.4 ± 11.2</td>
<td>73.3 ± 9.4**</td>
<td>97.2 ± 3.5</td>
</tr>
<tr>
<td>10</td>
<td>90.7 ± 24</td>
<td>69.6 ± 9.4</td>
<td>79.4 ± 5.7</td>
<td>70.0 ± 12.3</td>
<td>86.8 ± 28.0</td>
</tr>
<tr>
<td>50</td>
<td>91.1 ± 12.6</td>
<td>65.6 ± 6.9</td>
<td>60.0 ± 5.0*</td>
<td>58.2 ± 2.4*</td>
<td>86.2 ± 13.0</td>
</tr>
<tr>
<td>100</td>
<td>99.7 ± 16.8</td>
<td>52.2 ± 6.9***</td>
<td>48.2 ± 7.5***</td>
<td>45.6 ± 3.9***</td>
<td>75.5 ± 5.2</td>
</tr>
<tr>
<td>200</td>
<td>85.7 ± 10.7</td>
<td>39.9 ± 1.5***</td>
<td>22.6 ± 8.0***</td>
<td>35.2 ± 3.6***</td>
<td>43.5 ± 2.0***</td>
</tr>
</tbody>
</table>
Table 7.3 a) *In-vitro* cytotoxicity of the gelatin-D-pen conjugate in HL-60 cells. a) naïve and cupric sulfate (10, 25, 50, 100 µM) pre-treated HL-60 cells were plated at a concentration of $3 \times 10^4$ cells/well. Cells were treated with gelatin-D-pen conjugate (20-2000 µg/mL) for 48 h. Data represents mean ± SD (n = 3). *p<0.05 compared to untreated cells for the corresponding gelatin-D-pen conjugate concentration.

<table>
<thead>
<tr>
<th>Gelatin-D-pen conjugate (µg/mL)</th>
<th>% viability HL-60 cells (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Un-treated (naïve)</td>
</tr>
<tr>
<td>2</td>
<td>84.5 ± 19.7</td>
</tr>
<tr>
<td>10</td>
<td>82.4 ± 20.8</td>
</tr>
<tr>
<td>50</td>
<td>83.3 ± 17.7</td>
</tr>
<tr>
<td>100</td>
<td>85.9 ± 10.6</td>
</tr>
<tr>
<td>200</td>
<td>79.9 ± 3.9</td>
</tr>
</tbody>
</table>
Table 7.3 b) Naïve and cupric sulfate (10, 25, 50, 100 µM) pre-treated HL-60 cells were plated at a concentration of 3×10^4 cells/well. Cells were treated with gelatin (20-2000 µg/mL) plus D-pen (2-200 µM) for 48 h. Data represents mean ± SD (n = 3). *p<0.05, **p<0.01 and ***p<0.001 compared to the un-treated cells for corresponding gelatin plus D-pen concentration.

<table>
<thead>
<tr>
<th>D-pen (µM) + Gelatin (µg/mL)</th>
<th>% viability HL-60 cells (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Un-treated (naïve)</td>
</tr>
<tr>
<td>2 + 20</td>
<td>97.1 ± 2.7</td>
</tr>
<tr>
<td>10 + 100</td>
<td>86.5 ± 0.4</td>
</tr>
<tr>
<td>50 + 500</td>
<td>90.6 ± 18.4</td>
</tr>
<tr>
<td>100 + 1000</td>
<td>93.3 ± 5.7</td>
</tr>
<tr>
<td>200 + 2000</td>
<td>93.7 ± 8.7</td>
</tr>
</tbody>
</table>
Figure 7.1 The intracellular copper and thiol status after cupric sulfate pre-treatment. Total cellular thiol content/mg protein as a result of cupric sulfate pre-treatment and copper (ng) per million cells as a result of cupric sulfate pre-treatment. Data represents mean ± SD (n = 3). **p<0.01 compared to untreated (naïve) cells.
Figure 7.2 In-vitro cytotoxicity of D-pen plus cupric sulfate in HL-60 cells Naïve HL-60 cells were plated at a concentration of $3 \times 10^4$ cells/well. Cells were treated with D-pen (1-200 µM) plus cupric sulfate (10 µM) for 48 h. Data represents mean ± SD (n = 3).
Chapter 8

Summary and conclusions

The focus of these studies was to 1) investigate the cytotoxic anti-cancer properties of the copper chelating agent D-penicillamine (D-pen) through the generation of hydrogen peroxide ($\text{H}_2\text{O}_2$) and other reactive oxygen species (ROS), and 2) to develop novel delivery strategies for enhancing its intracellular uptake in order to potentially improve its \textit{in-vivo} therapeutic efficacy as an anti-cancer agent. Therefore, the hypotheses driving this research were: 1) Copper catalyzed D-pen oxidation will lead to the concentration dependent generation of $\text{H}_2\text{O}_2$ 2) $\text{H}_2\text{O}_2$ generated from D-pen in the presence of copper will cause oxidative stress in human cancer cells and result in cytotoxicity 3) A novel polymer-D-pen conjugate could be synthesized and the conjugate would be able to deliver D-pen intracellularly and cause cytotoxicity in cancer cells and 4) Copper pre-treatment of human cancer cells in culture would simulate the elevated \textit{in-vivo} copper status and augment the cytotoxicity of D-pen.

