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# ROLE OF OXIDATIVE REACTIVE SPECIES AND ANTIOXIDANTS IN METABOLISM AND TRANSPORT OF THERAPEUTIC DRUGS

Svetlana Verenich
*Virginia Commonwealth University*

School of Pharmacy

Virginia Commonwealth University

This is to certify that the thesis prepared by Svetlana Verenich entitled
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has been approved by his or her committee as satisfactory completion of thesis or dissertation requirement for the degree of Master of Science in Pharmaceutical Science

Phillip M. Gerk, Pharm. D., Ph.D., School of Pharmacy

Jürgen Venitz, M.D., Ph.D., School of Pharmacy

Joseph K. Ritter, Ph.D., School of Medicine

Peter R. Byron, Ph.D., Chairman of the Department of Pharmaceutics, School of Pharmacy

Victor A. Yanchick, Ph.D., Dean, School of Pharmacy

F. Douglas Boudinot, Ph.D., Dean of the Graduate School

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

SVETLANA VERENICH
M.S. in Chem. Eng., Lappeenranta University of Technology, Finland, 1998
Ph.D. in Chem. Eng., Lappeenranta University of Technology, Finland, 2003

Director: Phillip M. Gerk, Pharm.D., Ph.D.
Assistant Professor, Department of Pharmaceutics

Virginia Commonwealth University
Richmond, Virginia
April, 2010

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# List of Abbreviations

| | |
|---|---|
| 2Me-E1 | 2-Methoxyestrone |
| 2Me-E2 | 2-Methoxyestradiol |
| 2Me-E2-3G | 2-Methoxyestradiol-3-glucuronide |
| 2Me-E2-17G | 2-Methoxyestradiol-17-glucuronide |
| 2OH-E1 | 2-Hydroxyestrone |
| 2OH-E2 | 2-Hydroxyestradiol |
| 3Ac-2Me-E2 | 3-Acetyl-2-methoxyestradiol |
| 4HNE | 4-Hydroxynonenal |
| ABC | ATP-binding cassette |
| ABCB1 | ATP-binding cassette transporter isoform B1 |
| ABCC1 | ATP-binding cassette transporter isoform C1 |
| ABCC2 | ATP-binding cassette transporter isoform C2 |
| ABCC3 | ATP-binding cassette transporter isoform C3 |
| ABCC4 | ATP-binding cassette transporter isoform C4 |
| ABCC5 | ATP-binding cassette transporter isoform C5 |
| ABCG1 | ATP-binding cassette transporter isoform G1 |
| ABCG2 | ATP-binding cassette transporter isoform G2 |
| Ac | Acetyl group |
| AD | Alzheimer’s disease |

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AMP | Adenosine monophosphate |
| ATP | Adenosine triphosphate |
| AUC | Area under the plasma drug concentration-time curve |
| BCRP | Breast cancer resistance protein |
| bid | Twice daily |
| $C_{max}$ | Maximum concentration of drug |
| Caco-2 | Human colon adenocarcinoma cells |
| CL | Clearance |
| $CO_2$ | Carbon dioxide |
| COMT | Catechol-O-methyltransferase |
| COPD | Chronic obstructive pulmonary disease |
| CYP 450 | Cytochrome P450 |
| CYP2E1 | Cytochrome P450 isoform 2E1 |
| CYP1A1 | Cytochrome P450 isoform 1A1 |
| CYP1A2 | Cytochrome P450 isoform 1A2 |
| CYP1B1 | Cytochrome P450 isoform 1B1 |
| CYP2C9 | Cytochrome P450 isoform 2C9 |
| CYP3A4 | Cytochrome P450 isoform 3A4 |
| CYP3A5 | Cytochrome P450 isoform 3A5 |
| Cys | Cysteine |

| | |
|---|---|
| DIR | Directionality ratio |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| E1 | Estrone |
| E2 | Estradiol |
| E2-3G | Estradiol-3-glucuronide |
| E2-17G | Estradiol-17-glucuronide |
| EDTA | Ethylenediaminetetraacetic acid |
| ER | Estrogen receptor |
| EV | Empty vector |
| GF120918 | Elacridar or N-[4-[2-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide |
| GLUT3 | Glucose transporter, member 3 |
| GSH | Reduced glutathione |
| GSTA4-4 | Glutathione-S-transferase A4-4 |
| HBSS | Hank's balanced salt solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| His | Histidine |
| HPLC | High-performance liquid chromatography |
| $IC_{50}$ | Half maximal inhibitory concentration |
| $K_i$ | Binding affinity of an inhibitor |

| | |
|---|---|
| $K_m$ | Substrate affinity |
| $L^{\bullet}$ | Lipid radical |
| LDL | Low-density lipoprotein |
| LH | Lipid molecule |
| $LOO^{\bullet}$ | Lipid peroxyl radical |
| LOOH | Lipid peroxide |
| LP | Lipid peroxidation |
| Lys | Lysine |
| MDA | Malondialdehyde |
| MDCK | Madin-Darby canine kidney cells |
| MDR1 | Multidrug resistance protein 1 |
| MK571 | (E)-3-[[[3-[2-(7-Chloro-2-quinolinyl) ethenyl] phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid |
| Met | Methionine |
| mRNA | Messenger ribonucleic acid |
| MRP1 | Multidrug resistance associated protein 1 |
| MRP2 | Multidrug resistance associated protein 2 |
| MRP3 | Multidrug resistance associated protein 3 |
| MRP4 | Multidrug resistance associated protein 4 |
| MRP5 | Multidrug resistance associated protein 5 |
| $NO^{\bullet}$ /$NO_2^{\bullet}$ | Nitric monoxide/nitrogen dioxide radicals |
| $ONOO^-$ | Peroxynitrite |

| | |
|---|---|
| $ONOOCO_2^-$ | Nitrosoperoxycarbonate anion |
| ONOOH | Peroxynitrous acid |
| $O_2^{\bullet -}$ | Superoxide |
| $OH^{\bullet}$ | Hydroxyl radical |
| $OH_2^{\bullet}$ | Hydroperoxyl radical |
| OS | Oxidative stress |
| $P_{app}$ | Apparent permeability |
| PD | Parkinson's disease |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| PUFA | Polyunsaturated fatty acid |
| RNS | Reactive nitrogen species |
| Qc | Quercetin |
| SD | Standard deviation |
| *Sf9* | *Spodoptera frugiperda* |
| SULT1A1 | Sulfotransferase isoform 1A1 |
| $t_{1/2}$ | Half-life |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| UDP | Uridine diphosphate |
| UGT | UDP-glucuronosyl transferase |
| UGT1A1 | UDP-glucuronosyl transferase isoform 1A1 |

| | |
|---|---|
| UGT1A3 | UDP-glucuronosyl transferase isoform 1A3 |
| UGT1A7 | UDP-glucuronosyl transferase isoform 1A7 |
| UGT1A8 | UDP-glucuronosyl transferase isoform 1A8 |
| UGT1A9 | UDP-glucuronosyl transferase isoform 1A9 |
| UGT1A10 | UDP-glucuronosyl transferase isoform 1A10 |
| UGT2B4 | UDP-glucuronosyl transferase isoform 2B4 |
| UGT2B7 | UDP-glucuronosyl transferase isoform 2B7 |
| UGT2B17 | UDP-glucuronosyl transferase isoform 2B17 |
| UDPGA | UDP-Glucuronic acid trisodium salt |
| $V_d$ | Volume of distribution |
| $V_{max}$ | Maximum rate of saturable transport or metabolism |
| WHCO3 | Human esophageal carcinoma cells |
| *h* | Hill coefficient |

# Abstract

ROLE OF OXIDATIVE REACTIVE SPECIES AND ANTIOXIDANTS IN METABOLISM AND TRANSPORT OF THERAPEUTIC DRUGS

By Svetlana Verenich, Ph.D.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Major Director: Phillip M. Gerk, Pharm.D., Ph.D.
Assistant Professor, Department of Pharmaceutics

Oxidative stress (OS) is a frequent complication of various disease conditions such as Alzheimer's and Parkinson's disease, atherosclerosis, preeclampsia, rheumatoid arthritis, diabetes including gestational diabetes, etc. OS is defined as an imbalance between the production of reactive species and the ability of an organism to detoxify the reactive intermediates and repair the damage. As a result of OS, the excess of reactive species such as oxygen superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$), 4-hydroxynonenal (4HNE), etc., have a tendency to react with nearby proteins/nucleic acids/lipids changing their functionality or inactivating them completely. The organism has many ways to protect itself from the harmful effects of oxidants. One strategy employs antioxidants introduced to the body with food. The purpose of this thesis was to investigate the effect of reactive species on the active transport mediated by

ABC efflux transporters as well as exploring the possibility of using antioxidants not as interceptors of reactive species but rather as inhibitors of metabolic enzymes and transporters.

The BCRP/ABCG2 efflux transporter was selected for the investigation of the effect of reactive anion, $ONOO^-$, generated during OS and the product of OS, 4HNE, formed after a series of chain reactions involving ROS. Experiments conducted with *Sf*9 membrane vesicles overexpressing BCRP/ABCG2 revealed that both species are capable of inactivating this ABC transporter with $IC_{50}$ being 31 ± 2.7 μM and 92 ± 1.4 μM for $ONOO^-$ and 4HNE, respectively. In presence of 4HNE, $V_{max}$ decreased 4-fold and $K_m$ remained unchanged, suggesting a noncompetitive inhibition mechanism. However, with addition of 4HNE, positive cooperativity was also observed. With $ONOO^-$, the situation was different: both $V_{max}$ and $K_m$ changed consistent with mixed type inhibition. Overall, OS-mediated BCRP/ABCG2 inactivation occurred at biologically relevant concentrations of the reactive species.

Antioxidants are substances that are known to reduce the amount of ROS/RNS accumulated during OS, but this research considered the use of antioxidants not only as interceptors of ROS/RNS but rather as inhibitors of metabolic enzymes. The effect of the dietary antioxidant, quercetin (Qc), on the metabolism of 2-methoxyestradiol (2Me-E2), a promising potential anticancer agent was investigated. Qc possesses five hydroxyl groups, several of which are targets for UDP-glucuronosyltransferases (UGTs). Thus, the simultaneous presence of Qc and 2Me-E2 could result in decreased glucuronidation of 2Me-E2. Using the LS180 intestinal human colon adenocarcinoma cell line,

glucuronidation of 2Me-E2 resulted in formation of only one major glucuronide, 2-Methoxyestradiol-3-glucuronide (2Me-E2-3G). Qc effectively reduced its formation ($IC_{50}$ = 7.8 ± 0.26 μM) to a minimum level. The decrease in the activity of UGTs increased the intracellular concentration of parent 2Me-E2. Additional increase in cellular concentration of 2Me-E2 was achieved when LS180 cells were pre-incubated with Qc prior the addition of 2Me-E2. Transwell experiments with MDCKII – BCRP cells revealed that BCRP/ABCG2 did not appear to transport 2Me-E2.

All in all, the present study showed that OS has a negative impact on active transport mediated by ABC transporters. This, in turn, can affect drug disposition and protection of endogenous organs and tissues. Antioxidants are one of the mechanisms that can effectively reduce the negative impact caused by oxidative species. Nevertheless, this research revealed that they can also be an effective tool to reduce the excessive metabolism of therapeutic drugs. Thus, Qc was found to be a dietary antioxidant that could reduce metabolism of 2Me-E2 and increase it intracellular concentration.

# CHAPTER 1

# Oxidative Stress and Active Transport of Drugs through Cell Membranes

## 1.1. Oxidative Stress

In healthy organisms, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is approximately balanced by the antioxidant defense system. A condition of serious imbalance between ROS/RNS and antioxidants is often referred as oxidative stress (OS). It can be a result of a) an increase in ROS/RNS production (e.g. due to tissues damage, inflammation) or b) depletion of antioxidants (e.g. due to the excessive presence of toxins or elevated amount of oxygen). The former mechanism is thought to be relevant to disease pathology. Some ROS/RNS make a significant contribution to the disease development and some do not; however in most cases OS is a consequence and not a primary cause of the disease process.

Halliwell and Gutterridge (1999) point out that radical species might be involved in disease states as rheumatoid arthritis, preeclampsia (Hubel, 1999), gestational diabetes mellitus (Coughlan et al., 2004), emphysema, hypoxia, Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, etc. For example, in rheumatoid arthritis decease in viscosity of joint fluid is cause by hypoxia conditions. This, in turn, leads to extra production of ROS which causes OS (Okamoto, 2005). In chronic obstructive pulmonary disease (COPD), oxidants inhaled (e.g. with cigarette

smoke) or generated from inflammatory immune and structural cells form peroxidation products. They are capable of damaging proteins in airways, alveolar epithelium and endothelium cells altering their function (Rahman, 2005). RNS modified tyrosine-containing proteins were found in neurodegenerative disorders such as AD and PD. Proteins modified by peroxidation products were detected in nigro-striatal dopamine neurons in PD and neurofibillary tangles in AD (Naoi et al., 2005). Nitrotyrosine residues as well as dityrosine formed in the presence ROS were detected in atherosclerotic plaques (Jang and Surh, 2005; Beckman et al., 1994). A more complete list of pathologies where the involvement of ROS/RNS was claimed to take place has been published elsewhere (Halliwell and Gutterridge, 1999).

Although OS is caused by ROS and RNS, it does not mean that they are unwanted by the organism; ROS/RNS actually play a variety of important physiological roles. Examples include but not limited to: phagocyte killing, control of blood pressure, biosynthesis of thyroid hormone, regulation of apoptosis process and platelet function, stimulation of cell proliferation (primarily by oxygen superoxide), etc. (Halliwell and Gutterridge, 1999). Due to selective reactivity of nitric oxide, hydrogen peroxide, and oxygen superoxide, they are often playing a role of ‘signal molecules’ or secondary messengers. Currently a variety of RNS/ ROS as well as nonradical species are known to exist in organisms. The list of some reactive species and their sources are presented in Table 1 (Halliwell and Gutterridge, 1999; Acworth et al., 1997).

Table 1: Examples of RNS, ROS, and Other Radicals Found in Biological Systems

| Name | Formula | Comments |
|---|---|---|
| **Reactive Oxygen Species (ROS)** | | |
| Hydroxyl | $OH^{\bullet}$ | Formed from hydrogen peroxide in sunlight-exposed skin; hydrochlorous acid reacting with superoxide |
| Superoxide | $O_2^{\bullet-}$ | Produced as a part of metabolism (e.g. from catabolism of xanthene to uric acid) and during the respiratory burst of phagocytic cells |
| Peroxyl/Alkoxyl | $RO_2^{\bullet}/RO^{\bullet}$ | Formed during the breakdown of organic peroxides |
| Hydrogen peroxide | $H_2O_2$ | Produced by enzyme reactions catalyzed by SOD, amine oxidase, urate oxidase, etc.; formed during redox cycling of catecholamines, from microsomal CYP450, respiratory bust of phagocytes, and mitochondrial respiration |
| Hypochlorous acid | HOCl | Produced by the enzyme myeloperoxidase in activated neutrophilis |
| **Reactive Nitrogen Species (RNS)** | | |
| Oxides of Nitrogen | $NO^{\bullet}/NO_2^{\bullet}$ | Nitric oxide is formed *in-vivo* from the amino acid L-arginine; nitrogen dioxide is formed in reaction of nitric oxide with oxygen |
| Peroxynitirite | $ONOO^-$ | Formed in the reaction with nitric oxide and superoxide |
| Nitrocarbonate | $O_2NOCO_2^-$ | Unstable anion nitrosoperoxycarbonate formed after reaction of peroxynitrite with carbon dioxide, which quickly rearranges to form nitrocarbonate |
| Nitrosoperoxycarbonate | $ONO_2CO_2^-$ | |
| **Miscellaneous** | | |
| Thiyl/Perthiyl | $RS^{\bullet}/RSS^{\bullet}$ | A group of radicals that have unpaired electrons residing on sulfur |
| Hydrogen Atom | $H^{\bullet}$ | The simplest free radical |
| Trichloromethyl | $CCl_3^{\bullet}$ | Formed during metabolism of $CCl_4$ in the liver; rapidly reacts with oxygen to form peroxyl radicals |

Control of ROS/RNS levels is a major challenge for the living organism. It has developed a variety of mechanisms to keep balance between antioxidants and reactive species; however, this balance is not 100 percent effective. In general, cells can tolerate mild OS by up-regulating the synthesis of antioxidant defense mechanisms. Otherwise, ROS/RNS can cause irreversible damage to all types of biomolecules, including DNA, proteins, and lipids (lipid peroxidation). It is not clear however, what is the primary target of ROS/RNS since injury mechanisms are widely overlapping. It depends on constitutes of the target, type of reactive species and its amount or, in other words, the severity of OS.

ROS and RNS can directly attack proteins or via 'secondary damage' involving by-products of OS such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE). Although different ROS/RNS are produced in the cells/organism, the attack by hydroxyl radical ($OH^{\bullet}$) is perhaps most common. This radical, which reacts at diffusion-controlled rate, is one of the most aggressive radicals found in the body (Acworth et al., 1997). Due to its non-selectivity toward biomolecules and aggressiveness, $OH^{\bullet}$ can oxidize protein backbones forming hydroxyl protein derivatives and even cause protein breakdown (Berlett and Stadtman, 1997). This might take place if $OH^{\bullet}$ attacks glutamyl, aspartyl, and prolyl side chains. All amino acids are susceptible to oxidation by this radical, but methionine (Met) and cysteine (Cys) are perhaps most prone to the oxidative modifications (Shacter, 2000). Both of these amino acids contain a vulnerable sulfur atom, and their oxidation leads to the formation of disulfides and also sulfoxides or sulfones as in case with Met. However, due to defensive mechanisms (e.g., disulfide

reductases), these modifications can be reversed and have little effect on their biological functions (Berlett and Stadtman, 1997). Other amino acids require more stringent conditions or the presence of transition metals for OS-mediated oxidations, and their structural changes are usually irreversible. Thus, for instance, tyrosine (Tyr) and tryptophan (Trp) are selective targets for peroxynitrite ($ONOO^-$), and their nitration is an irreversible process. The presence of a nitro group in Tyr (i.e., 3-Nitrotyrosine) leads to significant changes in protein functioning. Nitrated Tyr cannot be phosphorylated and this, in turn, inhibits signal transduction (Berlett and Stadtman, 1997). The list of amino acids that are susceptible to oxidative modifications and their possible products are shown in Table 2 (Berlett and Stadtman, 1997; Shacter, 2000).

Amino acid residues are also capable of forming adducts with MDA and 4HNE. These reagents covalently bind to lysine (Lys), histidine (His) and Cys, thus giving them an additional aldehyde moiety (Shacter, 2000). This is an indirect way of protein oxidation. 4HNE and MDA are by-products of lipid peroxidation (LP) initiated by $OH^\bullet$, $HO_2^\bullet$, $ONOO^-$, or peroxyl radicals formed during LP (Halliwell and Gutterridge, 1999; Acworth et al., 1997). This process is an important indication of OS and will be discussed below in greater detail.