To test the generation of $\text{H}_2\text{O}_2$ as a result of D-pen oxidation a novel simple, sensitive and rapid HPLC assay was developed to detect and quantitate D-pen, its major oxidation product D-pen disulfide and $\text{H}_2\text{O}_2$ simultaneously in a single run. With the aid of the HPLC assay it was conclusively shown that in the presence of cupric sulfate, D-pen was oxidized to D-pen disulfide while simultaneously generating $\text{H}_2\text{O}_2$. The ratio of D-pen oxidized to $\text{H}_2\text{O}_2$ generated was shown to be 2:1 mole/mole at low D-pen concentrations ($<500 \ \mu\text{M}$). At higher concentrations (1 mM), D-pen was shown to react with $\text{H}_2\text{O}_2$ being produced and thus the ratio of D-pen oxidized to $\text{H}_2\text{O}_2$ generated was
much lower. The formation of H$_2$O$_2$ due to D-pen oxidation was confirmed with the H$_2$O$_2$ specific enzyme, catalase. Cupric sulfate at a range of concentrations (nanomolar to micromolar) was shown to catalyze D-pen oxidation. Also, it was shown that the rate of copper catalyzed D-pen oxidation was greater than that compared to iron catalyzed D-pen oxidation. Further, the rate of D-pen oxidation was shown to increase with temperature, with the rate of D-pen oxidation at 37ºC > 25ºC > 4ºC. D-pen oxidation was inhibited in the absence of oxygen (anaerobic) conditions even if cupric sulfate was added at micromolar concentrations. Copper chelator such as EDTA and the Cu(I) specific chelator, BCS, were shown to inhibit copper catalyzed D-pen oxidation. The inhibition of the oxidation in the presence of BCS suggests that Cu(II) added to the reaction in the form of cupric sulfate was converted to Cu(I) during its interaction with D-pen.

To investigate the potential cytotoxicity of H$_2$O$_2$ being produced as a result of D-pen oxidation, D-pen was incubated in the presence of cupric sulfate with human cancer cells. Breast cancer cell lines differing in their expression of HER2 protein were employed in these studies (MCF7: HER2 negative and BT474: HER2 positive) and leukemia cell lines differing in their sensitivity to anthracycline drugs (HL-60: wild type and resistant cell lines, HL-60/VCR (P-gp) and HL-60/ADR: (MRP1) were employed. Increasing concentrations of D-pen (1-400 µM) in the presence of cupric sulfate (10 µM) resulted in concentration dependent cytotoxicity in the wild type HL-60 cells and also in the mildly resistant HL-60/VCR cells, but was not cytotoxic in the highly resistant HL-60/ADR cells. This was interesting as it suggested a relationship between the ROS mediated cytotoxicity and the mechanism of action of cytotoxic agents such as
anthracyclines. These results support the hypothesis that $\text{H}_2\text{O}_2$ and other ROS might be involved in the mechanism of cytotoxicity of chemotherapeutic drugs.

Similar concentrations of D-pen in the presence of cupric sulfate were less cytotoxic in human breast cancer cells. The involvement of $\text{H}_2\text{O}_2$ was indirectly confirmed, since in the presence of catalase, D-pen mediated cytotoxicity was inhibited. Additionally, direct proof of intracellular $\text{H}_2\text{O}_2$ and ROS generation was shown with the aid of the intracellular oxidation of cell permeable, non-fluorescent $\text{H}_2\text{DCFDA}$ into deacetylated, oxidized, fluorescent DCF. Significantly higher DCF was shown to be formed in D-pen plus cupric sulfate treated cells compared to D-pen, cupric sulfate and un-treated cells. Additionally, a direct correlation between the intracellular ROS generation and the increasing D-pen concentration was also shown and this correlation was shown to improve over time. The total cellular thiol levels were significantly lower after treatment with D-pen plus cupric sulfate compared to either D-pen or cupric sulfate alone. It should be pointed out that the incubation of cancer cells with either D-pen or cupric sulfate alone did not result in any of the above cytotoxic effects. This result indicated that the cytotoxicity was due to the interaction of D-pen with copper.

To improve the intracellular D-pen concentrations, a novel disulfide gelatin-D-pen conjugate was synthesized and characterized. D-pen is highly hydrophilic and is reported to be not taken up by cells. Therefore, to increase its intracellular levels, we synthesized a gelatin-D-pen conjugate with the aid of heterobifunctional cross-linker SPDP. The synthesis was performed in two steps; gelatin was modified with SPDP followed with conjugation with D-pen. In-vitro release of D-pen from the conjugate was examined in the presence of glutathione. These studies demonstrated that the disulfide bond between
gelatin and D-pen is biologically reversible as glutathione (10 mM) was able to release D-pen in 2 h. A novel modified assay was developed to analyze glutathione and D-pen simultaneously. While D-pen alone was shown not to be taken up by cancer cells, the intracellular uptake of the conjugate was clearly exhibited with confocal microscopy. The fluorescein-gelatin-D-pen conjugate exhibited a slow in-vitro uptake in the human leukemia cells, with progressive uptake seen from 48 h to 72 h. The process of cell uptake was similar to a fluid phase or pinocytosis process rather than the punctuate endocytosis observed with particulate delivery systems. However, the intracellular localization of the conjugate was not determined in these studies.

To simulate the enhanced copper levels in cancer cells, human leukemia cells (HL-60) were pre-treated with cupric sulfate for 96 h. The total cellular copper amount, cell number, total thiol status were evaluated after the copper pre-treatment. Copper levels were shown to be significantly higher after cupric sulfate treatment compared to untreated (naïve) cells. The copper pre-treated leukemia cells were then incubated with either free D-pen or gelatin-D-pen conjugate. The studies demonstrated that both the free D-pen and the D-pen conjugate alone were able to cause cytotoxicity selectively in the copper pre-treated cells and not in the naïve leukemia cells. D-pen alone exhibited concentration dependent cytotoxicity, while the conjugate was shown to have only moderate cytotoxicity. The concentration dependent cytotoxicity of free D-pen was explained with the aid of copper exchange between the intracellular and extracellular media, once the copper containing media is removed. The moderate conjugate cytotoxicity was explained with the aid of previously described above was slowly taken up in the cells and after uptake has to be reduced to release D-pen
In conclusion, these studies demonstrated the potential application of D-pen as cytotoxic anti-cancer agent. The proof of concept of D-pen to produce H$_2$O$_2$ both in the presence of cupric sulfate and in cupric sulfate pre-treated cancer cells combined with the successful intracellular delivery of D-pen into cancer cells with the aid of polymer conjugate were promising results when combined with the well documented higher copper levels known to be present in various malignancies. Therefore, these studies provide a proof of concept as well as an excellent opportunity to selectively target cancer cells with D-pen resulting in both copper chelation and subsequent cytotoxic effect with the aid of H$_2$O$_2$ generation as a result of the copper chelation by D-pen.

The studies presented in the dissertation have established the mechanism of action of D-pen for its use as an anti-cancer agent and provided novel delivery strategies to improve its therapeutic effectiveness. Along these lines, future studies would focus on selection of improved biocompatible polymers such as poly-glutamic acid or chitosan for enhanced D-pen delivery. Furthermore, the mechanism of intracellular uptake and localization of the polymer conjugate also needs to be characterized in order to, 1) optimize the release and release rate of D-pen from the conjugate, and 2) the interaction of the released D-pen with protein-bound intracellular copper. Ultimately, in-vivo studies would be necessary to assess the improvement in the half-life and biological activity of the D-pen formulation compared to free D-pen. Combined these additional studies may revel further improvements or alternative formulation approaches in the delivery of D-pen as an anti-cancer agent.

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Appendices

This section contains the following additional experiments:

- **Appendix A**: Chemical structures of compounds employed in this dissertation
- **Appendix B**: Development of a HPLC-Fluorescence assay for the determination of D-pen in biological samples
- **Appendix C**: Preparation and characterization of D-pen nanoparticles
- **Appendix D**: Synthesis of monostearyl ester of D-pen disulfide, a prodrug approach for the potential D-pen delivery
- **Appendix E**: Preparation and characterization of the Herceptin-D-pen immunoconjugate
Appendix A

Figure A1. Structure of thiols and disulfides employed in the studies.
Figure A2. Structure of chelators and reducing agents used in these studies.
Figure A3. Structure of cross-linker and lipid used in these studies.

a) Structure of the heterobifunctional cross-linker, Sulfosuccinimidyl 6-(3'(2-pyridyldithio)-propionamido) hexanoate (Sulfo-LC-SPDP) m.w: 527.57, spacer arm Length: 15.6 Å and

b) the functionalized phospholipid, 1,2-Dipalmitoyl-\textit{sn}-Glycero-3-Phosphoethanolamine-N-(3-(2-pyridyldithio)propionate) (Sodium Salt) (PDP-PE) m.w: 911.2
Figure A4. Mechanism of Ellman’s and TNBS assay.

a) Mechanism of Quantification of sulfhydryl groups with 5,5’-Dithio-bis (2-nitrobenzoic acid) DTNB, and b) Mechanism of quantification of amino groups with trinitrobenzene sulfonate (TNBS) assay.
Figure A5. Mechanism of intracellular ROS and superoxide radical assay.

a) Mechanism of quantification of intracellular reactive oxygen species (ROS) generation with 2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) m.w: 487.3. H$_2$DCFDA is deacetylated and oxidized in the presence of ROS to dichlorodihydrofluorescein (DCF) ($\lambda_{\text{exc}}$: 485 $\lambda_{\text{emis}}$: 525 nm) and b) the mechanism of intracellular superoxide ($O_2^{\cdot-}$) generation with Hydroethidine (HE) ($\lambda_{\text{exc}}$: 365 $\lambda_{\text{emis}}$: 420 nm)
Figure A6. Structure of the amino reactive fluorescein, (NHS-Fluorescein).
Appendix B

Development of a HPLC-Fluorescence assay for the determination of D-pen in biological samples

Despite its wide use in the treatment of Wilson’s disease and rheumatoid arthritis, the pharmacokinetics of D-pen in humans is not well established. This is partly due to the lack of suitable detection methods [115, 116]. There have been a number of reports over the years regarding development of suitable assay for D-pen utilizing gas, cation exchange chromatography and radioimmunoassay [165, 171, 184, 468]. Sensitivity, reproducibility, time consuming are some of the limitations of these assays.