Table 2: Amino Acids and Their Oxidation Product/Modifications

| **Amino Acids** | **Oxidation Products/Modifications** |
|---|---|
| Cysteine | Disulfides, Glutathiolation, lipid peroxidation-adducts |
| Methionine | Methionine sulfoxide, Methionine sulfone |
| Tryptophan | 2-, 4-, 5-, 6-, or 7-Hydroxytryptophan, Nitrotyptophan, Kynurenine, 3-Hydroxykynurenine, N-formylkynurenine |
| Phenylalanine | 2-, 3, or 4-Hydroxy- and 2,3- Dihydroxyphenylalanine |
| Tyrosine | Dityrosine, Chlorotyrosine, Nitrotyrosine, crosslinked nitrotyrosine, LP-adducts |
| Histidine | 2-Oxohistidine, Asparagines, Aspartic acid, LP-adducts |
| Arginine | γ-Glutamylsemialdehyde |
| Lysine | α-Aminoadipic semialdehyde, LP-adduct, Glycoxidation adduct, Chloramines, Deamination |
| Proline | 2-Pyrrolidone, 4- or 5- Hydroxyproline, γ-Glutamylsemialdehyde |
| Threonine | Amino-ketobutyrate |
| Glutamyl | Oxalic acid, Pyruvic acid |

Major constituents of biomembranes are proteins and lipids. Unsaturated phospholipids and cholesterol are prominent targets for oxidation not only by ROS/RNS but also by non-radical species such as $ONOO^-$ leading to LP. This process is initiated by any species that can abstract a hydrogen atom. The primary target for hydrogen

abstraction is electron rich unsaturated covalent bonds which are present in polyunsaturated fatty acids (PUFAs). The presence of double bond weakens the attachment of hydrogen atom adjacent to it, which leads to the formation of a PUFA radical. Once formed, this lipid radical ($L^{\bullet}$) is stabilized by a molecular rearrangement to a conjugated diene. From this point, this radical can undergo variety of reactions. Conjugated $L^{\bullet}$ might collide with another lipid radical ($L'^{\bullet}$) within a membrane forming a dimer and cross-link the fatty acids. At this point the LP is terminated. However, most probably, $L^{\bullet}$ combines with molecular oxygen to form a peroxyl radical ($LOO^{\bullet}$). The peroxyl radical is capable of abstracting hydrogen atom from another lipid molecule (LH), thus, propagating the LP process. The abstraction of hydrogen gives lipid hydroperoxide or simply lipid peroxide (LOOH). The more unsaturated bonds are in PUFA, the more LOOHs are going to form during the initiation of LP. In the presence of transition metals (e.g., iron or cooper) LOOH can decompose further to form aldehydes and hydrocarbon gases (Halliwell and Chirico, 1993; Schnelder et al., 2008). In another scenario, $LOO^{\bullet}$ can also attack a double bond in the same lipid molecule forming cyclic peroxide radicals. After cascade of reactions in presence of oxygen, PUFAs get fragmented to a number of different aldehydes such as pentanal, 4HNE, MDA, 2-hexanal, etc. (Esterbauer et al., 1991; Halliwell and Gutterridge, 1999). Continuous oxidation of PUFAs and their fragmentation to aldehydes and hydrocarbons eventually leads to loss of membrane integrity. Prior to that LP can also be terminated if lipid to protein ratio decreases sufficiently so that $LOO^{\bullet}$ has to react with proteins (Acworth et al., 1997). The molecules that enter into LP cascade of reactions are shown in Figure 1 (Halliwell and Gutterridge, 1999; Muriel, 1997; Kubala and Lojek, 2008).

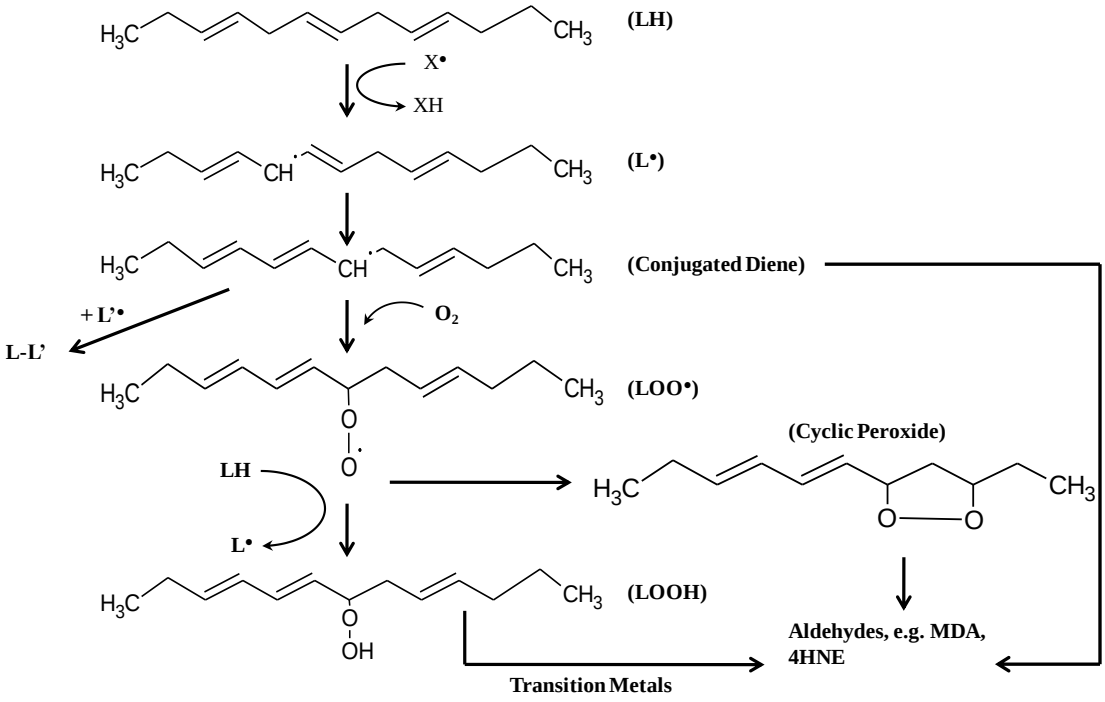



Figure 1: Mechanism of lipid peroxidation (LP)

LP has devastating consequences for living organism. The overall effect of LP are impairment of the membrane functionality by decreasing its fluidity, to make it easier for phospholipids to exchange between monolayers, increase the 'leakiness' of the biomembranes to the molecules that previously had to use specific channels/transporters to cross a membrane (Halliwell and Gutterridge, 1999; Muriel., 1997). Crosslinking of membrane lipids (L-L') also decreases lateral and rotational mobility of proteins (Halliwell and Gutterridge, 1999). The final products of LP are MDA and 4HNE which are known mutagenic and carcinogenic aldehydes. The latter is a less mutagenic compound than MDA but more toxic. It can react with proteins, change their functions or

even inactivate them. More detailed properties of 4HNE and its reactivity with biomolecules are discussed in Chapter 1.1.2.

### 1.1.1. Peroxynitrite

Peroxynitrite is readily formed from reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$. These two radicals react at near diffusion-controlled rates with a reaction rate constant reported to be between $3.4 \times 10^7$ to $6.7 \times 10^{10}$ $M^{-1}$ $s^{-1}$ (Acworth et al., 1997; Portugal and Kohen, 2008). The formation of peroxynitrite ($ONOO^-$) depends on the concentration of both reactants. $NO^{\bullet}$ is highly diffusive radical having biological half-life ($t_{1/2}$) of several seconds whereas $t_{1/2}$ for $O_2^{\bullet-}$ is less than 1 ms, (Portugal and Kohen, 2008). This means that $ONOO^-$ should form near the sites of $O_2^{\bullet-}$ formation. The rate of $ONOO^-$ formation *in vivo* was estimated to be about 0.8 to 1.7 μM $s^{-1}$ (Szabó at al., 2007). Despite that relatively short $t_{1/2}$ (~ 10 ms), it can easily cross membrane and damage cells in a distance from its formation site, approximately 5 – 20 μm (Szabó at al., 2007).

$ONOO^-$ is a pH-dependent anion which is highly stable at basic pH. However, at physiological pH, it is rapidly protonated to form peroxynitrous acid (ONOOH) with $t_{1/2}$ between 0.1 to 1 second (Portugal and Kohen, 2008; Acworth et al., 1997). For instance, at pH 7.4 the ratio of $ONOO^-$ to ONOOH was reported to be approximately 3 to 7 (Portugal and Kohen, 2008). From this point, ONOOH can react directly with different types of biomolecules or undergoes homolytic fission forming $NO_2^{\bullet}$ and $OH^{\bullet}$. One should mention that the fate of $ONOO^-$ is also depends on the amount of carbon dioxide ($CO_2$) present in the system. $CO_2$ readily reacts with $ONOO^-$ (rate constant of $4.6 \times 10^4$ $M^{-1}$ $s^{-1}$), resulting in nitrosoperoxycarbonate anion ($ONOOCO_2^-$) with $t_{1/2}$ of about 1μs

(Alvarez et al., 2004; Halliwell and Gutterridge, 1999; Portugal and Kohen, 2008). Further homolysis of this anion leads to formation of two highly reactive radicals: carbonate ($CO_3^{\bullet-}$) and $NO_2^{\bullet}$. These species readily react with different biomolecules causing secondary OS. Partially these reactions are also may be attributed to action of $ONOO^-$, e.g. nitration of tyrosine (Halliwell and Gutterridge, 1999).

Many biomolecules are oxidized or nitrated by ONOO-derived radicals such as DNA, PUFAs, amino acid residues of proteins (see Table 2). Perhaps the most studied reaction is the conversion of Tyr to 3-nitrotyrosine. The modification of this amino acid residue can cause protein crosslinking, inactivation of enzymes, and also interfere with signal transduction (Halliwell and Gutterridge, 1999). Intensive formation of nitrotyrosine residues was evident in patients with such diseases as AD, PD, COPD, asthma, and atherosclerosis (Beckman et al, 1994; Naoi et al, 2005; Hanazawa et al., 2000). Besides Tyr, $ONOO^-$ also modifies other amino acids such as phenylalanine, Trp, Met, and Cys, attacks deoxyribose, guanine, gluthathione transferases, actin, manganese superoxide dismutase, unsaturated fatty acids (Acworth et al., 1997; Halliwell and Gutterridge, 1999). More detailed discussion of pathophysiology of peroxynitrite is published by Szabó at al. (2007).

### 1.1.2. 4-Hydroxynonenal

4-hydroxynonenal (4HNE) is formed during the lipid peroxidation of linoleic and arachidonic fatty acids by cleavage of LOOH in the presence of transition metal ions, e.g. iron or copper (Schnelder et al., 2008; Sayre et al., 2006).

4HNE generated *in vivo* rapidly reacts with amino acids of proteins and does not remained free. This aldehyde owes its reactivity mainly to three functional groups: aldehyde group, the double bond, and hydroxyl group (Figure 2). The double bond (C3) serves as a site for the Michael addition of sulfur atom of Cys, imidazole nitrogen of His and to amine group of Lys (Figure 3a-c). The 4HNE reactivity with Lys is much less than with the former two amino acids, and it is even lesser with nitrogen of arginine (Grimsrud et al., 2008). Because 4HNE also contains a reactive aldehyde group, it can form Schiff bases with amino groups of His and Lys (Figure 3d). If a Schiff base formed with Lys residues, this adduct of 4HNE can also co-exist in dynamic equilibrium with its cyclic form as shown in Figure 3e (Sayre et al., 2006). Moreover, after formation of Michael adducts, 4HNE can covalently bind (through C1) to adjacent Lys residues producing intra- or intermolecular cross-linked amino acids. Cyclization of 4HNE adducts can be reversible or irreversible (Esterbauer et al., 1997). Thus, after Michael addition of 4HNE to amine group of deoxy-guanosine, this adduct rapidly undergoes cyclization as shown in Figure 3f. The ability of 4HNE to react rapidly with thiol groups at physiological pH accounts for much of its cytotoxicity: this aldehyde quickly reacts with glutathione depleting the cells of their defensive mechanism.

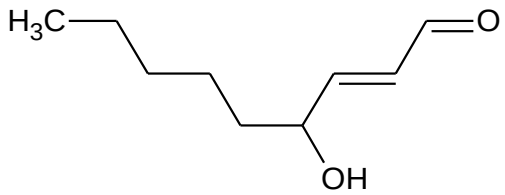



Figure 2: Structure of 4-hydroxynonenal (4HNE)

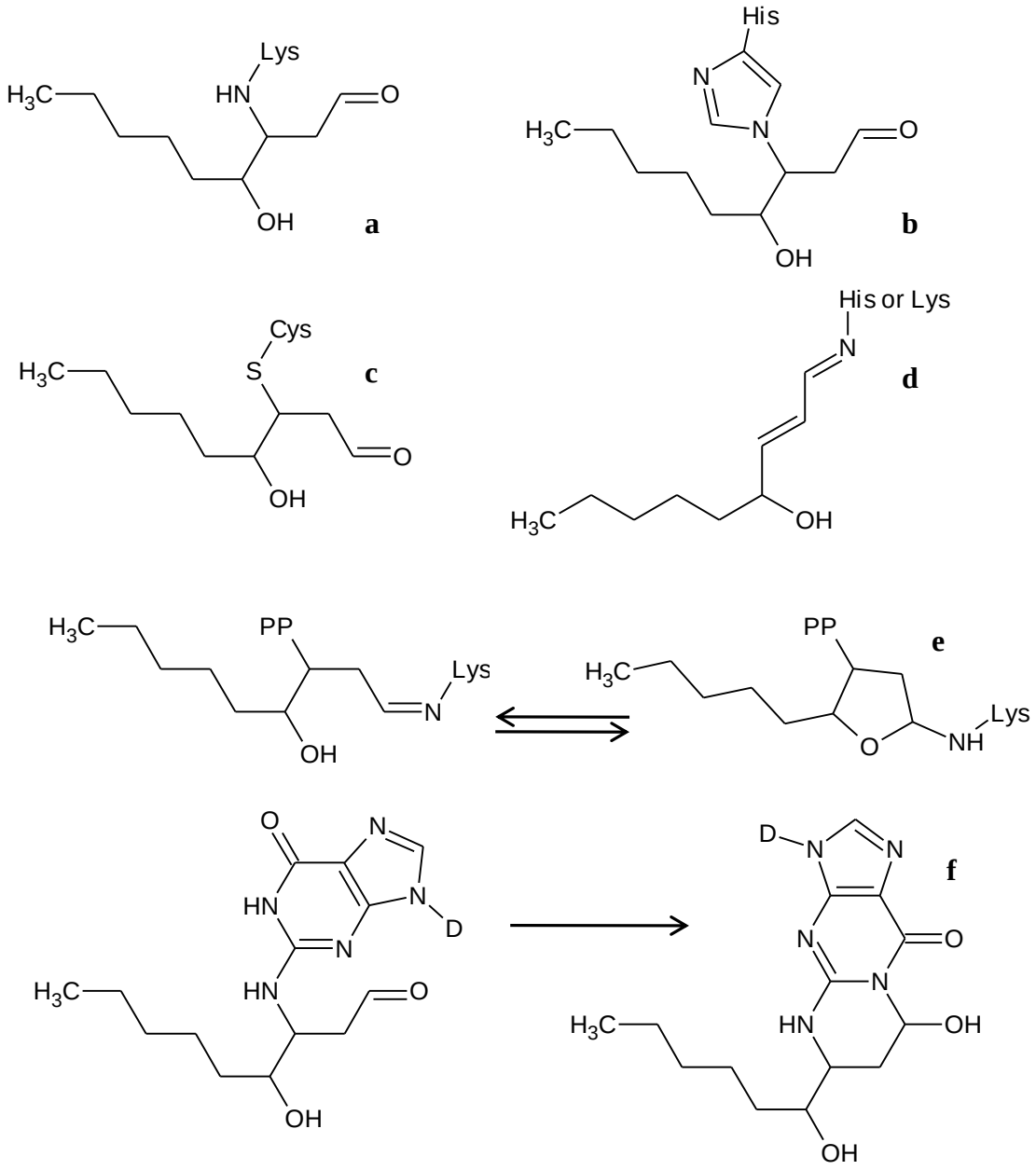



Figure 3: Examples of reactions of 4-hydroxynonenal (4HNE) with amino acids/nucleosides. **a**: With lysine (Lys); **b**: With histidine (His); **c**: Cysteine (Cys); **d**: Formation of Schiff base with histidine (His) or lysine (Lys). **e**: Formation of Schiff base directly or after formation of Michael adduct with amino acid residues of proteins (PP), with possible cyclization afterwards; **f**: Reaction with deoxy-guanosine, with formation of Michael adduct followed by rapid cyclization. 'D' corresponds to deoxyribose ring (Sayre et al., 2006; Esterbauer et al., 1991; Kinter, 1996; Siegel et al., 2007).

Because of high reactivity toward Cys, His, and Lys protein residues, the most common effect of 4HNE is alteration or inactivation of enzyme or protein. Thus, it was reported that 4HNE has been able to inactivate $Na^+$-$K^+$-ATPase (Miyake et al., 2003) and the GLUT3 transporter (Mark et al., 1997). Modifications of the arginine residue of glutathione peroxidase amplify oxidative stress by inhibition of this enzyme's protective activity against peroxide, thereby allowing the peroxide level to rise (Grimsrud et al, 2008). The extent of adducts formed with Cys, His, and Lys residues of protein in airways and alveolar epithelial and endothelial cells was found to correlate with forced expiratory volume 1 in COPD patients (Rahman, 2005). 4HNE is also capable of modifying 45 Lys, 7 His, 23 serine, 53 Tyr residues, and 2 SH-groups in low-density lipoprotein (LDL) (Esterbauer et al, 1991), and the presence of 4HNE-modified LDL was identified in atherosclerotic humans and animals (Halliwell and Gutterridge, 1999). Crosslinked 4HNE-modifed amyloid β peptides implicated in neurotoxic processes were found to form during the course of AD (Speigel et al., 2007). 4HNE-modified proteins were also detected in nigro-striatal dopamine neurons in PD patients (Naoi et al., 2005).

Generally, the consequences of 4HNE presence in the cells/organism can vary depending on its concentration and can be divided into the following three categories (Halliwell and Gutterridge, 1999; Esterbauer et al., 1991; Grimsrud et al, 2008):

a. At concentrations of 100 μM and above, 4HNE exhibits acute toxic effects and causes rapid cell death, inhibition of mitochondrial function and anabolic processes such as DNA, RNA and protein synthesis. Such concentrations can be achieved *in vivo* but only locally, close to peroxidizing membranes.

b. At concentrations between 1 to 20 μM, 4HNE induces cell cycle arrest, might partly inhibit DNA and protein synthesis, ADP-ribosyl transferase, CYP2E1, and CYP1A1, but stimulates phospholipase $A_2$. The effect of 4HNE can vary at this concentration range depending on the defensive mechanisms present in each cell.
c. Concentrations of 1 – 0.1 μM and lower represent the basal level of 4HNE in the organism and probably exist in protein-bound forms. Below 0.1 μM 4HNE has simulative properties. For example, it stimulates the activities of guanylate-cyclase, phospholipase C, and adenylate cyclase.

A variety of antioxidant mechanisms exist to reduce the toxicity of 4HNE and remove it from the system. For instance, aldehyde dehydrogenase, alcohol dehydrogenase and aldehyde reductase convert 4HNE to carboxylic acid and alcohol, respectively (Grimsrud et al., 2008). However, the major route of 4HNE detoxification is formation of 4HNE-glutathione adduct by glutathione-S-transferase. Glutathione-S-transferase A4-4 (GSTA4-4) exhibits specific activity toward 4HNE aldehyde (Bruns et al., 1999). Once the adduct is formed, it can be eliminated from the cells by the RLIP76 transporter, multidrug resistant proteins 1 (MRP1) or 2 (MRP2) (Grimsrud et al., 2008; Renes et al., 2000; Reichard et al., 2003).

## 1.2. ABC Transporters: an ATP-dependent Efflux of Drug Molecules

ATP-binding cassette (ABC) transporters are one of the largest families of integral membrane proteins that responsible for the active transport of wide variety of endogenous

compounds and xenobiotics, their metabolites and conjugates. To date, forty nine human ABC transporters have been characterized and categorized into seven subfamilies (ABCA to ABCG) (Velamakanni et al., 2007). ABC transporters utilize the energy released after ATP hydrolysis to pump the substrates/drugs out of the cells, often against the concentration gradient. Most ABC transporters contain two ATP-binding domains, also known as nucleotide binding domains, and two putative transmembrane segments. Typically, each transmembrane domain consists of six membrane spanning α-helices, e.g. multidrug resistance (MDR1) or ABCB1 transporter, MRP4/ABCC4 and MRP5/ABCC5. The exceptions are breast cancer resistance protein (BCRP) or ABCG2, MRP1/ABCC1, MRP2/ABCC2 and MRP3/ABCC3 (Schinkel and Jonker, 2003). All of them have the same basic structure, but the former is a half transporter as it contains only one transmembrane segment of six helices, whereas MRP1/ABCC1 through MRP3/ABCC3 transporters have a third segment containing five α-helices. Some of the extracellular loops in ABC transporters are heavily N-glycosylated. The N-glycosylation does not affect their transport properties, but it is most like responsible for the protein folding and its stability within the cell membrane (Schinkel and Jonker, 2003). Nucleotide binding domains contain two highly conserved sequence elements, Walker A and Walker B, separated by about 120 amino acids (Velamakanni et al., 2007). These motifs are common to most ATP-binding proteins. The third element, signature or C motif, is specific only to ABC transporters and located between Walkers A and B. Its exact role is not known, but it was implicated in recognition, binding, and hydrolysis of ATP (Leslie et al., 2005). All these three elements are required to bind ATP, hydrolyze it, and use the released energy to transport a drug molecule across the membrane.

Amongst ABC transporters, MDR1/ABCB1, BCRP/ABCG2, and MRP/ABCC 1 – 5 are implicated in the transport of clinically relevant drugs (Schinkel and Jonker, 2003, Leslie et al., 2005). There is a significant overlap between drug-substrates among these transporters considering their structural differences. Thus, MDR1/ABCB1 can transport or extrude a wide variety of compounds, the majority of which are bulky hydrophobic molecules with molecular weight ranging from about 200 to 1900 Da. They are typically uncharged or slightly positively charged compounds that might contain either aromatic or non-aromatic groups (Schinkel and Jonker, 2003; Bodó et al., 2003). Some acidic compounds, e.g. methotrexate, can be also transported although at lower rates (Schinkel and Jonker, 2003). These are some classical substrates of MDR1/ABCB1: colchicine, doxorubicin, vinblastine, digoxin, cisplatin, paclitaxel, anthracyclines, epipodophyllotoxins. Just as MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2 and MRP3/ABCC3 can also extrude uncharged hydrophobic or weakly basic molecules; however, their main distinguished feature is the ability to extrude xenobiotics as glutathione, glucuronide, and sulfate conjugates (Leslie et al., 2005; Jedlitschky et al., 2006). MRP2/ABCC2 and MRP3/ABCC3 are the major players in transport of conjugates of organic anions, whereas MRP4/ABCC4 and MRP5/ABCC5 have special functions as transporters of nucleosides such as 9-(2-phosphonyl methoxyethyl) adenine and purine analogs, and may play role in cGMP secretion (Bodó et al., 2003; Dean et al., 2001).

Tissue distribution and cell localization of ABC-efflux transporters play an important role in drug absorption, distribution, metabolism, excretion, and protection against toxins/xenobiotics. Thus, MRP1/ABCC1 is expressed in most tissues and can be

found, for instance, in the lung, testis, small and large intestines, kidney, and liver (Leslie et al., 2005; Schinkel and Jonker, 2003). MRP1/ABCC1 is primarily localized on the basolateral cell membrane indicating that it is responsible for the efflux of the substrates into the blood. In the blood-brain barrier however, MRP1/ABCC1 located on the apical side of capillary endothelium (Leslie et al., 2005). MDR1/ABCB1 and MRP1/ABCC1 have quite extensive but not complete overlap in tissue distribution. Unlike MRP1/ABCC1, the former protein is primarily located on the apical membrane surface (Bodó et al., 2003; Schinkel and Jonker, 2003). MRP2 and BCRP/ABCG2 have more limited tissue distribution than MRP1/ABCC1 or MDR1/ABCB1. Both these proteins can be found in blood-brain barrier, placenta (syncytiotrophoblast), liver (canalicular membrane of hepatocytes), and villous membranes of small intestines; however, there are other tissues where only one of these transporter is expressed (Jedlitschky et al., 2006; Allen and Schinkel, 2002). Like MDR1/ABCB1, these proteins are localized in the apical membrane surface (Schinkel and Jonker, 2003). The localization and tissue distribution of MDR1/ABCB1, MRP2/ABCC2, and BCRP/ABCG2 point out that they can modulate the bioavailability of orally administered drug in addition to the protective functions and xenobiotics/metabolites excretion from the body.

### 1.2.1. Breast Cancer Resistant Protein (BCRP)

BCRP/ABCG2 is a half transporter of 72 kDa, composed of 655 amino acids (Schinkel and Jonker, 2003; Velamakanni et al., 2007). It consists of only one transmembrane domain of six α-helices and one ATP-binding cassette. However, it is likely to function as a homodimer bridged by a disulfide bond of Cys residues.

BCRP/ABCG2 protein contains eleven Cys residues, three of which are located on the external loop. These three Cys residues form two intra- and one intermolecular disulfide bonds, responsible for ATP activity, protein stability and cellular localization (Wakabayashi et al., 2006).

BCRP/ABCG2 shares less than 20% amino identity with MDR1/ABCB1, MRP1/ABCC1 and MRP2/ABCC2. Nevertheless, there is considerable overlap in substrate specificity between these proteins. BCRP/ABCG2 transports a narrower range of anticancer drugs (Leslie et al., 2005). Some of them are anthracyclines, doxorubicin, mitoxantrone, and topotecan. On the other hand, epipodophyllotoxins, paclitaxel, vincristine, verapamil are not substrates of BCRP/ABCG2 (Allen and Schinkel, 2002; Doyle and Ross, 2003). Like MDR1/ABCB1, BCRP/ABCG2 is a primary transporter of parent drug substrates and similar to MRPs/ABCCs, it can also transport sulfate and glucuronide conjugates of organic anions, e.g., estrone sulfate and SN38-glucuronide. Several members of ABCG subfamily are known to transport steroids. BCRP/ABCG2 was primarily characterized as multidrug resistance transporter and yet this protein might also transport estrone and 17β-estradiol (Janvilisri et al., 2003; Imai et al., 2002).

Its plasma membrane location differentiates BCRP/ABCG2 protein from almost all half transporters, except ABCG1 (Doyle and Ross, 2003). It was found to be expressed in apical membranes of lung, blood-brain barrier, placenta (syncytiotrophoblast), liver (canalicular membrane of hepatocytes), duct and lobules of the breast, and villous membranes of small and large intestines (Maliepaard et al., 2001; Allen and Schinkel, 2002; Leslie et al., 2005). The greatest expression of BCRP/ABCG2 was found, however, in the placenta, about 100 times higher than in brain, small intestine or liver (Doyle and

Ross, 2003). Protection from xenobiotics and excretion of steroid conjugates is one of the main functions of BCRP/ABCG2 (e.g. in placenta and blood-brain barrier), however its hepatic and intestinal presence infers its role in limiting bioavailability of orally administered drugs.

# CHAPTER 2

# Role of Dietary Antioxidants in Drug Glucuronidation

## 2.1. Dietary Antioxidants

There is no exact definition for 'antioxidant' but it can be roughly described as agents of low molecular mass or more complex structure, such as enzymes, that are capable of direct reaction with free radicals or ROS/RNS, decreasing their concentration or indirectly, via prevention of formation of oxidative species. Generally, in order to be called 'antioxidant', an agent must be able to donate an electron or hydrogen atom to prevent an oxidative process (Packer, 2005). Antioxidants can act in different ways by metal chelating, scavenging free radicals and other reactive species, acting as chain breakers, being as a part of the redox antioxidant network, and/or regulating gene expression (Packer, 2005).

Antioxidants come from different sources. They can be present *in vivo* (superoxide dismutase, transferrin, peroxidase), be synthesized *in vivo* such as GSH and uric acid, or come from exogenous exposure. This last group of antioxidants includes dietary or natural antioxidants that get absorbed with food. The major natural antioxidants include vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), polyphenols (quercetin, naringin, apigenin) and carotenoids (carotene, lutein, zeaxanthin). Vitamins C and E act as redox substances that can be oxidized and reduced

(Packer, 2005; Halliwell and Gutterridge, 1999). Both of them can scavenge $LO_2^{\bullet}$, $OH_2^{\bullet}$, $O_2^{\bullet-}$, and react with $OH^{\bullet}$ at diffusion control rates. Due to the hydrophilic nature of vitamin C, it can also scavenge hydrophilic peroxides. Carotenoids act as free radical sinks forming carotenoid radicals that can undergo either dismutation or interception with another free radical producing non-radical compounds (Halliwell and Gutterridge, 1999). Flavonoids, a subgroup of polyphenols, have several mechanisms protecting organism from the unwanted oxidative species (Packer, 2005):

a. They chelate the transition ions (i.e. $Fe^{2+}$, $Cu^{+}$) preventing formation of new free radicals and reactive species.
b. They scavenge the radicals and ROS/RNS directly.
c. They induce enzymes that participate in detoxification and cancer prevention, e.g. manganese superoxide dismutase and gamma-glutamylcysteine synthetase.

### 2.1.1. Quercetin

Quercetin (Qc) is a major representative of dietary flavonol, a subgroup of flavonoids. This compound is found ubiquitously in food of plant origin such as onion, lettuce, broccoli, cranberry, apple skin, olive, red wine, and tea (Halliwell and Gutterridge, 1999). The exact amount of Qc in food is not known but it was roughly estimated to be 2 to 250 mg/kg of fruits, 0 to 100 mg/kg of vegetables, 4 to 16 mg/l in red wine, and 10 to 25 mg/l in tea (Manach et al., 1998).

Qc was widely studied because of its antioxidative properties exhibited via chelating iron ions and reactivity with lipid peroxyl radicals (Afanasev et al., 1989). It

was found to be effective in preventing atherosclerosis by halting oxidation of low density lipoproteins (LDL) (Manach et al., 1998; de Whalley et al., 1990). Qc has been also reported to inhibit *in vitro* the growth of several human cancers which includes gastric, breast, esophagus, lung and nasopharyngeal cancers (Choi et al., 2001; Cheong et al., 2004; Kuo et al., 2004; Ong et al., 2004, Yoshida et al, 1990).

Little is known about absorption and distribution of Qc or quercetin aglycone in the body, but a number of experiments were conducted to establish the extent of oral absorption and bioavailability of Qc glucosides, a typical form of Qc present in food. Studies on healthy volunteers have established that $C_{max}$ was achieved after about 3 hrs of food ingestion containing 60 – 90 mg of Qc with $t_{1/2}$ being 0.87 hrs, 3.8 hrs and 16.8 hrs for absorption, distribution and elimination phases, respectively (Hollman et al, 1996; Manach et al., 1998). $C_{max}$ for Qc aglycone (in a pure form, not in food) was reached with some delay, after 1.9 to 4.8 hr, for doses between 8 to 50 mg (Erlund et al., 2000). Bioavailability of Qc glucosides can be as high as 52%, whereas quercetin aglycone had bioavailability of only 24% (Hollman et al., 1995).

Once Qc is absorbed, it undergoes extensive metabolism. Due to the presence of five hydroxyl groups (Figure 4), it forms methylated, glucuronidated or/and sulfated conjugates (Erlund et al., 2000; Manach et al., 1998; Van der Woude et al., 2004). Analysis of plasma from volunteers fed with a quercetin-supplemented diet shows that quercetin is mainly circulating as quercetin glucuronides (Yang et al., 2006). The major sites for Qc metabolism are 7-, 3-, 3'- and 4'-hydroxyl moieties with UGT1A1, UGT1A3, UGT1A8 and UGT1A9 being the most active UGT isoforms (Boersma et al., 2002; Chen et al., 2005). The apparent $K_m$ for the formation of 7-, 4'-, and 3'-

glucuronides determined with liver cell-free extract were found to be 6.5 ± 0.9 μM, 0.6 ± 0.2 μM, and 0.8 ± 0.2 μM, respectively (Day et al., 2000). However, incubations with recombinant UGTs resulted in values of about 10-fold higher (Chen et al., 2005). 3'- and 4'-hydroxyl groups are the first moieties attacked by UGTs, and interestingly these groups are primarily responsible for the pro-oxidant properties of this flavonoid (Boersma et al., 2002; Day et al., 2000).

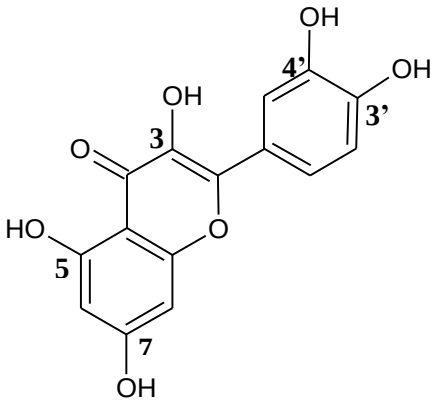



Figure 4: Structure of quercetin (aglycone).

Being absorbed through the intestine wall, Qc forms glucuronide conjugates which tend to be excreted back into the gut via active transport. It was established that Qc glucuronides, especially 7-O-glucuronosyl Qc, are likely to be transported via MRPs and are capable of inhibiting MRP1/ABCC1 and to the lesser degree MRP2/ABCC2 (van Zanden et al., 2007;). Additionally, Qc is a an inhibitor for MDR1/ABCB1, MRP1/ABCC1 and BCRP/ABCG2 transporters (Wang et al., 2004; Choiprasert et al, 2010; Yoshikawa et al., 2004). $K_i$ values were determined to be 0.33 μM, 1μM, and 0.28 μM for MDR1/ABCB1, MRP1/ABCC1, and BCRP/ABCG2, respectively (Choiprasert et al, 2010; Yoshikawa et al., 2004).

## 2.2. Phase II Metabolism: Drug Glucuronidation

Phase II metabolism includes what is known as conjugation reactions. In general, the conjugation reactions with endogenous substrates occur on the metabolites of a parent compound after Phase I metabolism; however, in some cases, the parent compound itself can be subject to Phase II metabolism. Typical conjugation reactions of Phase II metabolism include glucuronidation, sulfonation, glutathione-conjugation, N-acetylation, methylation, and conjugation with amino acids (amidation). The enzymes that are involved in these reactions, respectively, uridine diphosphate-glucuronosyltransferases (UGTs), sulfotransferases, N-acetyltransferases, glutathione S-transferases, methyltransferase, and amidotransferases.

Glucuronidation is quantitatively the most important conjugation reaction of xenobiotics/drugs. It is generally considered to be a low-affinity and high-capacity reaction in which glucuronic acid is conjugated with alcohols, phenols, amines, amides, carboxylic acids and thiols. By addition of glucuronic acid, the conjugates become more polar, get ionized at physiological pH ($pK_a$~ 4.0) and increase their molecular weight by 176 Da (Remmel et al., 2008). These modifications result in excretion of glucuronides via the kidney either by glomerular filtration, or active secretion, or both. Glucuronide formation is generally considered to be the final metabolic step. However, recent evidence suggests that monoglucuronides of nonsteroidal anti-inflammatory agents might also be substrates for CYP2C9 oxidation, and monoglucuronides of Qc can undergo further glucuronidation, sulfation or methylation (Remmel et al., 2008; O'Leary et al., 2003).

There are more than 60 UGTs identified in mammalian species, and 16 functional UGTs were found in humans (Albert et al., 2000; McCarver and Hines, 2002). Those found in human are present in the liver, lung, kidney, stomach, intestine, skin, spleen, thymus, heart and brain (Kwon, 2001). UGTs can be subdivided: hepatic (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, 2B17), extra-hepatic (2B4, 2B17) and gastro-intestinal (1A7, 1A8, 1A10) (Coleman, 2005). Being well-expressed in liver, mRNA of UGT1A1 was also found in duodenum, ileum, and stomach at relative levels of approximately 10:4:1, respectively (Basu et al., 2004a). UGT1A7 is present in nearly every tissue with high levels in esophagus, thyroid and adrenal glands, with the highest being in esophagus (Basu et al., 2004b). mRNA of UGT1A8 was found at moderate to high levels in esophagus, duodenum ileum, colon, and stomach. mRNA of UGT1A9 was detected in high levels in distal parts of GI tract such as jejunum, ileum, colon and rectum. The highest amounts, however, were found in kidney and liver (Basu et al., 2004b). UGT1A10 appeared to be present in duodenum, ileum, colon, stomach, and esophagus at relative levels of 9:8:3:2:1, respectively (Basu et al., 2004a). Therefore, orally administered drug can theoretically undergo extensive metabolism starting from esophagus by UGT1A7, followed by UGT1A8 in stomach (empty), then in duodenum by UGT1A1, 1A7, 1A9, and 1A10, in ileum by UGT1A1, 1A9 and 1A10, in colon by UGT1A7, 1A9, and 1A10, and of course, in the liver.

### 2.2.1. 2-Methoxyestradiol

2-Methoxyestradiol (2Me-E2) is an endogenous and nonpolar metabolite of estradiol (E2) that undergoes extensive Phase II metabolism. Generally, formation and

metabolism of estrogens are rather complex; thus, Figure 5 illustrates only 2Me-E2 formation and its possible pathways of elimination (Martucci, 1983; Lakhani et al. 2003; Dubey and Jackson, 2009; Parl et al., 2009). Estrone (E1) and E2 are substrates for the phase I enzymes, CYP1A1 and 3A4. Oxidative metabolism by these enzymes leads mainly to the formation 2-hydroxyestrogens with 4-hydroxyestrogens being minor products of CYP- mediated metabolism (Lee et al., 2003). Catechol-O-methyltransferase or (COMT) subsequently O-methylates the 2-hydroxyl group to produce 2Me-E2 or 2-methoxyestrone (2Me-E1). In the presence of CYP enzymes or methoxyestrogen demethylase (Paul et al., 1983) methoxyestrogens can be transformed back to hydroxylated form. It is believed that glucuronidation is a major pathway of 2Me-E2 elimination. The presence of hydroxylated and dimethylated metabolites of 2Me-E2 was also detected *in vitro*, but as a minor fraction (Lakhani et al., 2007).

Many tissues can actively produce 2Me-E2, since COMT is a ubiquitous enzyme found in different organs such as liver, kidney, intestine, stomach, spleen, brain, pancreas, lung, etc. (Männisto and Kaakkola, 1999). The exact endogenous 2Me-E2 concentrations in tissues are not known and even the serum concentrations of 2Me-E2 were frequently reported in combination with 2-methoxyestrone (Berg et al., 1983). Recently, Xu et al. (2007) have developed an analytical method to detect different methoxyestrogens and reported total concentration of 2Me-E2 in the blood serum to be about 9.4 ± 1.3, 10.6 ± 7.9, and 2.5 ± 0.6 pg/ml in luteal, follicular and postmenopausal women, respectively. The concentrations of unconjugated form of 2Me-E2 were about half of the above values. 2Me-E2 concentrations however, might increase by 1000 during last months of pregnancy (Berg et al., 1983).