Yusof et al. previously described an assay where D-pen was conjugated with N-pyrenyl maleimide (NPM) through a Michael’s reaction to form a stable fluorescent D-pen-NPM adduct (Figure B1) [171]. We have modified the assay described by Yusof et al. The assay we developed consisted of a HPLC system (Thermoquest) comprised of a model P4000, spectra pump, injection valve with 100 μL loop and a FL3000, fluorometer operating at an excitation/emission wavelength of 330/380 nm. The HPLC column (YMC Pack Pro 150 × 4.6 mm i.d.) contains 5 μm particles of C18 packing material. Peaks were quantified with Chromoquest®, Chromatography Workstation software. The mobile phase consists of 60% water, 40% acetonitrile and 1 mL/L of acetic acid, 1 ml/L o-phosphoric acid. The sample injection volume is 20 μL and is eluted at a flow rate of 0.5 mL/min.

Both the samples and standards were pre-column derivatized to form the NPM-D-pen adduct as follows. Tris-EDTA buffer (240 μL) and 1 mM NPM (750 μL) was added to diluted samples (10 μL). The resulting solution was vortexed and incubated at room
temperature for 30 min after which it is acidified with 5 μL of 1/6 M HCl to stop the reaction. Samples are then filtered through 0.2 μm acrodisc and subsequently injected in the HPLC column. The NPM-D-pen adduct peak (retention time: 2.90 min) was effectively separated from NPM hydrolysis peaks (retention time: 5.75, 6.59 and 7.38 min) within a short runtime of 12 min (Figure B2a).

A calibration curve of D-pen was constructed by injecting 20 μL of NPM derivatized standards. Excellent linearity was observed when the standard curve was performed in either water or in rat brain homogenate. Linearity was observed over the entire range of concentration (15-400 ng/mL), \( r^2 = 0.9992 \) and 0.9998 in water and rat brain homogenate, respectively (Figure B2b). The lower limit for detection was established to be 2 ng/mL (S/N = 3) while the lower quantitation limit was 15 ng/mL (S/N = 10). Inter-day and intra-day variations for the assay were <3%. Spike recovery studies of D-pen in rat brain homogenate were also performed in the concentration range of 15-400 ng/mL. Additionally, the stability of the NPM-D-pen adduct stored at 4ºC was investigated. Adduct was observed to be stable for at least 4 weeks with RSD <3% for all the concentrations analyzed (Figure B3a). Additionally, the time needed for derivatization reaction to be complete was confirmed as follows, D-pen was incubated with NPM and Tris buffer for 10, 15, 20, 30 and 40 min and reaction was stopped using 10 μL of 1/6 M HCl, as shown in Figure B3b, the reaction was complete in 30 min.

Although, this assay has been validated for D-pen, a discrete assay development for a thiol like D-pen should ideally incorporate reduction of drug from its disulfides or any relevant mixed disulfide to account for total D-pen. This holds true for D-pen, as it will most likely be present in the form of a mixed disulfide (with cysteine and/or
glutathione) or as D-pen disulfide in the biological samples. Therefore, reduction of D- pen-disulfide was investigated with strong reducing agents such as Dithiothreitol (DTT) and Tri (2-carboxyethyl) phosphine (TCEP). DTT is a thiol containing reducing agent as compared to TCEP which is a non-thiol reducing agent. However, DTT and TCEP are known to interfere with reactions involving maleimide chemistry (derivatization of D-pen with NPM). TCEP was found to interfere with the derivatization reaction at all ranges (500 µM-50 mM) compared to DTT, which interfered only at concentrations >10 mM. The reduction of D-pen in the presence of excess reducing agents such as DTT was found to be incomplete, as a maximum of 5-10% of D-pen-disulfide could be reduced. Further modifications in the assay are required to optimize the assay in order to detect and quantify D-pen disulfide simultaneously with D-pen and other mixed D-pen disulfides.
Figure B1. Structure of N-pyrenyl maleimide (NPM). Structure of NPM and the reaction of NPM with D-pen to form fluorescently tagged NPM-D-pen derivative ($\lambda_{\text{exic}}$: 330 and $\lambda_{\text{emis}}$: 380 nm)
Figure B2. HPLC-Fluorescence assay of D-pen. a) HPLC chromatograph of NPM-D-pen derivative elution. b) Recovery of D-pen spiked in brain homogenate.
**Figure B3. Stability of NPM-D-pen adduct.**

**a)** Stability of D-pen-NPM adducts (14.9-402.3 ng/mL) stored in solution at 4°C for 4 weeks. Data represents mean ± SD (n = 3).