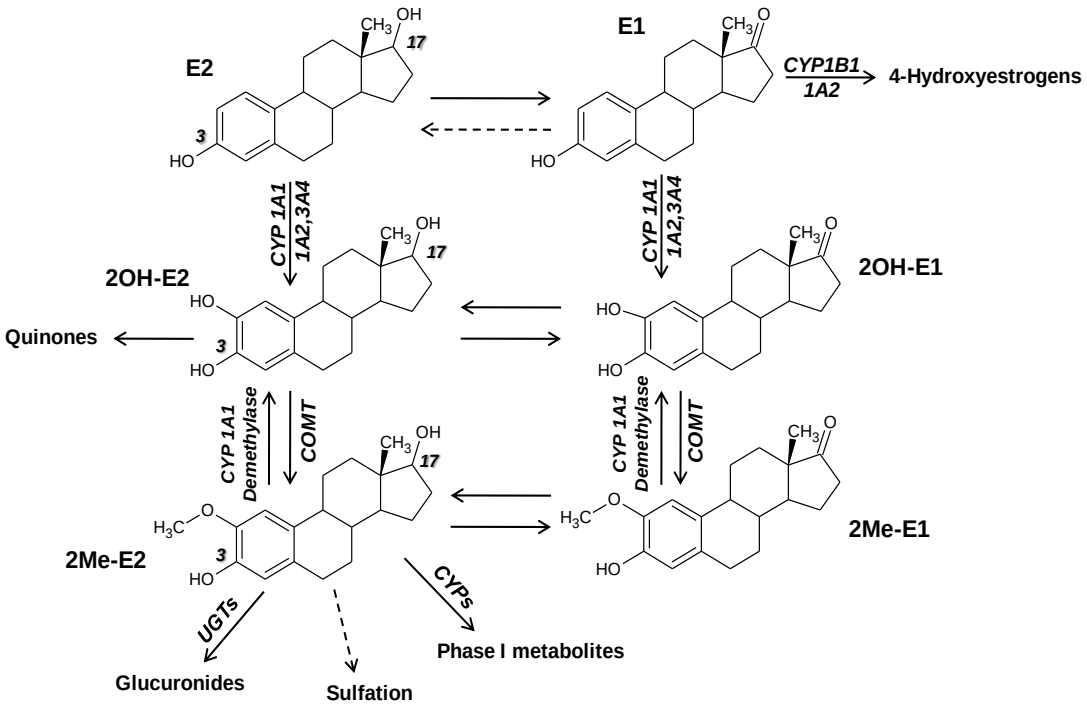



Figure 5: Pathways of 2Me-E2 formation and elimination.

During the last decade 2Me-E2 has received attention due to its anticancer activities. Phase I and II clinical trials revealed that orally administered 2Me-E2 is well tolerated by patients with only grade 2 and 3 toxicities observed (Dahut et al., 2006; Sweeney et al., 2005; James et al., 2006; Rajkumar et al, 2007). No maximum tolerated dose, however, was determined. It was also observed that during the treatment 2Me-E2 concentrations in plasma remained in the nanogram per milliliter range, partially due to its extensive presystemic metabolism. As for *in vitro* antiproliferative activity, the majority of 60 cell lines from NCI are found to be sensitive to 2Me-E2 with inhibitory concentrations between 0.08 to 5.0 μM (Pribluda et al., 2000; Papathanassiu et al., 2001). The list of the tumor cells that respond to the treatment with 2Me-E2 continues to increase. For instance, recent studies on esophageal (WHCO3), nasopharyngeal (CNE2)

carcinoma cells, and tumor-derived uterine leiomyoma cell lines (ELT3 and huLM) revealed to be an effective antiproliferative agent with inhibitory concentrations between 1 to 20 μM (Thaver et al., 2009; Zhou et al., 2004; Salama et al., 2006).

It is believed that 2Me-E2 acts on tumor growth directly by reducing cell proliferation or by inducing apoptosis, and also by inhibiting angiogenesis (Pribluda et al., 2000). Apoptosis induced by 2Me-E2 occurs by upregulating death receptor 5 (DR 5), increasing expression of STAT1 protein, and activating p53, caspases 9, 8, and 3 (Chamaon et al., 2005, Pribluda et al., 2000, Abou El Naga et al., 2009, Li et al., 2004). The increase in STAT1 expression and activation of caspase are required as apoptosis is often blocked by caspases inhibitor Z-VAD-FMK. The interference in tumor microtubule formation takes place via inhibiting the tubulin polymerization rate by competitively inhibiting colchicine-binding sites. In comparison with other competitive inhibitors of colchicine-binding sites, 2Me-E2 is atypical since its $IC_{50}$ was found to be one or two magnitudes lower than other inhibitors (Pribluda et al., 2000). The $K_i$ value 2Me-E2 inhibition of tubulin polymerization is 22 μM (Papathanassiu et al., 2001). Thus, by binding to the colchicine-binding site, 2Me-E2 inhibits the tubule formation or alters its stability. Generally, apoptosis requires much lower 2Me-E2 concentrations than those to attain interference in tubulin polymerization (Chamaon et al., 2005).

Most investigators observed that the antiproliferative activity of 2Me-E2 is time- and concentration-dependent, whereas it is estrogen receptor (ER) independent. The affinity of 2Me-E2 to ER α and β was established to be about 0.5% and 0.008% of estradiol (E2), respectively (LaVallee et al., 2002; Pribluda et al. 2000; Papathanassiu et al., 2001). However, Lui and Zhu (2004) have observed that 2Me-E2 affinity to both ER

might be much higher than that estimated earlier, about 1 to 2% of estradiol. Moreover, they detected both mitogenic and antiproliferative properties of 2Me-E2. In agreement with previous findings antiproliferative properties were ER independent, whereas mitogenic ones were ER dependent. The latter were attributed to the residual 2Me-E2 estrogenic activity which was observed only at very low 2Me-E2 concentrations, up to 750 nM, and when 2Me-E2 antiproliferative activity was not yet observed.

Besides antitumor properties, 2Me-E2 possesses other biological activities. Thus, it was reported that 2Me-E2 induces the expression of endothelial nitric oxide synthase and production of nitric oxide (Tsukamoto et al., 1998; Xiao et al., 2001). The latter besides being vasodilator is also known to have antithrombogenic properties, thereby preventing platelet aggregation and adhesion, and it also enhances endothelium barrier properties via decreasing monocytes and macrophages infiltration. Therefore, 2Me-E2 may alleviate the symptoms of atherosclerosis via decreasing plaque formation (Kurokawa et al., 2007). In addition to barrier-improving properties, 2Me-E2's ability to decrease proliferating cells and inhibit collagen synthesis can attenuate hypertension, pulmonary hypertension, glomerulosclerosis, brain injury, etc. (Dubey and Jackson, 2009; Dubey et al., 2007).

To achieve limited positive response on tumor treatment with 2Me-E2, large orally administered doses were required, up to 3000 mg bid (Dahut et al., 2006; Sweeney et al. 2005; James et al., 2006) Even at these doses, the plasma concentration of methoxylated estradiol remained in lower nanogram per milliliter range. Unphysiologically high apparent CL and $V_d$ were estimated with median $t_{½}$ of about 1 to 2 days. Such a long half-life may be attributed to a much higher affinity of 2Me-E2 to

plasma proteins. Consequently, the drug binding to plasma proteins in humans was found to be about 98%, which is also concentration independent at 2Me-E2 concentrations of 1 to 30 ng/ml (Lakhani et al., 2006). The binding affinity was highest for albumin (4.29 (±0.420) × $10^4$/mol/l), then $\alpha_1$-acid glycoprotein (4.41 (±0.139) × $10^4$/mol/l) and sex-hormone-binding globulin (1.5 × $10^6$ (±2.6) × $10^5$/mol/l).

In addition, clinical trials revealed that a linear relationship was not found between $C_{max}$ or AUC and the 2Me-E2 dose (Dahut et al. 2006; Sweeney et al., 2005). The orally administered drug also showed high inter- and intrapatient variability and highly variable $T_{max}$.

Lakhani et al. (2007) has found less than 0.01% of the unchanged 2Me-E2 in urine and about 1% as glucuronides after oral administration of 800 or 2200 mg bid. The latter indicates that glucuronidation might be a major pathway of 2Me-E2 metabolism. The estimated hepatic clearance of 2Me-E2 could be as high as 712 ml/min, about half of hepatic blood flow (Lakhani et al., 2007). This value includes known Phase I and II metabolism of 2Me-E2 but not biliary excretion. Hence, 2Me-E2 clearance could be as high as hepatic blood flow which makes 2Me-E2 a high extraction ratio drug. This leads to the conclusion that first pass metabolism is also major player in 2Me-E2 disposition (Lakhani et al., 2007, Martucci, 1983).

Lakhani (2005) also reported that 2Me-E2 solubility in phosphate buffer (pH 6-7) is only about 2.3 to 3.3 μg/ml (7.6 – 10.9 μM), whereas its high apparent permeability ($P_{app}$) of 28.8 ± 4.9 ×$10^{-6}$ cm/s allows the drug to easily penetrate gastrointestinal wall. This makes 2Me-E2 to fall into Class 2 of Biopharmaceutical Classification System (BCS) - a drug with poor GI solubility.

#### 2.2.1.1. Metabolism of 2-Methoxyestradiol

2Me-E2 contains two hydroxyl groups at the positions C-3 and C-17 and thus, it is a potential substrate for UGTs. Using transfected cell lines, Basu et al. (2004a) and Lépine et al. (2004) have reported that several UGT 1A and some 2B isoforms extensively transformed 2Me-E2 to the glucuronidated forms, as summarized in Table 3. The relative contribution to the metabolism of 2Me-E2 by some UGTs is somewhat controversial. Basu et al. (2004a) implied that UGT1A10 is the main isozyme contributing to 2Me-E2 glucuronidation; whereas Lépine et al. (2004) have emphasized that UGT1A8 has the highest rate and intrinsic clearance followed by 1A3 and 1A1. Although $K_m$ (12 μM) for UGT1A10 implies that this isozyme has a high affinity to 2Me-E2, its intrinsic clearance was estimated to be quite low. Glucuronidation of 2Me-E2 took place at higher rates than methoxylated or hydroxylated estrones (Basu et al., 2004a; Lépine et al., 2004). Both groups agreed that UGT2B7 was not involved in the formation of 2Me-E2 glucuronides, and there was no glucuronidation that occurred at the C-17 position. Even for primary estrogens like E2, the involvement of UGT2B7 or other isozymes in the formation of estradiol-17β-glucuronide is not quite clear (Lépine et al., 2004; Ritter et al., 1990; Gall et al., 1999). On the other hand, incubation with pooled human liver microsomal proteins resulted in formation of two types of glucuronides, presumably 2Me-E2-17- and -3-glucuronides (Lakhani et al., 2007). Therefore, the mechanism of formation, conditions, and type of the enzymes participating in the formation of 17-glucuronide still need to be clarified.

Table 3: Rate of 2-Methoxyestradiol (2Me-E2) Glucuronidation by Human UGTs

| | **pmol/h/mg protein** | |
|---|---|---|
| **UGTs** | Basu et al., 2004a | Lépine et al., 2004 |
| 1A1 | 160 ± 30 | 1306 – 2612 |
| 1A3 | 621 ± 21 | 261 – 1305 |
| 1A7 | 2504 ± 95 | - |
| 1A8 | 2045 ± 77 | 1306 – 2612 |
| 1A9 | ~52.5 | 261 – 1305 |
| 1A10 | 7922 ± 267 | 261 – 1305 |
| 2B4 | - | 131 – 260 |
| 2B7 | - | - |
| 2B17 | - | 26 – 130 |

Lakhani et al. (2007) have also emphasized that Phase I metabolism can play an important role in metabolism of 2Me-E2, but not as extensively as glucuronidation. Although no oxidative metabolites were found in urine samples of patients taking 2Me-E2, *in vitro* experiments showed the presence of five Phase I metabolites such as 2Me-E1, 2-hydroxyestradiol, 2-hydroxyestrone, 2,3-dimethoxyestrone, and 2,3-dimethoxyestradiol. The incubation of 2Me-E2 with different recombinant CYP450 enzymes demonstrated that the following isoforms are responsible for hepatic oxidation of the drug: CYP 1A1, 1A2, 3A4, 3A5 and 2E1 (Lakhani, 2005). Neither the urine samples nor *in vitro* studies showed the presence of sulfated metabolites of 2Me-E2,

indicating that 2Me-E2 metabolism by sulfotransferase is limited or does not take place. However, it was reported that 2Me-E2 can undergo sulfonation catalyzed by SULT1A1 (Spink et al., 2000). Being a low-capacity and high-affinity enzyme, SULT1A1 may be effective in 2Me-E2 biotransformation in nanomolar range; at very high concentrations UGTs become the major route of 2Me-E2 biotransformation.

#### 2.2.1.2. Active Transport of 2-Methoxyestradiol

No studies have been published so far to reveal the role of drug transporters, especially ABC transporters, on the disposition of 2Me-E2. Some research however, was done to identify an ABC transporter that might be responsible for the movement of E2 though the lipid bilayer. Considering the fact that E2 and 2Me-E2 are structurally similar, one might also apply the results obtained with E2 to 2Me-E2. Because E2 is a very hydrophobic compound it would be reasonable to assume that E2 is a substrate for MDR1/ABCB1, known to transport variety of hydrophobic compounds (Schinkel and Jonker, 2003). However, Fujise et al. (2002) have shown via *in vitro* experiments that estradiol is not one of them, whereas uptake experiments with inside-out membrane vesicles and BCRP/ABCG2-transduced cells showed that E2 could be a competing substrate for BCRP/ABCG2 (Janvilisri et al., 2003; Imai et al., 2002). Furthermore, with $P_{app}$ of 28.8 ± 4.9 $\times 10^{-6}$ cm/s (Lakhani, 2005), one would expect that any biological transport of 2Me-E2 would be overwhelmed by very rapid passive diffusion.

# CHAPTER 3

# Significance, Hypothesis and Specific Aims

## 3.1. Effect of OS-generated Reactive Species, $ONOO^-$ and 4HNE, on the Activity of BCRP/ABCG2 Transporter

Oxidative stress (OS) refers to the imbalance between reactive oxidative species (ROS) and antioxidant protective mechanisms. The formation and harmful effect of oxidative species have been observed in such diseases as Alzheimer's disease, Parkinson's disease, chronic obstructive pulmonary disease, preeclampsia, gestational diabetes mellitus, rheumatoid arthritis, atherosclerosis. OS is often a consequence of the disease state rather than its cause; nevertheless, their presence can redouble the deteriorating conditions. Oxidative species are capable of reacting with various biomolecules including DNA, protein, lipid, and carbohydrates. By the reaction with protein residues ROS, especially peroxynitrite ($ONOO^-$) and 4-hydroxynonenal (4HNE), are capable of modifying enzyme or protein function or shut them down, e.g. GLUT3 or glutathione peroxidase. ABC efflux transporters may be subject to oxidative modifications as well.

ABC efflux transporters, BCRP/ABCG2 in particular, play an important role in drug absorption, distribution, metabolism, excretion as well as protection against toxins

and xenobiotics. This recently discovered transporter is highly expressed in placenta, where it plays a role as a first line of defense pumping drugs/toxins away from the fetus, back into the mother's blood. BCRP/ABCG2 was also found in the liver, intestine, lung, and kidney. This implies that BCRP/ABCG2 can also diminish the bioavailability of orally administered drugs. This thesis investigates the effect of ROS on the activity of BCRP/ABCG2 transporter, which may reflect fetal protection as well as drug bioavailability.

**Hypothesis and Specific Aims**

Pathologic oxidants associated with OS such as $ONOO^-$ and 4HNE are detrimental to the function of BCRP/ABCG2 transporter.

To address this hypothesis, the following specific aims were addressed:

- Determine the exposure-effect relationships between prototypical oxidants, $ONOO^-$ and 4HNE, and activity of BCRP/ABCG2 transporter using riboflavin as a substrate;
- Determine whether changes in $V_{max}$ and/or $K_m$ for the transporter occur upon exposure to $ONOO^-$ and 4HNE.

## 3.2. Effect of Qc, an Antioxidant, on the Disposition of 2Me-E2, a Highly Metabolized Drug-Candidate

As OS develops, the excess of ROS/RNS can cause damage to various biomolecules. One of the mechanisms that can prevent accumulation of ROS/RNS is a substantial presence of endogenous and exogenous antioxidants. These substances can not only intercept ROS/RNS but also inhibit metabolic transformations of therapeutically important drugs/compounds. For instance, preeclampsia is a medical condition associated with hypertension in pregnant women. Typically this syndrome is linked with hypoxia, which, in turn, causes OS. There is no known treatment for this condition but either Cesarean section or induced labor. However, Kanasaki et al. (2008) have reported that pregnant women with preeclampsia had lower levels of 2-methoxyestradiol (2Me-E2) in comparison with normotensive controls. This means that 2Me-E2 may be used as a therapeutic agent in preventing preeclampsia. Moreover, this compound maybe useful for other medical conditions, including inhibition of cancer cell proliferation and angiogenesis (Dubey and Jackson, 2009; Dubey et al., 2007; Lakhani et al., 2003). However, 2Me-E2 is highly susceptible to activities of UDP-glucuronosyltransferases (UGTs) resulting in low oral bioavailability and high systemic clearance. The excessive metabolism of 2Me-E2 could be lessened by an antioxidant that is also a substrate for UGTs, such as quercetin (Qc). Qc is a dietary antioxidant that is intensively metabolized by UGTs, sulfotransferases and methyltransferases. It also exhibits inhibitory effects on the BCRP/ABCG2, MDR1/ABCB1, MRP1/ABCC1, and MRP2/ABCC2 transporters that might limit drug bioavailability from GI tract and/or drug penetration to the target

tissues. Therefore, it is reasonable to investigate whether Qc can inhibit the activity of ABC transporters and UGT enzymes to increase therapeutic efficacy of extensively metabolized drugs such as 2Me-E2.

**Hypothesis and Specific Aims**

Qc will significantly inhibit the activity of ABC transporters and UGT enzymes involved in the efflux and metabolism of 2Me-E2, i.e. it will increase 2Me-E2 intracellular enterocyte cell concentration.

To address this hypothesis, the following specific aims were addressed:

- Determine the extent to which Qc inhibits UGT-mediated 2Me-E2 metabolism;
- Determine factors that might affect Qc inhibition of 2Me-E2 metabolism;
- Determine the role of BCRP/ABCG2 in 2Me-E2 efflux and the effect of Qc on its activity.

# CHAPTER 4

# Materials and Methods

## 4.1. Materials

### 4.1.1. Chemicals

Effect of $ONOO^-$ and 4HNE on BCRP/ABCG2 Activity: Riboflavin was obtained from MP Biomedicals, Inc. (Solon, OH). Peroxynitrite ($ONOO^-$) and 4-hydroxynonenal (4HNE) were purchased from Cayman Chemical (Ann Arbor, MI) and stored at $-80^{o}$ C. The concentration of ONOO was determined spectrophotometrically after each thawing as described by the manufacturer. All other reagents and solvents were obtained from Sigma-Aldrich (St. Louis, MO).

Effect of Quercetin on Metabolism of 2Me-E2: 2Me-E2 and Quercetin (purity 99%) were purchased from Steraloids, Inc., (Newport, RI) and Acros Organics (Morris Plains, NJ), respectively. Series of inhibitors were used during this study such as indomethacin (Hawkins Pharmaceutical Group, Minneapolis, MN), ritonavir and GF120918 (Toronto Research Chemicals, Inc., North York, ON, Canada), MK571 (Alexis Biochemicals, San Diego, CA). Glacial acetic acid was obtained from J.T. Baker Chemical Co.

(Phillipsburg, NJ). Methanol (reagent grade) was from GJ Chemicals, Inc., (Newark, NJ). Hank's Balanced Salt Solution (HBSS), Ascorbic acid (purity >99%), and acetonitrile (HPLC grade) were purchased Sigma-Aldrich (St. Louis, MO). HEPES and dimethyl sulfoxide (DMSO) was obtained from Fisher Chemicals (Fair Lawn, NJ).

Synthesis of 2Me-E2 Glucuronides: β-glucuronidase (EC 3.2.1.31), alamethicin, Tris base (purity 99.9%), porcine esterase (E2884-1KU), D-saccharic acid 1,4-lactone monohydrate were obtained from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid and sodium sulfate anhydrous were from EMD chemicals, Inc. and EM Science (Gibbstown, NJ), respectively. Acetic anhydride (purity >97%) and magnesium chloride were purchased from Fisher Chemicals (Fair Lawn, NJ). UDP-Glucuronic acid trisodium salt (UDPGA) was obtained from Calbiochem, EMD Chemicals, Inc. (La Jolla, CA). Human liver fraction S9 was provided as a gift from Dr. Joseph K. Ritter (Department of Pharmacology and Toxicology, School of Medicine, VCU).

Transwell Experiments with MDCK-BCRP Cells: Prazosin (purity 99%) and Hank's Balanced Salt Solution (HBSS) were obtained from Sigma-Aldrich (St. Louis, MO).

### 4.1.2. Cell Cultures

#### 4.1.2.1. *Sf9* Cells

The *Sf9* insect cell line, a clonal isolate derived from the parental *Spodoptera frugiperda* cell line, with passages between 1 to 30 was purchased from Invitrogen Corp., (Carlsbad, CA). These cells were cultured in GIBCO® Sf-900 II SFM (1×) medium

(Invitrogen Corp., Grand Islands, NY). *Sf9* cells were maintained as a suspension in 125 ml Nalgene flasks with loosened caps to allow oxygenation, and maintained in a non-humidified 27º C incubator shaker rotating at 140 rpm. Once cell density reached 3 – 4.5 $\times 10^6$ viable cells/ml, they were ready to be split. Cells were reseeded at 0.6 to 0.75 $\times 10^6$ viable cells/ml. Medium was typically changed every 48 hours.

#### 4.1.2.2. LS180 Cells

The LS180 intestinal human colon adenocarcinoma cell line (passage between 42 and 55), was provided as a gift from Dr. Joseph K. Ritter (Dept. Pharmacology and Toxicology, VCU). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1X) supplemented with 10% (v/v) fetal bovine serum and 1% non-essential amino acids (Mediatech, Inc., Manassas, VA). Cells were seeded into 75 $cm^2$ tissue culture flask (CELLTREAT Scientific Products, Shirley, MA) containing 15 ml of medium and maintained in a humidified 37º C incubator with a 5% carbon dioxide in air atmosphere. Medium was changed every 48 hours. The cell line was passaged by removing spent medium, adding fresh medium and dislodging the cells from the surface of the culture flask with a sterile scraper. The cell suspension was pipetted up and down in sterile pipette as well as passed 6 times through a 23G1 needle to break up clumps, and then dispensed into new culture flasks.

#### 4.1.2.3. MDCK II-BCRP Cells

The BCRP/ABCG2 transfected Madin Darby canine kidney (MDCKII-BCRP) cell line (passage between 14 and 27), was provided as a gift from Dr. Piet Borst

(Netherland Cancer Institute, Amsterdam, The Netherlands). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1×) supplemented with 10% (v/v) fetal bovine serum, 1% non-essential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Mediatech, Inc., Manassas, VA). Cells were seeded into 25 $cm^2$ cell culture flask (Corning, Inc., Corning, NY) containing 5 ml of medium and maintained in a humidified 37º C incubator with a 5% carbon dioxide in air atmosphere. Medium was changed every 48 hours. After MDCK cells reached 80% confluence, spent medium was removed and cells were washed 2 times with warm DPBS. 1 ml of 0.25% trypsin solution containing 1mM EDTA was added to the flask and let sit for 30 seconds. After 0.7 ml of trypsin solution was removed, the flask was incubated for 5 – 8 min, until the cell deattached from the flask surface. Trypsin was deactivated by adding 3 ml of medium. Cells were then diluted and dispensed into new culture flasks.

## 4.2. Methods

### 4.2.1. *Sf9* Membrane Vesicle Preparation and Transport Experiments

Inside-out *Sf9* membrane vesicles overexpressing human BCRP/ABCG2 or empty vector (i.e., lacking ABC transporters) were prepared as previously described (Ito et al., 2001; Vaidya and Gerk, 2006). Briefly, a suspension of *Sf9* cells was infected with titered viral stocks of BCRP or empty vector (EV) at a multiplicity of infection of 4. 48 hours later, *Sf9* cells were harvested, lysed and homogenized, then their membranes were isolated by fractionation over 38% sucrose. Isolated membranes were vesiculated, snap frozen in liquid nitrogen, and stored at – 80º C. The membrane protein concentrations

were determined by the modified Lowry protein assay with bovine serum albumin used as a standard (Lowry et al., 1951).

Transport experiments were carried out as described in Gerk et al. (2004) with small modifications in the procedure. Thus, Tris-sucrose or triethanolamine buffers contained 5 mM ATP or AMP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, 10 mM $MgCl_2$, riboflavin in deionized water or dimethyl sulfoxide (0.5%) with or without reduced glutathione (1mM). ATP-dependent transport of riboflavin was conducted in 96-well plate with a reaction volume of 60 μl at 37º C between 2 to 60 minutes. The experiment was stopped by transferring 50 μl of the reaction buffer to a 96-well plate with pre-wetted 0.45 μm filters (Millipore Corporation, Bedford, MA) and by quickly adding ice-cold stop buffer (3 x 200 μl). After stop buffer was filtered, 100 μl of water/DMSO mixture (75/25) was added to the filter plate and centrifuged for 8 min at 1200 rpm. The filtrate was collected in a flat bottom 96-well plate (Costar 3631, Corning Inc., Corning, NY). The amount of riboflavin was determined by fluorescence detection using a microplate reader Synergy$^{TM}$ 2 (BioTek Instruments, Inc., Winooski, VT) at 450/528 (ex/em) wavelengths. The ATP-dependent transport of riboflavin was estimated a difference between the values obtained in the presence of ATP and AMP. For the experiments with reactive species, the membrane vesicles were pre-incubated with $ONOO^-$ or 4HNE at 37º C for 30 minutes prior to the transport experiments. The reaction was quenched by setting the pre-treated membrane vesicles on ice.

### 4.2.2. Synthesis of 2-Methoxyestradiol Glucuronides

2Me-E2 Acetylation at Position C-3: This synthesis was carried according to Rao et al. (2008). Briefly, a solution of 24.8 mg of 2Me-E2 in 488 µl of 2-propanol was mixed with 139.4 µl of 2M sodium hydroxide and 22.3 µl of acetic anhydride. The reaction mixture was stirred at room temperature for 2 hours. The reaction was quenched with methanol, diluted with water and concentrated in a vacuum concentrator. The residue was acidified with 4.2 ml of 3M hydrochloric acid and extracted 3 times with 4 ml of ethyl acetate. The collected organic fractions were washed with 3ml of water and 3ml of saturated solution of sodium chloride. Finally, the organic fraction was dried with 0.5 g of sodium sulfate. The treated solution was again concentrated in vacuum concentrator to yield 3-acetyl-2-methoxyestradiol (3Ac-2Me-E2) (~15 mg) as a white powder (yield of > 52%). The white powder was transferred into a centrifuge tube (1.5 ml) and stored at -20ºC. The powder residue was then redissolved in DMSO for further purification. The liquid sample was purified with Waters HPLC system using Alltima HP C18 column (100 mm × 4.6 mm × 3µm) by Alltech (Deerfield, IL). The separation was performed using acetonitrile with 1% acetic acid in water (40:60) as a mobile phase at a flow rate of 1 ml/min. The sample fractions were detected with Waters 2487 dual wavelength ultraviolet absorbance detector at 279 nm. The fraction (F1) eluted between 9.5 to 12.1 minutes was collected for further analysis and deemed to be 3Ac-2Me-E2.

Glucuronidation Assay: Glucuronidation of 2Me-E2 and 2Ac-2Me-E2 (F1) were carried out with S9 human liver fraction according to the procedure established earlier in our laboratory. Briefly, 100 µl of S9 human liver fraction (final concentration of about 4

mg/l) was added to a solution containing 50 µl of 200 mM magnesium chloride, 190 µl of 400 mM Tris Base (pH 7.5), 50 µl of 2mg/ml alamethicin, 10 ul of 100 mM ascorbic acid, 60 ul of 100 mM saccharolactone, 120 µl of 25 mM UDPGA, 5 ul of 20 mM of 2Me-E2 or 5 ul of 3Ac-2Me-E2 solution, and 415 µl of deionized water. The reaction mixture was incubated at 37º C for 120 minutes stirring every 30 minutes. After 2-hr reaction, the samples were extracted 3 times with 1 ml of 2% acetic acid in ethyl acetate. The collected organic factions were vacuum dried. The residue was resuspended in methanol for further analysis and purification with HPLC. Methanolic solutions of glucuronidated products were analyzed with Alltima HP C18 column using acetonitrile with 1% acetic acid in water (30:70) as a mobile phase at a flow rate of 1 ml/min. The 2Me-E2 glucuronides were detected using Waters 2475 dual wavelength fluorescence detector at excitation/emission wavelengths of 275/315 nm. Fractions eluted within 2.2 to 3.1 minutes (F2) and 4.1 to 4.9 minutes (F3) were collected for further studies.

Hydrolysis of 3Ac-2Me-E2 and Its Acetylated Glucuronides: Hydrolysis of the acetylated glucuronide was carried out according to Moen et al. (2005) and Zhang et al. (2004). Briefly, the remaining (from the above described procedure) methanolic solution of glucuronides (S1) was dried again only to resuspend the residue in DMSO. Then 101 µl of 0.67 mg/ml porcine liver esterase solution was added to 0.2 M phosphate buffer (pH 7.0) containing 30 µl of S1. The reaction mixture was incubated at 37º C for 2 hours stirring every 30 minutes. The reaction was quenched by putting the mixture on ice. The sample was extracted with 3 times with 1 ml of 2% acetic acid in ethyl acetate and vacuum dried. The residue was resuspended in 2% acetic acid in methanol for further

analysis with Waters HPLC system. Methanolic solutions of glucuronidated products were analyzed with Alltima HP C18 column using acetonitrile with 1% acetic acid in water (30:70) as a mobile phase at a rate of 1 ml/min. The 2Me-E2 and its glucuronides were detected using Waters 2475 dual wavelength fluorescence detector at excitation/emission wavelengths of 275/315 nm.

β-Glucuronidase Assay: This assay was conducted according to Lakhani (2005). Briefly, 1.6 µl of β-glucuronidase solution (400 units/ml) was added to 20 µl of 0.13 M phosphate buffer (pH 6.5) already containing 50 µl of deionized water and 50 µl of either fraction F2 or F3 (collected from glucuronidation assay). The reaction mixture was incubated in water bath at 52º C for 2 hours. After the reaction time elapsed, the samples were extracted 3 times with 0.5 ml of 2% acetic acid in ethyl acetate. The collected organic fractions were dried in vacuum concentrator and the residues were then redissolved in 2% acetic acid in methanol. The methanolic solutions were analyzed with Waters HPLC system using Alltima HP C18 column (Alltech; Deerfield, IL). The analytes separation was performed using acetonitrile with 1% acetic acid in water (30:70) as a mobile phase at a flow rate of 1 ml/min. 2Me-E2 and its glucuronides were detected with Waters 2475 dual wavelength fluorescence detector at excitation/emission wavelengths of 275/315 nm. Under these conditions, F2, F3 and 2Me-E2 eluted at 2.50 ± 0.02 minutes, 4.48 ± 0.27 minutes, and 14.9 ± 0.71 minutes, respectively.

### 4.2.3. Glucuronidation of 2-Methoxyestradiol Using LS180 Cells

For metabolic studies, cells were seeded onto 12-well plates (CELLTREAT Scientific Products; Shirley, MA) at a density of $0.5 \times 10^4$ cells/cm$^2$ (or 414 ± 5.2 mg protein/per well) and cultured for 4 days until confluence near 70%. At the end of this period, wells were washed twice with HBSS containing 10 mM of HEPES and adjusted to pH 7.4. Cells were equilibrated with HBSS at 37º C for 15 – 20 minutes. After equilibration period, wells were refilled with 0.5 ml of warm HBSS containing 2Me-E2 (0.24% DMSO and 100 μM ascorbic acid) with or without inhibitors. At this point, the reaction was deemed to start. After the designated period of time, 12-well plates were set on ice and samples of spent HBSS containing 2Me-E2 metabolites were rapidly collected for further analysis. The procedure for reaction quenching and extraction of metabolites from the cells were was adopted from Faijes et al. (2007). Briefly, the metabolic reaction was quenched with 1 ml of ice cold solution of methanol with 70 mM of HEPES (pH 5.5) (60:40). Wells were then refilled with 1ml of ice cold 2% acetic acid methanol. Cells were scraped, transferred into 1.7 ml centrifuge tube and vortexed for 20 seconds. After centrifugation for 5 min, methanol was collected into glass test tube. This procedure was repeated two more times. The collected methanol (~ 3 ml) was vacuum dried, and the residue was resuspended with 35 μl of 2% acetic acid in methanol for further analysis with Water HPLC system. The separation of 2Me-E2 and its metabolite was performed with Alltima HP C18 column (Alltech; Deerfield, IL) using acetonitrile in 1% acetic acid in water (30:70) as a mobile phase at a flow rate of 1 ml/min. 2Me-E2 and its glucuronides were detected with Waters 2475 dual wavelength fluorescence detector at

excitation/emission wavelengths of 275/315 nm. Under these conditions, 2Me-E2-3G and 2Me-E2 eluted at 2.6 ± 0.05 minutes and 14.9 ± 0.71 minutes, respectively.

### 4.2.4. Transwell Experiments Using MDCKII-BCRP Cells

For transport studies, cells were seeded onto polyester Transwell #3460 membranes (0.4 μm pore diameter, 1.12 $cm^2$ surface area; Corning, Inc., Corning, NY) at a density of $0.5 \times 10^4$ cells/$cm^2$ and cultured for 7 days. At the end of this period, monolayers were washed two times with HBSS containing 10 mM of HEPES and adjusted to pH 7.4. Cell monolayers were equilibrated with HBSS at 37º C for 30 minutes. After equilibration, 2Me-E2 transport was measured for a period of time of 60 minutes in the apical to basolateral (A to B) and basolateral to apical (B to A) directions under 'sink' conditions. Samples (200 μl) were withdrawn at 15, 30 and 60 minutes from receiver side and replaced with 200 μl of pre-warmed HBSS solution. 2Me-E2 transport across cell monolayer was carried out in 3 parallel wells for each direction. The concentrations of 2Me-E2 in collected samples were analyzed with Alltima HP C18 column using acetonitrile with 1% acetic acid in water (40:60) as a mobile phase at a flow rate of 1 ml/min. The 2Me-E2 was detected using Waters 2475 dual wavelength fluorescence detector at excitation/emission wavelengths of 275/315 nm. Under these conditions no metabolites were observed and 2Me-E2 eluted at 4.6 ± 0.01 minutes.

2Me-E2 permeability in each direction was measured as apparent permeability $P_{app}$ (cm $s^{-1}$) estimated according to the following equation:

$$P_{app} = \frac{dQ/dt}{C_d \times A} \qquad (Eq.\ 1)$$

where the numerator is the amount of 2Me-E2 appeared in received per unit of time (pmol $s^{-1}$), $C_d$ is the concentration of 2ME in the donor compartment (pmol/$cm^3$), and A is the area of membrane ($cm^2$).

The directionality ratio (DIR) was then calculated as a ratio of $P_{app}$ from receiver to donor (B to A) to $P_{app}$ from donor to receiver (A to B):

$$DIR = \frac{P_{app,BA}}{P_{app,AB}} \qquad (Eq.\ 2)$$

DIR values higher than 2 were considered as a cut offs to identify a substrate for the transporter.

### 4.2.5. Statistical Analysis

All data values were expressed as a mean of 3 or 4 independent determinations ± standard deviation S.D. The data were compared by analysis of variance (one-way ANOVA) followed by Dunnett's post-test or unpaired *t*-test. Whenever different treatments were employed the data were compared with two-way ANOVA followed by Bonferroni's post test. A value of $p < 0.05$ (2-sided) was considered statistically significant. Goodness of fit of different models was compared using Akaike information criterion (AIC).

### 4.2.6. Data Analysis for Enzyme Kinetics

Kinetic model parameters were estimated from curve fitting using GraphPad Prism for Windows (version 5, GraphPad Software Inc., San Diego, CA) by means of unweighted nonlinear regression. The following equations were used:

Michaelis-Menten equation with variable slope (Hill coefficient):

$$v = \frac{V_{max} \times [S]^h}{{K_m}^h + [S]^h} \quad \text{(Eq. 3)}$$

For inhibitory experiments, sigmoid dose-response equation:

$$v = B_{min} + \frac{B_{max} - B_{min}}{1 + (I/IC_{50})^h} \quad \text{(Eq. 4)}$$

Substrate inhibition equation:

$$v = \frac{V_{max}[S]}{K_m + [S] + \frac{[S]^2}{K_i}} \quad \text{(Eq. 5)}$$

where $v$ is the velocity of the transport, S and I is substrate and inhibitor concentrations, $K_m$ is affinity constant for saturable transport, $B_{max}$ and $B_{min}$ are either maximum and minimum reaction/transport velocities or maximum or minimum amounts of 2Me-E2 formed, $h$ is Hill coefficient, $V_{max}$ is maximum reaction velocity, $IC_{50}$ is a half maximal inhibitory concentration, and $K_i$ is inhibition constant for second substrate molecule.

# CHAPTER 5

# Results and Discussion

## 5.1. BCRP-mediated Transport of Riboflavin

In this study ATP-dependent uptake of riboflavin (vitamin $B_2$) was investigated using inside-out *Sf9* membrane vesicles overexpressing BCRP/ABCG2. van Herwaarden et al. (2007) and Ifergan et al. (2009) have demonstrated that riboflavin is a substrate of BCRP/ABCG2. Additionally, riboflavin is a fluorescent compound, which allows tracing its transport into *Sf9* membrane vesicles. Thus, Figure 6A depicts that in the presence of ATP, the membrane vesicles overexpressing BCRP/ABCG2 showed a time-dependent transport of riboflavin; whereas EV membrane vesicles demonstrated only minimal with time transport. This figure also demonstrates that the transport of riboflavin was linear within first 5 minutes. Therefore, all the following BCRP/ABCG2-mediated experiments were carried out for 5 minutes. Figure 6B shows that ATP-dependent riboflavin transport occurs into an osmotically sensitive space, with minimal binding.