**b)** Effect of incubation time of D-pen with NPM on to changes in peak area.
Appendix C

Preparation and characterization of D-pen nanoparticles

C1. Preparation of D-pen nanoparticles

In the past decade, Dr Mumper’s lab has developed a novel technology to formulate stable nanoparticles from microemulsion precursors. These nanoparticles have been shown to be successfully employed for tumor targeting [469-472], brain targeting [473, 474], and dendritic cell targeting [469, 475-477]. The technology is based on the spontaneous formation of a warm microemulsion that can be used as a template to form nanoparticles. The formulation involves melting a pharmaceutically acceptable matrix polymer emulsifying wax and a surfactant polyoxy 20-stearyl ether (Brij 78) at 50-60°C to form a slurry of the melted material in water under constant stirring. Simple cooling of the heated microemulsion results in the formation of stable nanoparticles less than 100 nm. NPs require no further purification, and once passed through a 0.2 micron filter for sterilization, are ready for injection.

Thiol (sulfhydryl, -SH) activated liposomes have been employed previously to attach antibodies for cell targeting [478]. We employed a similar strategy to attach D-pen to sulfhydryl reactive NPs. D-pen has a free sulfhydryl group that may be conjugated to NPs that are made to have reactive thiol group at their surface. The resulting disulfide bond would be stable in normal physiologic conditions, but be reduced in the reducing environment of the cells. In preliminary studies, sulfhydryl reactive NPs (PDP-NPs) were prepared with functionalized lipid, PDP-PE (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(3-(2-pyridylthio)propionate); (Avanti Polar Lipids,
Birmingham, AL. Briefly, various amounts of PDP-PE (0-25% w/w) were added during the formulation of empty nanoparticle (EpS) to incorporate PDP-PE in the NPs to formulate PDP-NPs. The PDP-PE is thought to be incorporated in NPs according to its specific orientation (the hydrophobic tail is embedded in the NP core and hydrophilic head group outside the NP) as shown in Figure C1 a and b. To maximize PDP-PE loading, while at the same time maintaining the NP size below 100 nm, the PDP-PE loading was optimized. As seen in Figure C2, the size of the NPs increased with increasing PDP-PE loading. Although PDP-PE loading of 15-25% resulted in particle sizes <100nm, but the results showed inconsistency from batch to batch. Therefore, a 10% w/w PDP-PE loading was selected for future studies as it resulted in NPs size consistently below 100nm.

D-pen was conjugated to PDP-NPs. Briefly; freshly prepared PDP-NPs were mixed with excess D-pen (1 mg/mL). The pH was adjusted to 8.0 with NaOH and the reaction was stirred under N2 for 2 h. After 2 h, NPs were separated from the unconjugated D-pen using Sepharose CL-4B (15 mm × 70 mm). Particle size was measured by photon correlation spectroscopy (PCS) using a Coulter N4 plus Particle Sizer at 90° for 200 s.

C2. Separation of free D-pen from D-pen-NPs

As described earlier, after the incubation of excess D-pen (1 mg/mL) with the PDP-NPs for 2 h (deduced as the time at which reaction is essentially complete from earlier experiments) under N2 to conjugate D-pen on the surface of PDP-NPs, the unconjugated D-pen was removed with gel permeation chromatography (GPC). Fractions (0.5 mL each) were collected from the Sepharose CL-4B column; fractions were analyzed for
particle intensity in the Coulter N4 Plus Submicron Particle Size Analyzer and for the D-pen concentration with HPLC assay described below. Based on particle intensity it was established that NPs were eluted in fractions 3-4 (as seen in Figure C3) while free D-pen eluted in fractions 6-20 (based on the HPLC assay of the fractions). Therefore a complete separation of bound and free drug was shown to be achieved with GPC. The experimental conjugation of D-pen (34.8 µg/mL) for Sepharose CL-4B was similar to the theoretical D-pen conjugation calculated based on the amount of PDP-PE added (e.g. for 10% w/w PDP-PE loading, the maximum theoretical D-pen conjugation would be 34 µg/mL).

C3. Stability of nanoparticles

The stability of D-pen-NPs was evaluated in biologically relevant media (Phosphate buffer saline (PBS), Fetal Bovine serum (FBS), sodium chloride (NaCl) and lactose) at 0 and 30 min. As seen in Figure C4 the particle size of the NPs remain relatively unchanged between 0 and 30 min. The PDP-NPs and D-pen-NPs showed an increase in particle size when stored at 4ºC (Figure C5a.) for 7 days.