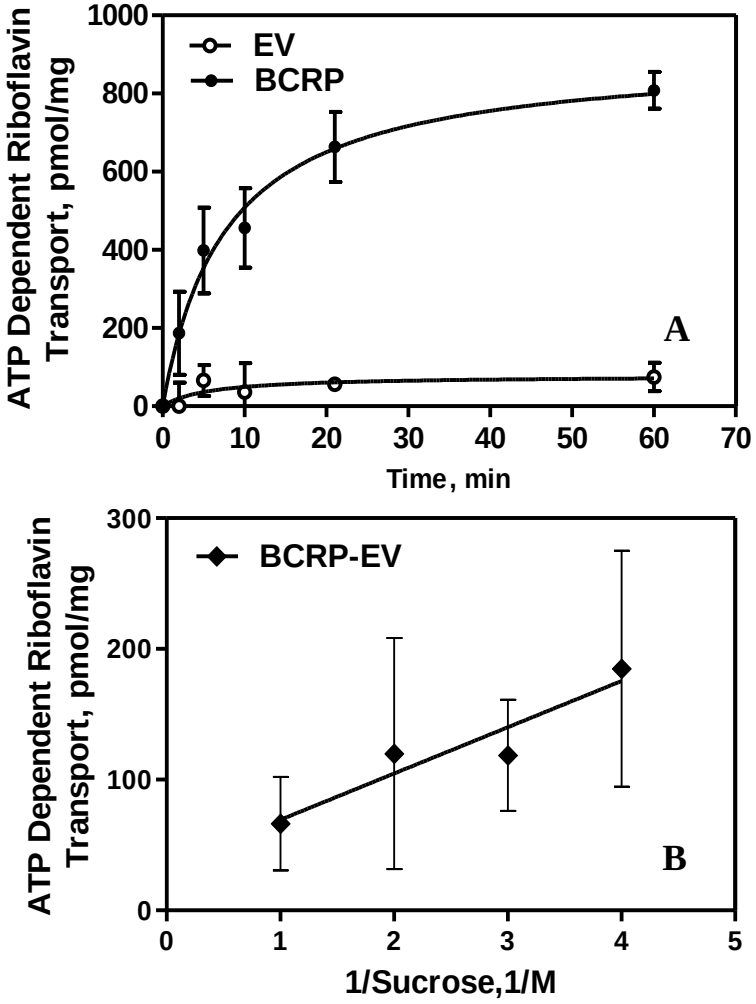



Figure 6: BCRP/ABCG2-mediated uptake of riboflavin into *Sf9* inside–out membrane vesicles. Each data point represents mean ± SD from four determinations. **A**: Time course of riboflavin (10 μM) uptake into *Sf9* membrane vesicles overexpressing BCRP/ABCG2 (filled circles) and EV vesicles (open circles). Protein content is 10 μg; **B**: Effect of sucrose (osmolarity) on ATP-dependent uptake of riboflavin (10 μM) into *Sf9* vesicles. The transport was determined in transport buffer after 5 min in the presence of 250 to 1000 mM of sucrose. Solid curves were fitted data for better representation of profiles.

The kinetic parameters for BCRP/ABCG2-mediated transport of riboflavin were assessed by fitting the Michaelis-Menten equation to the data obtained at different concentration of riboflavin (Figure 7A). Due to the limited solubility of riboflavin complete saturation of BCRP/ABCG2 was not possible to achieve. The maximum concentration of riboflavin tested was limited to 300 μM. Based on the available data, $K_m$ and $V_{max}$ were estimated to be 296 ± 71 μM and 1407 ± 193 pmol/mg protein/min ($r^2$ = 0.965), respectively, with Hill slope of 1.0. These data indicate that riboflavin is a relatively low affinity BCRP/ABCG2 substrate.

### 5.1.1. Effect of 4-Hydroxynonenal (4HNE)

The results of four independent measurements of riboflavin uptake at different concentrations and in the presence of 200 μM of 4HNE, a product of lipid peroxidation, are shown in Figure 7A. The estimated kinetic parameters in the presence of 4HNE are 168 ± 32 μM and 342 ± 43 pmol/min/mg ($r^2$ = 0.939) for $K_m$ and $V_{max}$, respectively. These parameters indicate that 4HNE is capable of inactivating the BCRP/ABCG2 transporter. $V_{max}$ was reduced 4-fold; whereas $K_m$ was remained unchanged (unpaired *t*-test, $p > 0.05$). Among obtained parameters Hill coefficient was estimated to be about 1.6 (using AIC), instead of initial 1.0, suggesting positive cooperativity. 4HNE has three main functional groups – aldehyde, carbon-carbon double bond, and hydroxyl group – which can react alone or in sequence of reactions (Esterbauer et al., 1991). Therefore one can speculate that 4HNE-protein adducts might additionally form chemical bonds with riboflavin, thus delaying its uptake by membrane vesicle and forming a sigmoidal curve.

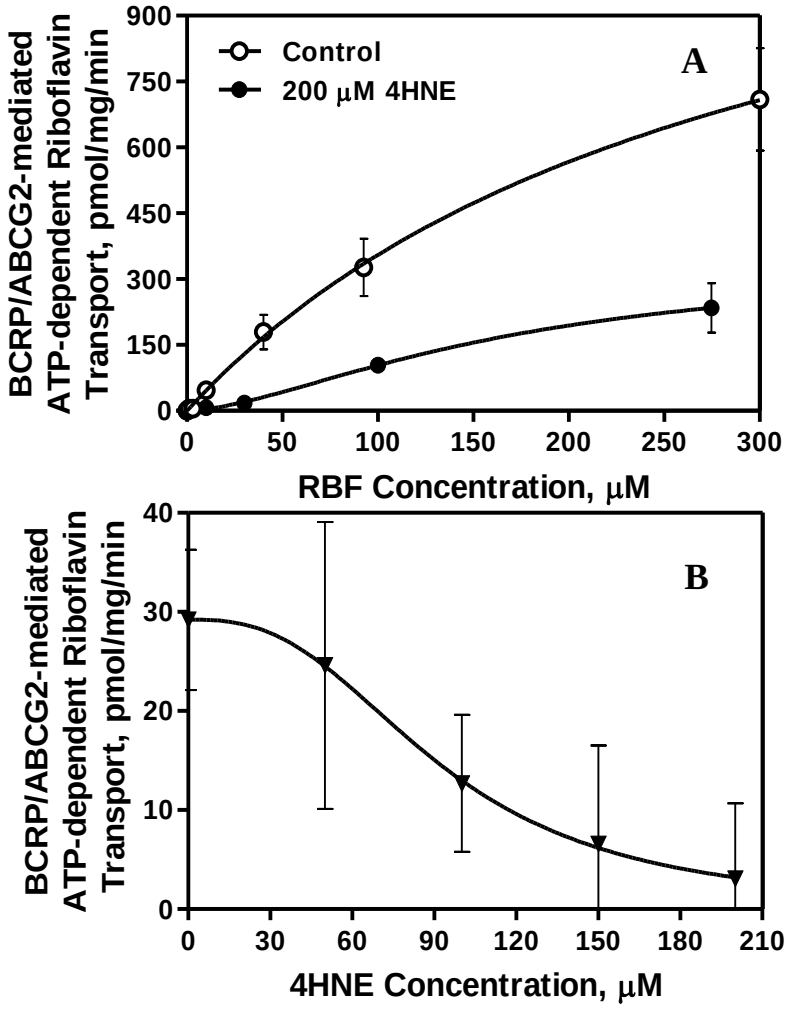



Figure 7: Effect of 4-hydroxynonenal (4HNE) on the transport of riboflavin into *Sf9*-BCRP/ABCG2 membrane vesicles. Data are mean ± SD from four determinations. **A**: BCRP/ABCG2-mediated transport of riboflavin in the presence of 200 μM of 4HNE (filled circles) vs. without 4HNE (open symbols); **B**: Inhibition of riboflavin uptake by 4HNE.

The inhibitory effect of 4HNE on the riboflavin uptake was tested within 0 to 200 μM of 4HNE concentration. The BCRP/ABCG2-mediated riboflavin uptake was tested within 5 min at 10 μg of protein. The 'dose-response curve' for ATP-dependent transport of riboflavin is illustrated in Figure 7B. The half maximal inhibitory concentration ($IC_{50}$) was estimated to be 92 ± 1.4 μM ($r^2$ = 0.999) with Hill coefficient of 2.7. Due to its lipophilic nature, 4HNE has a tendency to accumulate within the lipid bilayer at the site of lipid peroxidation and reach concentrations up to 4.5 mM (Koster et al., 1986). Thus, the obtained $IC_{50}$ could be achieved *in-vivo*. However, at concentrations ranging from 1 to 20 μM, 4HNE can already inhibit DNA and protein synthesis, and, at about 100 μM and higher, cells already undergo rapid death due to unspecific cytotoxic effects (Halliwell and Gutterridge, 1999; Esterbauer et al., 1991; Grimsrud et al, 2008). The inhibition of BCRP/ABCG2 activity occurred slower and concurrently with inhibition of other vital cell processes. One must note however that in present experiments 4HNE was added to the aqueous buffer. This would require some time for 4HNE to diffuse into the bilayer; whereas *in vivo* 4HNE is usually generated within lipid bilayer. Thus, the $IC_{50}$ values obtained in this study could be smaller due to possible reactions of 4HNE with other biomolecules before reaching a target transporter (Koster et al., 1986).

### 5.1.2. Effect of Peroxynitrite ($ONOO^-$)

The kinetic parameters for ATP-dependent riboflavin transport at various concentrations and in the presence of 50 μM of $ONOO^-$ were determined using the Michaelis-Menten equation. Because $ONOO^-$ anion is highly unstable at physiological pH, it is usually stored at highly basic pHs (in sodium hydroxide solution). Therefore, to

sustain the reaction buffer at the desired pH of 7.4, a buffer containing triethanolamine (pKa ~ 7.6) instead of Tris HCl was utilized. Because of the changes in buffer constituent, additional experiments were carried out without $ONOO^-$ to account for the effects of sodium hydroxide and triethanolamine. The kinetic parameters from these experiments were then compared to those obtained in the presence of 50 μM of $ONOO^-$. The results are shown in Figure 8A. In the case of the 'control' experiments, i.e. without $ONOO^-$, $K_m$ and $V_{max}$ were estimated to be 685 ± 167 μM and 2663 ± 486 pmol/min/mg ($r^2$ = 1.0), respectively. This compares to $K_m$ of 90 ± 39 μM and $V_{max}$ of 141 ± 23 pmol/min/mg ($r^2$ = 0.813), obtained with 50 μM $ONOO^-$. As can be seen, in the presence of 50 μM $ONOO^-$, both $V_{max}$ and $K_m$ were drastically reduced, by 19- and 7.5- times, respectively, suggesting a mixed type of inhibition.

The effect of $ONOO^-$ on the riboflavin uptake by *Sf9* membrane vesicles was tested only at $ONOO^-$ concentrations of up to 100 μM. The inhibition curve for BCRP/ABCG2-mediated riboflavin transport is shown in Figure 8B. $IC_{50}$ was estimated to be 31 ± 3 μM ($r^2$ = 0.991) indicating that $ONOO^-$ is a much more potent inhibitor of BCRP/ABCG2 than 4HNE. Although peroxynitrite anion is a relatively short-lived ROS, it causes not only lipid peroxidation, but also diffuses inside the lipid bilayer and oxidizes or nitrates membrane integral proteins. At physiological conditions, the rate of $ONOO^-$ production can be as high as 50 to 100 μM per minute, and its steady-state concentrations that can be sustained for a long period of time are usually in the nanomolar range (Szabó et al., 2007). Thus, a tissue can virtually be exposed to very high amounts of $ONOO^-$. Grover et al. (2003), for instance, have reported that $Ca^{2+}$ pumps (SERCA) can be inhibited by 50% at $ONOO^-$ concentrations of about 50 μM (*in vitro*). Hence $IC_{50}$ of

$ONOO^-$ obtained in this study can be easily observed at the physiological conditions and inactivation of BCRP/ABCG2 by $ONOO^-$ could possibly occur at lower concentrations than inhibition of other cellular processes.

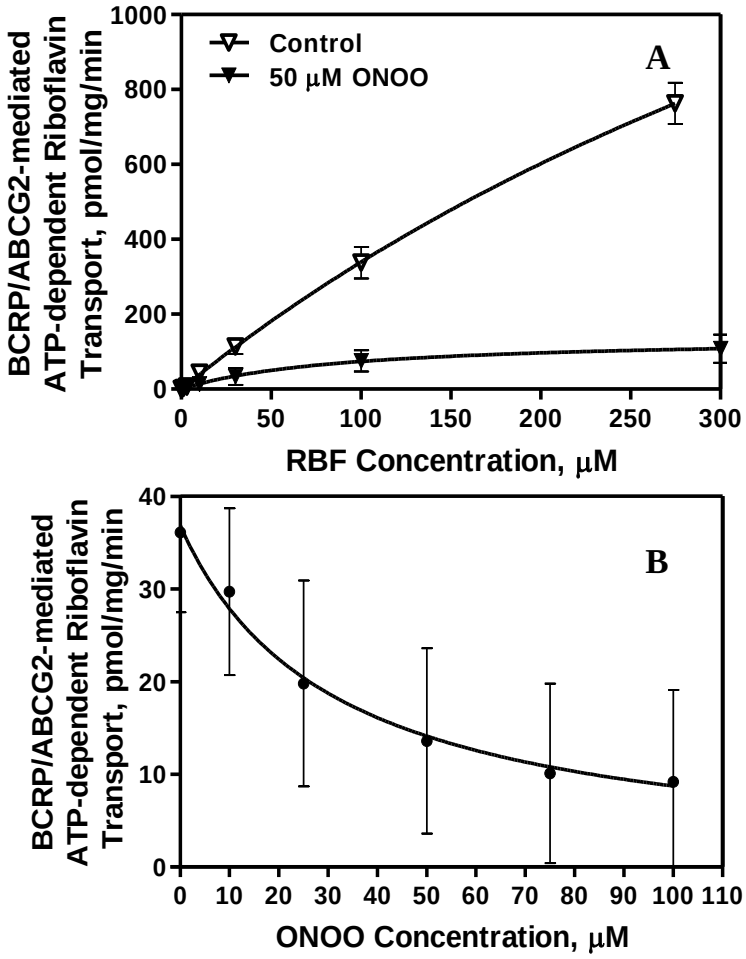



Figure 8: Effect of peroxynitrite ($ONOO^-$) on uptake of riboflavin into *Sf9*-BCRP/ABCG2 membrane vesicles. Data are mean ± SD from four determinations. **A**: BCRP-mediated transport of riboflavin in the presence of 50 μM of $ONOO^-$ (filled symbols) vs. control (open symbols); **B**: Inhibition of riboflavin uptake by $ONOO^-$ at its different concentrations.

### 5.1.3. Conclusions

The present study was conducted to investigate the effect and potency of peroxynitrite ($ONOO^-$) and 4-hydroxynonenal (4HNE), a product of lipid peroxidation, on the activity of BCRP/ABCG2. This investigation revealed that $ONOO^-$ ($IC_{50}$ = 31 ± 3 μM) and 4HNE ($IC_{50}$ = 92 ± 1.4 μM) inactivate BCRP/ABCG2 at biologically relevant concentrations. In addition, the formation of 4HNE-adducts caused changes in the transport of riboflavin demonstrated by increase in Hill coefficient to 1.6. Since, the formation of excess products of oxidative stress often takes place during different stages of various disease conditions; they are capable of decreasing the activity of ABC transporter, which, in turn, can affect drug disposition.

## 5.2. Glucuronidation and Transport of 2-Methoxyestradiol

### 5.2.1. Validation of 2-Methoxyestradiol Glucuronides

Incubation of 2Me-E2 with human liver fraction S9 and UPGA as a co-factor revealed that 2Me-E2 is metabolized, forming a number of metabolites. Chromatographic separation performed with HPLC showed appearance of two polar peaks (*F2* and *F3*) eluting at 2.50 ± 0.02 and 4.48 ± 0.27 minutes (Figure 9). Drawing an analogy to elution times for glucuronides of estradiol (E2), it was suspected that these two peaks should belong to glucuronides of 2Me-E2: 2Me-E2-3-glucuronide (2Me-E2-3G) and 2Me-E2-17-glucuronide (2Me-E2-17G), respectively.

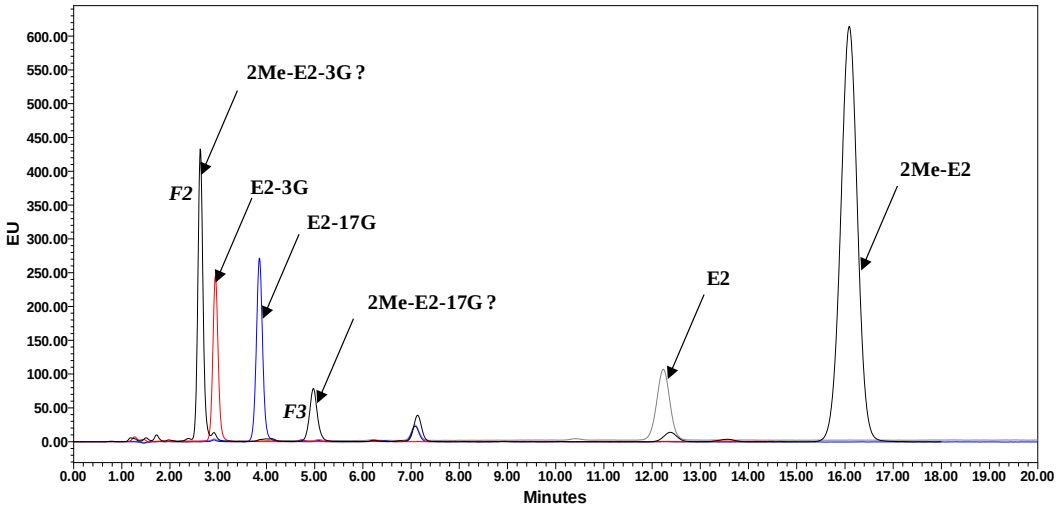



Figure 9: Representative chromatogram for detection of 2Me-E2 and its metabolite after incubation with human liver fraction S9. Comparison between elution times of 2Me-E2 metabolites (*F2* and *F3*) and solutions of 3- (E2-3G) and 17-glucuronides of estradiol (E2-17G).

To confirm the nature of these peaks/metabolites, the fractions F2 and F3 (see 'Materials and Methods' section) were collected and incubated with β-glucuronidase for 2 hours. Subsequently, the analysis with HPLC showed that the expected peaks at 2.50 ± 0.02 and 4.48 ± 0.27 disappeared, and the concentration of 2Me-E2 increased accordingly. This assay confirmed that peaks *F2* and *F3* are indeed glucuronides (Figure 10).

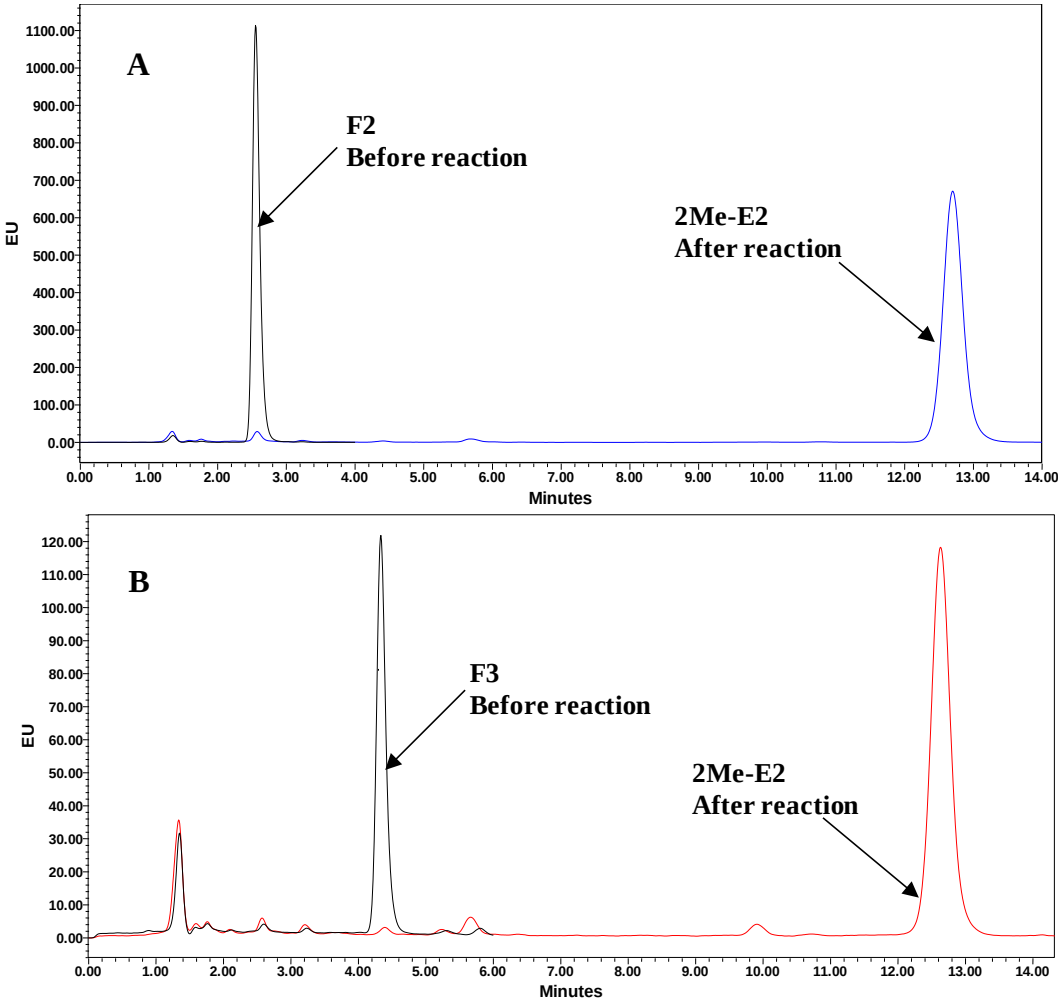



Figure 10: Representative chromatograms before and after the reaction with β-glucuronidase. **A**: Conversion of fraction F2 into 2Me-E2 by β-glucuronidase. **B**: Conversion of fraction F3 into 2Me-E2 by β-glucuronidase.

To identify which of these peaks, *F2* or *F3*, is the 17-glucuronide of 2Me-E2, a series of steps were undertaken. Firstly, 2Me-E2 was regioselectively converted to 3Ac-

2Me-E2 with the purpose of protecting this position from further attack by UGTs. By the end of this step, 3Ac-2Me-E2 was chromatographically separated (S1) (see Chapter 3, section 4.2.1) to carry out its glucuronidation with human liver fraction S9. During this reaction a number of metabolites were formed including 3Ac-2Me-E2-17G, 2Me-E2-3G and 2Me-E2-17G, likely due to the presence of esterase activity in the human liver S9 fraction. In the last step, the metabolite mixture from step 2 was hydrolyzed with porcine liver esterase to introduce hydroxyl (OH) group instead of Ac at the position 3, and to compare the changes in peak heights for *F2* and *F3* before and after hydrolytic reaction. As porcine liver esterase unmasked the 3-OH group, it was expected that the amount of 2Me-E2-17G would increase, whereas the amount of 2Me-E2-3G should remain unaffected. Therefore, if a peak height ratio (*F2/F3)* decreased after hydrolysis, then *F3* can be identified with 2Me-E2-17G, and if the ratio increased then *F3* can be assigned to 2Me-E2-3G. The chromatograms before the hydrolysis and after are shown in Figure 11. The peak ratio *F2* to *F3* before and after hydrolysis was estimated to be 3.8 and 1.6, respectively. Thus, the decrease in the peak height ratio confirmed that peak *F3* can be associated with 2Me-E2-17G, and peak F2 can be associated with 2Me-E2-3G.

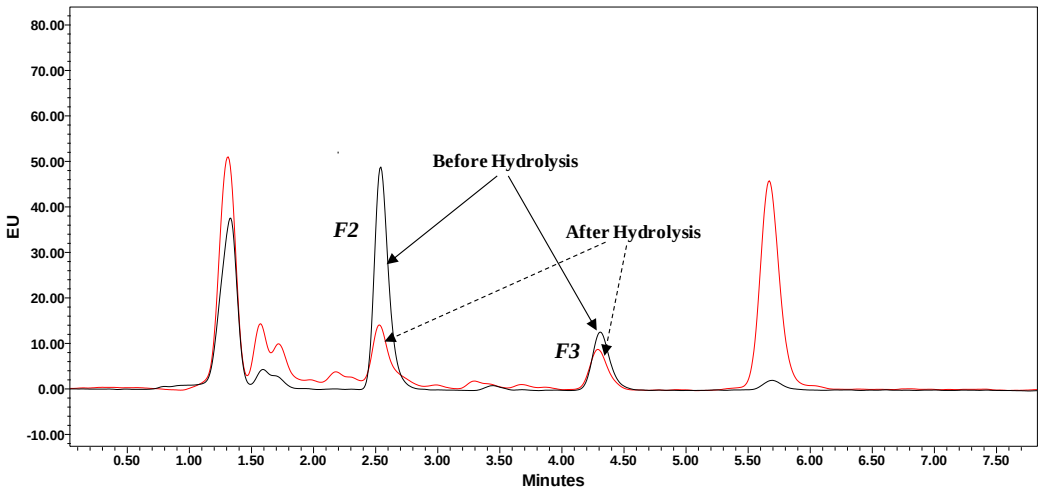


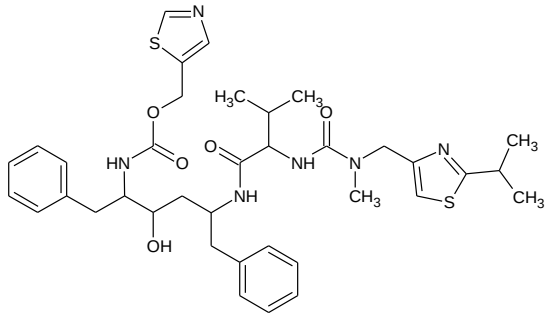

Figure 11: Representative chromatogram for detection of 2Me-E2 glucuronides (*F2* and *F3*) before and after hydrolysis with porcine liver esterase.

### 5.2.2. Effect of Different Inhibitors of UGT and ABC Efflux Transporters

This section describes the experimental results obtained with different inhibitors of UGT and ABC efflux transporters with the purpose of identifying possible UGTs and ABC transporters that might be involved in metabolism and transport of 2Me-E2 and comparing the effects of quercetin (Qc) to these inhibitors.

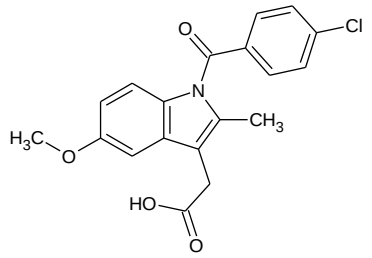
The LS180 intestinal human colon adenocarcinoma cell line was selected for these experiments. RT-PCR analysis (reported in literature) revealed that this cell line highly expresses CYP 3A4 and wide array of UGT 1A and 2B enzymes, including those ones reported earlier to catalyze metabolism of 2Me-E2, i.e. UGT 1A1, 1A3, 1A7, 1A8, 1A9, 1A10, 2B4, and 2B17 (Pfrunder et al., 2003; Nakamura et al., 2008; Lépine et al.,

2004; Basu et al., 2004a). Pfrunder et al. (2003) and Gutmann et al. (2005) have also reported that LS180 cells express a number of ABC transporters such as MDR1/ABCB1, BCRP/ABCG2, and MRP1/ABCC1 through MRP5/ABCC5 with MRP1/ABCC1 and MRP3/ABCC3 being highly expressed, and MRP2/ABCC2 and BCRP/ABCG2 being expressed the least. The expressions of these transporters however, were found to be much less in LS180 cells than in cell lines traditionally used in transport studies, e.g. Caco-2 (Pfrinder et al. 2003). Therefore, the LS180 model may be better suited for induction and metabolism studies rather than for the transport ones. Nevertheless, the preliminary experiments included ABC transport inhibitors such as MK571 (50 μM), GF120918 (1 μM) along with UGT inhibitors such as indomethacin (100 μM), and ritonavir (25 μM). Their structures and inhibitory properties are shown in Figure 12.



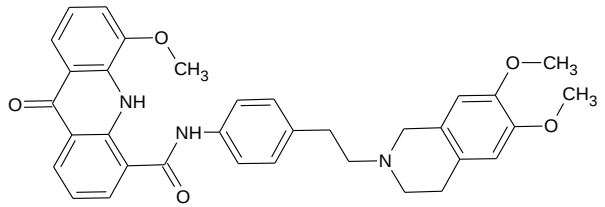
**Ritonavir:**

MDR1/ABCB1: $IC_{50}$ =26.4 μM Bachmeier et al. 2005
MRP-related: $IC_{50}$ = 2.9 μM Bachmeier et al. 2005
CYP3A4: $IC_{50}$ = 28.2 μM Granfors et al. 2006
UGT 1A1: $IC_{50}$ = 19.0 μM Zhang et al. 2005
UGT 1A3: $IC_{50}$ = 6.3 μM Zhang et al. 2005
UGT 1A4: $IC_{50}$ = 2.0 μM Zhang et al. 2005



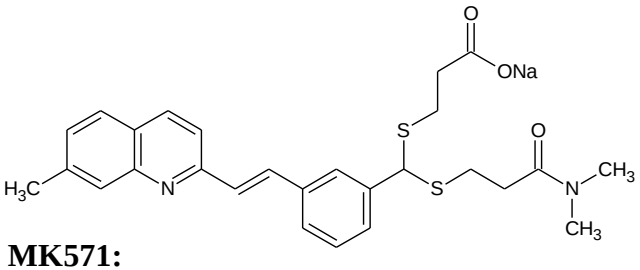
**Indomethacin:**

UGT 1A9: $IC_{50}$ = 34.1 μM Mano et al. 2006
UGT 1A1: $IC_{50}$ = 51.5 μM Mano et al. 2005
UGT 2B7: $IC_{50}$ = 34.0 μM Mano et al. 2007
MRP1/ABCC1: $IC_{50}$ = 44 - 55 μM Dekkers et al. 2000



**GF120918:**

MDR1/ABCB1: $IC_{50}$ = 0.3 μM Matsson et al. 2009



**MK571:**

MRP2/ABCC2: $IC_{50}$ = 10 μM Matsson et al. 2009
MDR1/ABCB1: $IC_{50}$ = 26 μM Matsson et al. 2009
BCRP/ABCG2: $IC_{50}$ = 50 μM Matsson et al. 2009
Inhibits glucuronidation of Naringenin Xu et al., 2009

Figure 12: Structures and inhibitory properties of ritonavir, MK 571, GF120918 and indomethacin.

For current experiments 100 μM 2Me-E2 was selected as a starting point with 60 minutes of reaction time. The results are depicted in Figure 13. By the end of the incubation period, HPLC analysis revealed that at the experimental conditions only one glucuronide of 2Me-E2, namely 2Me-E2-3G, was formed.

As can be seen in Figure 13A, the addition of UGT/CYP inhibitors, ritonavir and indomethacin, decreased total amount of 2Me-E2-3G formed. MK571 (50 μM) showed significant decrease in the formation of 3-glucuronide of 2Me-E2. This outcome can be explained in part by the presence of amide and carboxyl moieties in the structure of MK571, which may be the sites for glucuronidation. Thus, MK571 can be a substrate for UGT and compete with 2Me-E2. Similar results were observed with 100 μM Qc. Only 0.4% of 2Me-E2-3G was formed from 2Me-E2 during 60 minutes of the treatment. GF120918 (1μM) did not exhibit any inhibitory effect on 3-glucuronide formation, in part because it does not have groups that can be attacked by UGTs.

Figure 13B shows similar trend with 2Me-E2-3G efflux to that observed with its formation. Efflux of 3-glucuronide was expressed as a ratio of 2Me-E2-3G concentration in HBSS to its concentration in cells. It is known that glucuronides are typically substrates of MRP1/ABCC1, MRP2/ABCC2, or/and MRP3/ABCC3; therefore 2Me-E2-3G can be a substrate for these transporters. Inhibition of the abovementioned transporters by Qc, ritonavir, indomethacin, and MK571 were suspected in diminished efflux of 2Me-E2-3G. Besides being inhibitors for MRPs/ABCCs transporters, ritonavir, indomethacin, MK571 can undergo metabolism by UGTs forming glucuronides. Their glucuronides can compete with MRPs/ABCCs for their efflux from the cells too.

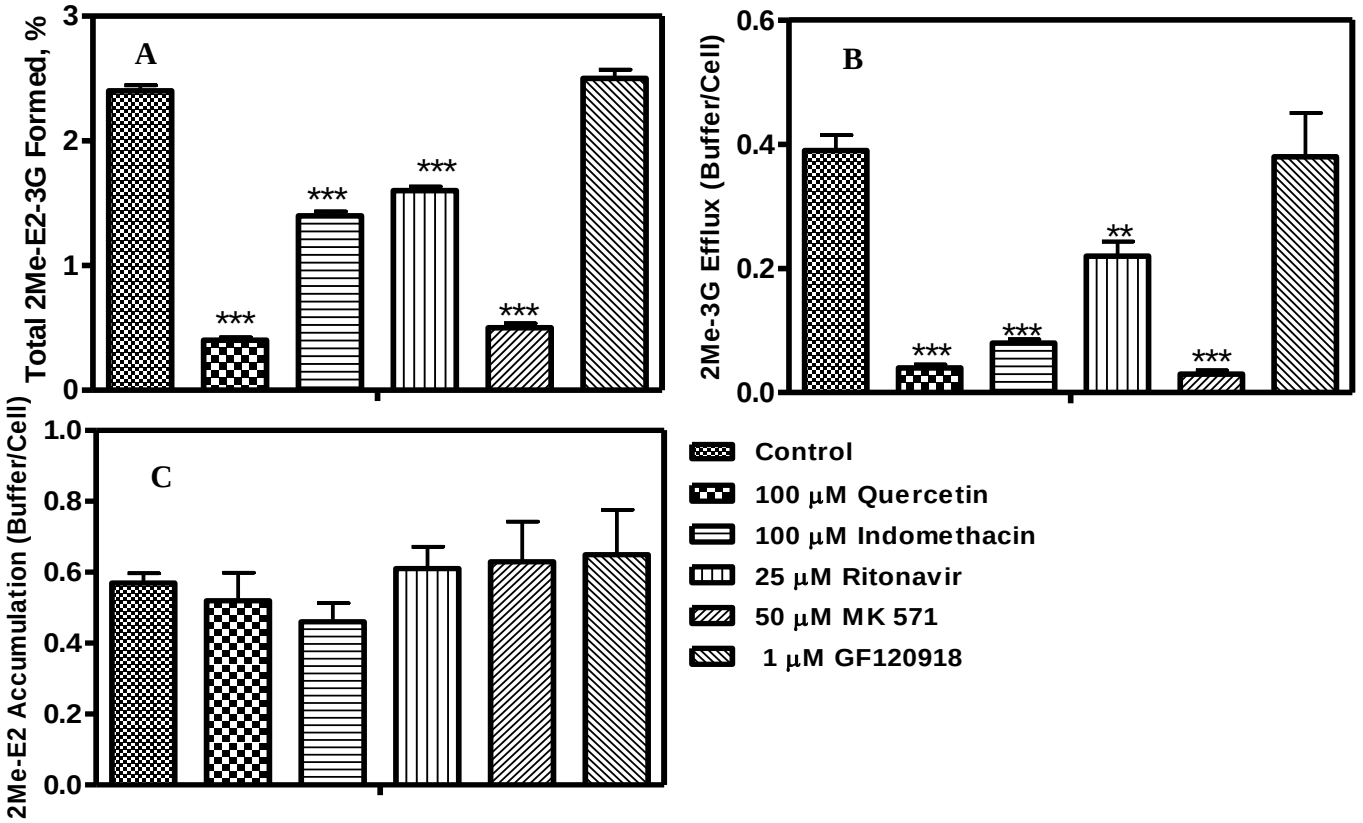



Figure 13: Effect of 25 μM ritonavir, 100 μM quercetin, 100 μM indomethacin, 50 μM MK571, and 1 μM GF 120918 on 2Me-E2-3G formation, its efflux and 2Me-E2 accumulation after incubation in HBSS for 60 min at 37º C. **A**: Formation of 2Me-E2-3G, calculated as a sum of the metabolite amount measured in the cells and buffer; **B**: Efflux of 2Me-E2-3G defined as a ratio of the metabolite concentration in the buffer to its concentration in the cells; **C**: 2Me-E2 accumulation in the cells, defined as a ratio of 2Me-E2 concentration in buffer to its concentration in cells. Data are expressed as mean ± SD (n= 3 – 4). The results were compared to controls with one-way ANOVA followed by Dunnett's post test, where ** and *** are $p < 0.01$ and $p < 0.001$, respectively.

Thus, ‘parent’ inhibitors and their glucuronides might effectively block or compete for MRPs/ABCCs with 2Me-E2-3G leading to a strong inhibition of efflux of 2Me-E2-3G. GF120918 did not affect 2Me-E2-3G efflux. This indicated that 2Me-E2-3G might not be a substrate for MDR1/ABCB1 transporter, as expected since E2-3G does not interact with MDR1/ABCB1 (Huang et al., 1998).

On the other hand, no significant changes in accumulation of 2Me-E2 were observed with all tested inhibitors (Figure 13C). It was expected that MK571 and Qc might exhibit some inhibitory effect toward 2Me-E2 accumulation as they are inhibitors of BCRP/ABCG2 transporter. It was reported earlier that E2 is a substrate of BCRP/ABCG2 (Janvilisri et al., 2003; Imai et al., 2002). Thus, it was assumed that 2Me-E2 might be a substrate of BCRP/ABCG2 as well. Either BCRP/ABCG2 expression is so low in LS180 that any active transport of 2Me-E2 cannot be detected or due to its highly lipophilic nature, 2Me-E2 passively diffuses though the cell membrane so very fast that BCRP/ABCG2-mediated transport cannot be observed at high concentrations of 2Me-E2.

### 5.2.3. Effect of Quercetin on 2Me-E2 Glucuronidation

This section describes data obtained with Qc present simultaneously with 2Me-E2.

#### 5.2.3.1. Condition Selection: Preliminary experiments

In the preliminary experiments with different inhibitors, 100 μM 2Me-E2 and reaction time of 60 minutes were selected as a starting point. Therefore, there was a need in a series of experiments to establish if 100 μM 2Me-E2 affects metabolic process in any

way and lower concentrations might need to be selected, or if 60 minutes is adequately long reaction time.

The experiments were conducted at varying concentrations of 2Me-E2 starting from 0.5 μM and ending with 100 μM. The results are shown in Figure 14.

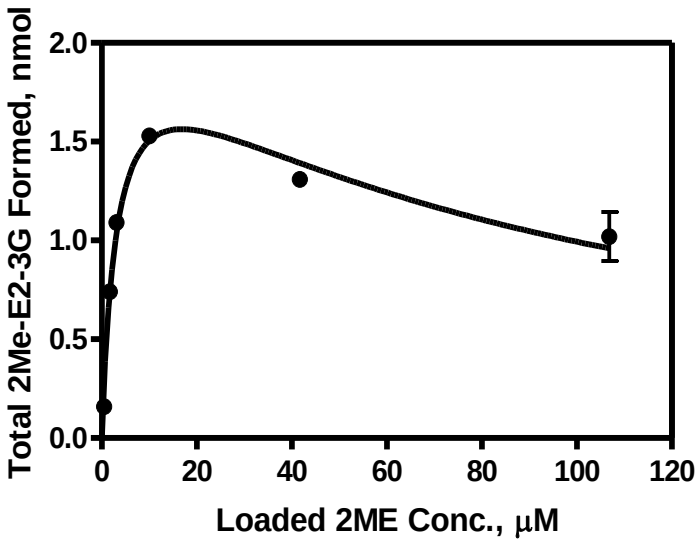



Figure 14: Effect of 2Me-E2 concentration on the formation of 2Me-E2-3G. Total amounts of metabolite (calculated as a sum of its amount in the cells and buffer) were measured after incubation time of 60 minutes at 37º C. Data are expressed as mean ± SD (n = 3 – 4).