To ascertain the stability of disulfide bond through which D-pen was bound to the NPs, the D-pen-NPs were incubated for 18 h at 25ºC with PBS buffer (5 mM), pH 6.0, 7.0 and 8.0, about 96% 92% and 85%, respectively, were still conjugated to the NPs. However, D-pen was shown to be cleaved from the NPs when incubated in presence of DTT, about 62% of D-pen was conjugated to the NPs (Table C5b).
Figure C1. Formulation of D-pen nanoparticles. a) Schematic of the formulation of D-pen nanoparticles, b) Schematic showing the conjugation of D-pen to PDP-NPs.
Figure C2. Effect of PDP incorporation on the size and PI of NPs.

a) Effect of increasing PDP-PE (% w/w) loading on NPs size (nm). Data represents mean ± SD (n = 3). b) Effect of increasing PDP-PE loading on particle size (nm) and Polydispersity index of three separate nanoparticle batches.
Figure C3. Gel permeation chromatography (GPC) separation of free D-pen from D-pen-NPs. Free D-pen (fractions 6-20) from D-pen associated with the NPs (fractions 3-4). Data represents mean ± SD (n = 3).
Figure C4. The short term stability of D-pen-NPs in biological media.
Table C1. Stability of D-pen-NPs. a) The stability of PDP-NPs and D-pen-NPs after storing at either 4 or 25°C for 7 days. b) The stability of D-pen bound to NPs after exposure to DTT at various pH.

a)

<table>
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<tr>
<th>pH 6.0 (5 mM PBS)</th>
<th>Original D-pen (µg/mL)</th>
<th>Remaining D-pen (µg/mL)</th>
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<td>33</td>
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<th>pH 7.0 (5 mM PBS)</th>
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<th>pH 8.0 (5 mM PBS)</th>
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<td>33</td>
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<th>Dithiothreitol DTT (DTT) in water</th>
<th>Original D-pen (µg/mL)</th>
<th>Remaining D-pen (µg/mL)</th>
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<tr>
<td>33</td>
<td>20.5</td>
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b)

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<th></th>
<th>Particle size (nm) ± SD</th>
<th>Polydispersity index</th>
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<tr>
<td>PDP-NPs</td>
<td>88 ± 35</td>
<td>0.430</td>
</tr>
<tr>
<td>PDP-NPs at 4°C for 1 day</td>
<td>105 ± 32</td>
<td>0.230</td>
</tr>
<tr>
<td>PDP-NPs at 4°C for 7 days</td>
<td>134 ± 40</td>
<td>0.453</td>
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<tr>
<td>PDP-NPs at 25°C for 1 day</td>
<td>137 ± 28</td>
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<td>PDP-NPs at 25°C for 7 days</td>
<td>265 ± 30</td>
<td>0.215</td>
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<tr>
<td>D-pen-NPs</td>
<td>108 ± 40</td>
<td>0.424</td>
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<tr>
<td>D-pen-NPs at 4°C for 1 day</td>
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<td>D-pen-NPs at 4°C for 7 days</td>
<td>158 ± 36</td>
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Appendix D

Synthesis of a monostearyl ester of D-pen disulfide, a prodrug approach for the potential D-pen delivery

D-pen is highly hydrophilic compound (log P: -0.35) [448], and thus its intracellular uptake is very limited [126]. Recently Chvapil et al. have reported on the synthesis of ester derivatives of D-pen to enhance its lipophilicity [448, 451]. We have investigated similar strategy to conjugate D-pen disulfide with stearyl alcohol to potentially form monostearyl ester of D-pen-disulfide. This lipophilic D-pen disulfide would then be entrapped in the emulsifying wax nanoparticles. Thus, the monostearyl ester of D-pen disulfide could be used as a potential lipophilic prodrug for D-pen delivery.

Briefly, mole to mole of stearyl alcohol dissolved in tetrahydrofuran was added to D-pen disulfide, which was then dissolved in a mixture of tetrahydrofuran and water. The pH of the reaction mixture was adjusted to 2.8 with the aid of H₂SO₄. The reaction was carried for 2 h at 70°C. After cooling to room temperature, a white oily precipitate was formed. The product was then extracted with dichloromethane/water solution to remove any unreacted D-pen. The dichloromethane was then removed by under nitrogen. The product was further dried in a rotovap to remove any dichloromethane and tetrahydrofuran for 8 h at 70°C. The product was then cooled to room temperature. A white powder was obtained. The product was confirmed to be monostearyl ester of D-pen disulfide by mass spectroscopy with the molecular ion peak present at 571 m/z.

However, our concurrent in-vitro studies to examine the reduction of D-pen disulfide to D-pen showed that the reduction was extremely slow even in the presence of
millimolar concentrations of DTT, TCEP and glutathione. This led us to reevaluate our approach of delivering D-pen disulfide as a prodrug for D-pen. It should be noted that \textit{in-vivo} the ester prodrug of D-pen disulfide would have to be converted to D-pen disulfide followed by its reduction to the bioactive form D-pen. This would certainly lead to the released D-pen concentrations to be below the minimum effective levels, and thus the strategy would not a good vehicle for D-pen delivery.
Figure D1. Schematic of the synthesis of monostearyl ester of D-pen disulfide

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\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{OH} + \text{D-pen disulfide (in THF/water)} \xrightarrow{\text{Low pH}} \text{Monostearyl ester of D-pen disulfide (m.p= 60-65°C)} \]

m/z: Product-Na+: 571

Stearyl Alcohol (in THF)
Figure D2. Mass spectrum of the purified monostearyl ester of D-pen-disulfide. The mass spectrum was obtained in a negative ion mode MALDI-TOFMS
Appendix E

Preparation and characterization of Herceptin-D-pen immunoconjugate

Herceptin (Trastuzumab, Genentech, Inc.) is a humanized monoclonal antibody that has been reported to selectively bind with high binding affinity ($K_d = 5 \text{ nM}$) in cell based assays to the extracellular domain of the human epidermal growth factor receptor 2 protein (HER2) [479, 480]. HER2 protein overexpression has been reported in about 20 to 30% of breast cancer patients [479]. The binding of Herceptin to HER2 receptor restricts the binding of growth factors needed by the breast cancer cells for growth and development and thus has been described to lead to cytostatic effect [480]. Herceptin is currently indicated either alone or in combination with chemotherapeutic agent, paclitaxel in HER2 positive metastatic breast cancer patients with no previous chemotherapy.

Immunoconjugate has been defined as monoclonal antibodies coupled to highly toxic agents, including radioisotopes and toxic drugs (ineffective when administered systemically alone) [481, 482]. The combination of the targeting specificity of monoclonal antibodies with the enhanced tumor-killing power of chemotherapeutic agents, immunoconjugate provide a selective treatment of malignant vs. normal tissue, resulting in fewer toxic side effects than most conventional chemotherapeutic drugs [481, 482]. Two radioimmunoconjugates, Ibritumomab Tiuxetan (Zevalin®) and Tositumomab-131I (Bexxar®), and one drug conjugate, Gemtuzumab Ozogamicin (Mylotarg®) are currently marketed immunoconjugates.
Mandler et al. [483, 484] reported on the synthesis of a novel Geldanamycin-Herceptin immunoconjugate synthesized with the aid of thiolation of Herceptin followed by attachment of a cytotoxic agent, geldanamycin with an irreversible maleimide bond [483, 484]. Bernstein [485], Hamann [486] et al. examined the CD-33 monoclonal antibody for the purpose of immunoconjugate synthesis, anti-CD33 antibody-calicheamicin conjugates have been described by the authors for effective targeting of the cytotoxic agent, calicheamicin and treatment of leukemia.

We decided to employ a similar strategy for the synthesis of Herceptin-D-pen immunoconjugate. Due to the availability of primary amino groups in lysine present in the heavy chain of the antibody Herceptin, it was decided to thiolate these amine groups to form a disulfide bond between the thiol group of D-pen and the thiol group introduced in Herceptin. The hypothesis was that the disulfide bond employed would be biologically reversible and lead to the release of D-pen under reducing conditions.

Herceptin was a kind gift from Genentech, Inc. (San Francisco, CA) as a part of their Research Contracts and Reagents Program. D-pen and traut’s reagent were purchased from Sigma Aldrich Co. (St Louis, MO). The Herceptin-D-pen immunoconjugate synthesis was performed in two separate steps. Briefly, in step I thiolation of Herceptin was accomplished and in step II D-pen conjugation to the thiolated Herceptin was performed. Briefly, traut’s reagent (133.3 µM) was incubated with Herceptin (13.3 µM, based on IgG molecular weight of 150 kDa) in PBS-EDTA (1 mM) buffer, pH 7.4 for 1 hr at room temperature. Traut’s reagent was in 10 fold molar excess to assure that on average 2-3 antibody amine groups would be thiolated (information from traut’s reagent thiolation protocol). After 1 hr, reaction mixture was
eluted through a dextran desalting column (molecular weight cut off: 5000 Da) in the presence of PBS-EDTA (2 mM) buffer, pH 7.4 and 10 fractions were collected. Each of these fractions was analyzed by Ellman’s assay to quantify thiol groups. The purified thiolated Herceptin was reacted with excess D-pen overnight at room temperature. The reaction mixture was again eluted through dextran column to separate the free D-pen or D-pen disulfide from Herceptin. Fractions were analyzed by coomassie assay to determine the antibody concentration and an HPLC assay was conducted for the concentrations of D-pen or D-pen disulfide. The coomassie assay confirmed the presence of Herceptin, thiolated Herceptin, Herceptin-D-pen conjugate in fraction 4 (void volume) while the HPLC assay confirmed the presence of D-pen and D-pen disulfide in the fractions 7-10. It should be noted that no free D-pen or D-pen disulfide was eluted in fractions 1-4.

The release of D-pen from the Herceptin-D-pen immunoconjugate was investigated in the presence and absence of DTT. Herceptin-D-pen immunoconjugate was lyophilized to obtain a concentrated sample for performing in-vitro release studies. This was done in order to achieve concentration of D-pen or D-pen disulfide released over the HPLC detection limits. The in-vitro release experiments were performed in PBS (phosphate buffer (100 mM), with NaCl (154 mM)) pH 7.4 at 37°C. For reducing conditions, a final concentration of DTT (20 mM) was employed as reducing agent in PBS and PBS alone was used in non-reducing conditions. Briefly, lyophilized immunoconjugate was weighed and resuspended in PBS as follows: 1) Immunoconjugate plus DTT (20 mM) (reducing conditions) in PBS, 2) Immunoconjugate in PBS (non-reducing conditions), 3) DTT (20 mM) (background control) in PBS, 4) PBS (negative control). Ellman’s and Coomassie
assays were performed both before and after the experiment to quantitative any reduction or loss of protein respectively.

Ellman’s assay before the reduction showed no free thiol, as expected. According to the coomassie assay the concentration of antibody was 2.9 mg/mL. This was equivalent to the presence of 19 μM of antibody in the lyophilized sample. If a theoretical conjugation of either 2:1 or 3:1 mole/mole is assumed between D-pen and Herceptin, then 38 and 57 μM of D-pen was expected to be conjugated to the antibody, respectively. The coomassie assay showed similar concentration of antibody presence as before reduction, thus indicating no loss of the antibody before and after reduction. The HPLC assay was then performed on both reduced and non-reduced immunoconjugate as well as DTT and PBS. D-pen concentration in the immunoconjugate sample subjected to reducing environment was calculated to be 36 ± 7 μM, while immunoconjugate incubated with PBS (non-reducing conditions) showed 0 μM of D-pen. This experiment confirmed that D-pen was conjugated to the antibody and the ratio of D-pen conjugated to the antibody was approximately 2:1 mole/mole. The studies also showed that the disulfide bond between D-pen and the antibody was reduced in the presence of reducing agent (DTT) and thus could be potentially biologically reversible in the presence of glutathione.

Although, the approach of synthesis of Herceptin-D-pen immunoconjugate was successful, there are two major obstacles in the potential in-vivo success of this delivery system: 1) internalization of the conjugate in the HER2 positive breast cancer cells. This is essential for the reduction of the disulfide bond between the antibody and D-pen, but is highly unlikely in the extracellular environment where the glutathione concentrations have been described to be 100-fold lower than that of the intracellular glutathione
concentrations. Based on the recently reported \textit{in-vitro} studies with the Geldanamycin-Herceptin, it is known that the internalization of Herceptin is low, quantified to be between 10-30\% therefore this would lead to extremely low reduction of D-pen and in turn lead to less than minimum effective concentration being released; 2) low drug loading on the antibody, as only two molecules of D-pen were conjugated per antibody. Higher loading, although possible, could limit the binding of the antibody to HER2 which would lead to loss of active targeting.
Figure E1. Schematic of the synthesis of Herceptin-D-pen immunoconjugate. Herceptin is thiolated with the aid of Traut’s reagent, followed by its conjugation with D-pen to form a stable disulfide conjugate.
Figure E2. Gel Permeation column (GPC) separation of Herceptin, thiolated Herceptin or the Herceptin-D-pen immunoconjugate.

a) Coomassie assay of fractions showing the elution of Herceptin in fraction 4.

b) Ellman’s assay on all fractions, showing thiolated Herceptin elution in fraction 4.
Figure E3. *In-vitro* release of D-pen from the Herceptin-D-pen immunoconjugate in non-reducing conditions and reducing (in presence of DTT: 20 mM) conditions after 2 h at room temperature. The D-pen concentration was analyzed with a HPLC assay.
References


68. Linder, M. C., Moor, J. R., Wright, K. *Tumori 1981*, 65, 331-338.


254. Foote, C. S. Mechanisms of photosensitized oxidation. There are several different types of photosensitized oxidation which may be important in biological systems. Science 1968, 162, (857), 963-970.


454. Thakor, D., Spigelman, I., Tabata, Y., Nishimura, I. Subcutaneous peripheral injection of cationized gelatin/DNA polyplexes as a platform


Vita

Anshul Gupte was born on March 30, 1978 in Indore, India. He received his Bachelor’s in Pharmacy degree (2001) from Rajiv Gandhi Technical University (Bhopal, India) graduating first in class of 60 students. He received his Master’s in Science (Pharmaceutical Sciences) degree (2003) from Temple University, School of Pharmacy, Philadelphia. Anshul worked under the supervision of Dr. Kadriye Ciftci in the Department of Pharmaceutical Sciences, School of Pharmacy at Temple University. His master’s thesis title was: Preparation and characterization of Paclitaxel vs. Docetaxel loaded PLGA microspheres for treatment of metastatic breast cancer. A poster summarizing his research was judged second in the Philadelphia Pharmaceutical Forum annual meeting. He joined the Department of Pharmaceutical Sciences Graduate Program at the University of Kentucky in the fall of 2003. Anshul was the recipient of AAPS Graduate Student Symposium in Biotechnology supported by Pfizer Biologics at the AAPS Annual Meeting in 2007. Anshul was awarded 2nd place at the Rho Chi Pharmacy Honor Society Research Day for his poster. In addition, Anshul was selected to present his research in the student symposia at the Annual Meeting of the Parental drug Association (PDA) in 2008. Anshul is an author and co-author of five peer reviewed publication, a book chapter, and one patent application. Additionally, Anshul has three manuscripts submitted for publication and will be a co-author of two other manuscripts that are currently in preparation.
Peer reviewed articles:


**Articles in preparation:**

1. Ma, P., **Gupte, A.**, Mumper, R. J. Preparation, characterization and *in-vitro* evaluation of Idarubicin nanoparticles. *In preparation*


**Book Chapters:**


**Patents:**

Mumper, R. J., **Gupte, A.**, Wadhwa, S., U.S. Patent Application No. 60/978,356 entitled “Polymer-metal chelator conjugates and uses thereof”.

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Author

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Date