As seen in Figure 14, the profile of 2Me-E2-3G formation is consistent with substrate inhibition kinetics unlike with Qc, where 2Me-E2 did not affect metabolism of the antioxidant (Appendix I). Parameters estimated from the equation for substrate inhibition (Eq. 5) are 0.036 ± 0.0018 nmol/min, 3.3 ± 0.36 μM and 87 ± 11.3 μM ($r^2$ = 0.962) for $V_{max}$, $K_m$ and $K_i$, respectively. The concentration of 10 μM 2Me-E2 was

selected for further experiments because at this concentration the maximum amount of 2Me-E2-3G was formed and the inhibition effect was roughly equal to the metabolic rate of glucuronidation. Secondly, lower concentrations of 2Me-E2 might result in a difficulty of detection of metabolite accumulation within the cells, especially in the presence of inhibitor such as Qc.

In the next step, a number of experiments were carried out to observe the rate of 2Me-E2-3G formation within 90 minutes of incubation. The results are depicted in Figure 15. The obtained profile might indicate about a zero-order reaction with slight inhibition in metabolite formation. Nevertheless, 60 minutes was considered to be an appropriate time for sufficient amount of product accumulation.

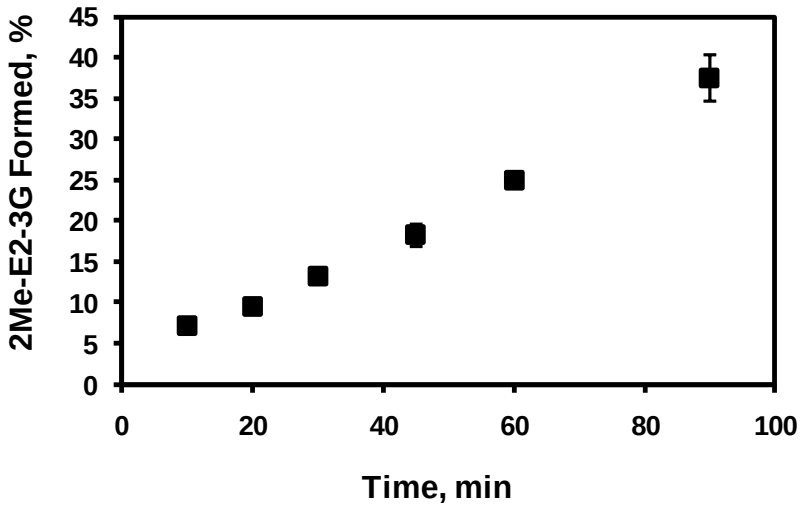



Figure 15: Formation of 2Me-E2-3G after incubation of 10 μM 2Me-E2 for 90 minutes. The quantity of metabolite formed was calculated as a sum of its amount measured in the cells and buffer. Data are expressed as mean ± SD (n = 3 – 4).

#### 5.2.3.2. Glucuronidation and Disposition of 2-Methoxyestradiol

To observe the effect of Qc on 2Me-E2 glucuronidation, its disposition, 0 to 100 μM of this flavonoid was added to the solution containing 10 μM 2Me-E2. Glucuronidation was carried out for 60 minutes and the results are depicted in Figure 16.

Figure 16A shows inhibitory properties of Qc on glucuronidation of 2Me-E2 with $IC_{50}$ being 7.8 ± 0.26 μM ($r^2$ = 0.996). The addition of more than 50 μM Qc did not further reduce the amount of 2Me-E2-3G formed. This implies that Qc could inhibit the majority of UGT activity responsible for the conversion of 2Me-E2 to 3-glucuronide, however the formation rate of metabolite plateaued above zero (~1 pmol/min), consistent with residual Qc resistant UGT activity. Figure 16B illustrates the concentration profile of 2Me-E2 in the cells and buffer (HBSS) with the addition of different concentrations of Qc. It was expected that inhibition of UGTs would results in accumulation of 2Me-E2 in the cells. However, this trend was observed only with the addition of Qc up to 15 μM. With further increase in Qc, 2Me-E2 cell concentration decreased to the value of the 'control' sample and remained unchanged.

In an attempt to explore the reasons behind these results, a series of time course experiments were carried out at 15 μM and 25 μM Qc and compared to the control samples. The results are shown in Figure 17.

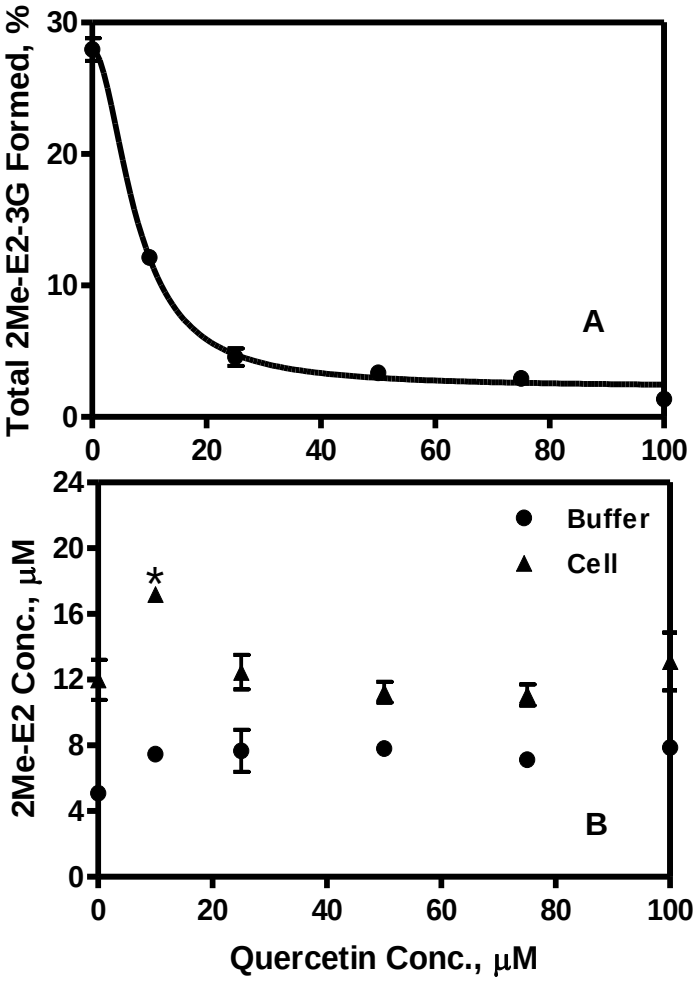



Figure 16: Effect of different concentrations of quercetin on 2Me-E2-3G formation and 2Me-E2 distribution between cells and medium after incubation in HBSS for 60 minutes at 37º C. **A**: Formation of 2Me-E2-3G, calculated as a sum of the metabolite amount measured in the cells and buffer; **B**: 2Me-E2 concentrations in cells and medium. Data are expressed as mean ± SD (n = 3 – 4). The results were compared to those obtained in the absence of quercetin with one-way ANOVA followed by Dunnett's post test, where * indicates $p < 0.05$.

As can be seen from Figure 17, within the first 30 minutes the concentration of 2Me-E2 within the cell was decreasing without significant differences whether Qc was added or not. Afterwards however, a gradual accumulation of 2Me-E2 in the cells took place, but only in the presence of Qc. As more Qc was added to the solution, more accumulation occurred.

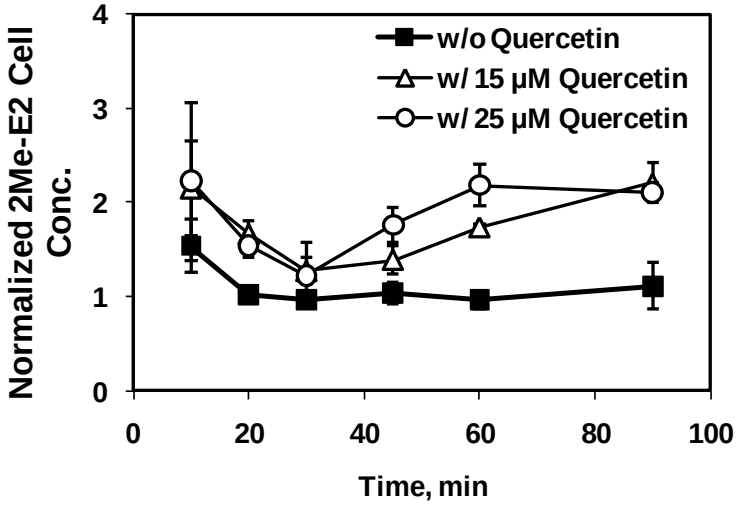



Figure 17: Effect of 15 µM and 25 µM Qc on accumulation of 2Me-E2 in LS180 cells (normalized to initial concentration of 2Me-E2) within 90 minutes. Data are expressed as mean ± SD (n = 3 – 4).

These results might indicate a delay in penetration of Qc into LS180 cells. Therefore, additional experiments were carried out with cells being pre-incubated in the medium containing Qc. LS180 cells were pre-incubated for 30 minutes with 0 µM, 15 µM, and 50 µM Qc followed by 60 minutes of incubation with HBSS containing 0 µM, 15 µM, or 50 µM Qc in presence of 10 µM 2Me-E2, respectively. The results shown in

Figure 18 revealed that pre-incubation with Qc did not affect the rate of glucuronidation (Figure 18A) but led to a drastic increase in 2Me-E2 cell concentration (Figure 18B). 2Me-E2 cell concentration increased up to 3 – 4 times. Thus, slow penetration of Qc can be overcome by pre-incubation with Qc prior the introduction of 2Me-E2.

If in the presence of Qc, with or without a pre-incubation period, the 2Me-E2 concentration in the cells can be affected, the same cannot be said about 2Me-E2 accumulation (Figure 19A and B). The addition of Qc with or without 30 minutes of pre-incubation did not affect the accumulation of 2Me-E2. The above observations might indicate that pre-incubation with Qc could lead to an additional inhibition of enzymes present in the cells, other than UGTs, and at the same time, it did not affect the 2Me-E2 transport, considering the fact that Qc inhibits several ABC transporters. Therefore, the reason for the accumulation of 2Me-E2 may not be associated with inhibition of efflux transporters, which is consistent with our previous results.

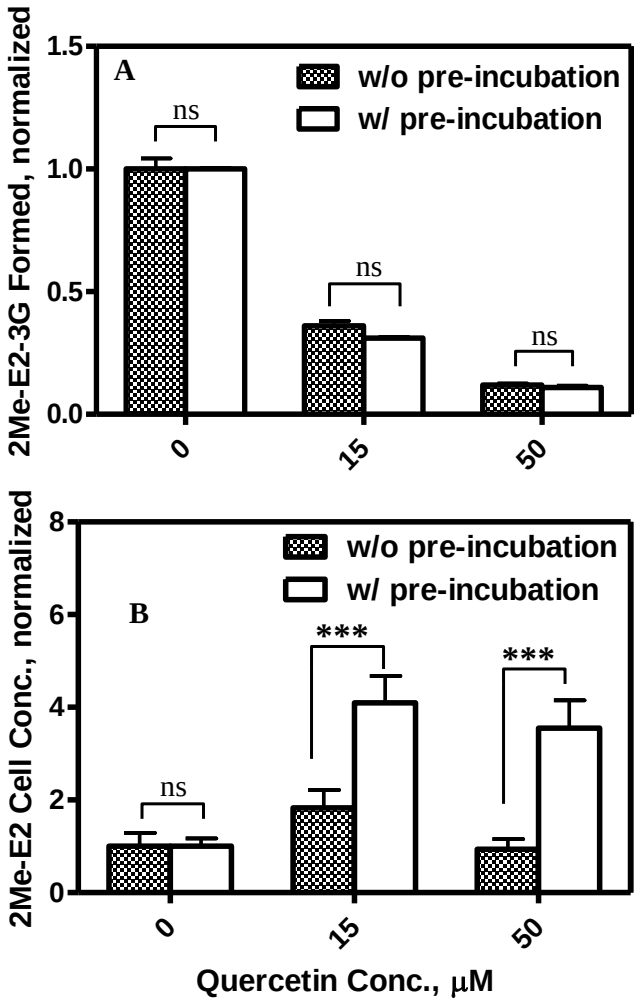



Figure 18: Effect of 30-minutes pre-incubation with Qc on 2Me-E2-3G formation and accumulation of 2Me-E2 in LS180 cells after 60 minutes. **A**: Formation of 2Me-E2-3G, calculated as a sum of metabolite amount measured in the cells and buffer. Data normalized to 52% and 28% – values obtained with and without pre-incubation in absence of Qc; **B**: 2Me-E2 cell concentration, normalized to 13.5 and 12 μM – values obtained with and without pre-incubation in absence of Qc; Data are expressed as mean ± SD (n = 3 – 4). The results were compared with two-way ANOVA followed by Bonferroni's post test, where *** indicates $p < 0.001$.

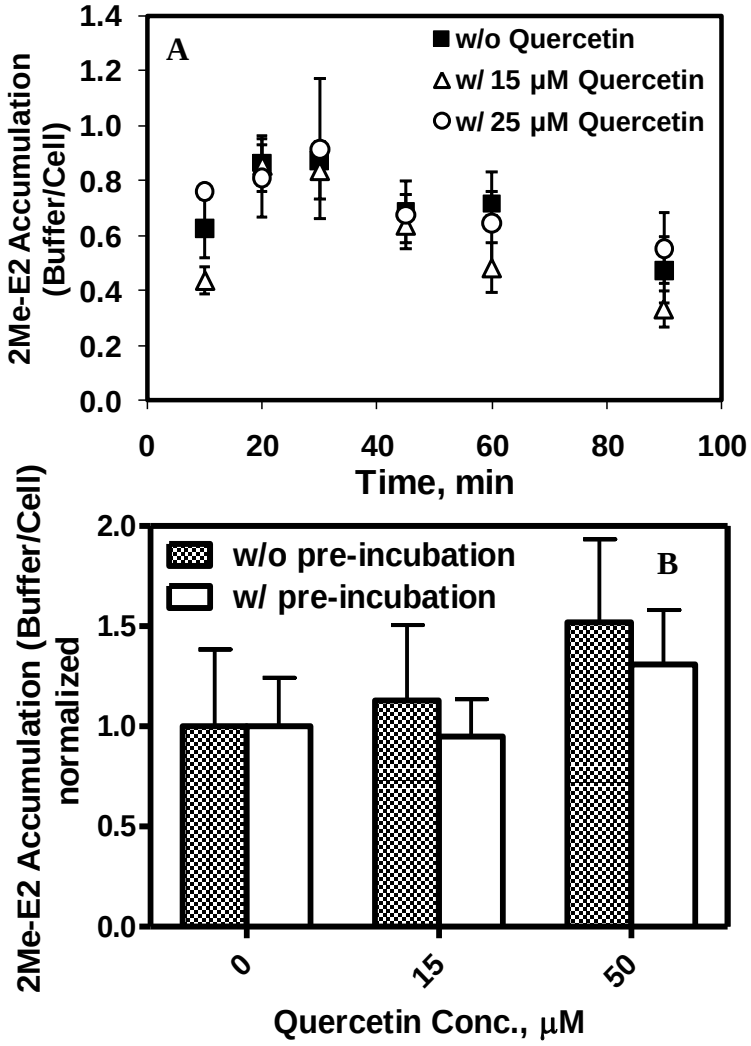



Figure 19: Effect Qc on 2Me-E2 accumulation. **A**: Accumulation of 2Me-E2 observed without 30 minutes pre-incubation for 90 minutes in the presence of 15 µM and 25 µM Qc or its absence; **B**: Effect of pre-incubation with 15 µM and 50 µM Qc on 2Me-E2 accumulation. 2Me-E2 accumulation is defined as a ratio of 2Me-E2 concentration in buffer to its concentration in cells. Data normalized to 0.12 and 0.43 obtained with and without pre-incubation in absence of Qc after 60 minutes; Data are expressed as mean ± SD (n = 3 – 4).

### 5.2.4. Efflux Transport of 2-Methoxyestradiol with BCRP/ABCG2

The above results indicated that cellular accumulation of 2Me-E2 was not affected by inhibition of ABC transporters that might be expressed in LS180 cell line (MDR1/ABCB1, MRP/ABCC 1 – 3). Previously reported data (Janvilisri et al., 2003; Imai et al., 2002) have shown that E2 is a substrate of BCRP/ABCG2. Thus, this section describes data obtained from a series of experiments conducted with MDCK-BCRP cells in attempt to determine, in isolation from the metabolic processes, if 2Me-E2 might be transported by BCRP/ABCG2 at the concentrations used in this study.

The experiments were carried out at 10 μM 2Me-E2, the concentration used throughout this study, for 30 minutes with Prazosin (20 μM) as a positive control for BCRP/ABCG2 activity. No metabolic activities were observed during this period of time. Thus, based on obtained data, directionality ratio (DIR) was estimated to be 11.9 ± 0.60 for the positive control; whereas DIR for 2Me-E2 was estimated to be only 0.8 ± 0.21. This result implies that, at this concentration of 2Me-E2, BCRP/ABCG2 does not significantly contribute to its efflux transport. However, 2Me-E2 is a very lipophilic compound and might very fast diffuse through MDCK cell monolayer so that BCRP/ABCG2 activity was not discerned at this 2Me-E2 concentration. Therefore, more experiments are proposed to be undertaken in the future at much lower concentrations of 2Me-E2, in nanomolar range, using more sensitive analytical technique than used in this study.

### 5.2.5. Conclusions

A series of experiments were conducted to investigate 2Me-E2 glucuronidation and the effect of Qc on its metabolism rate and disposition with LS180 cells expressing a wide array of UGTs. In such a system, the glucuronidation reaction of 2Me-E2 proceeded with the formation of 2Me-E2-3G as a main reaction product. High concentrations of 2Me-E2, up to 100 μM, caused inhibition of 3-glucuronide formation exhibiting substrate inhibition kinetics, with $K_i$ being 87 ± 11.3 μM. On the other hand, the addition of 2Me-E2 did not affect the glucuronidation of Qc (Appendix I).

The presence of Qc in the medium effectively, although incompletely, inhibited UGT activity ($IC_{50}$ = 7.8 ± 0.26 μM). The presence of Qc in the medium however, did not lead to immediate increase of 2Me-E2 in the cells. This delay could be associated with slow penetration of Qc into the cells. Therefore, the experiments carried out with pre-incubation of the cells with Qc revealed an increase in 2Me-E2 concentration up to 3 – 4 times in comparison to the experiments without pre-incubation. It was also determined that efflux transporters might not interfere with 2Me-E2 accumulation in the cells at the concentrations used in this study.

# CHAPTER 6

## Summary and Future Work

This research was conducted to investigate if OS can have a negative effect on transport processes mediated by efflux transporters. This interference, in turn, might affect drug absorption and protective properties of several endogenous tissues/organs. For the purpose of this study, two reactive species involved in OS, $ONOO^-$ and 4HNE, were selected and their effects on BCRP/ABCG2 were observed. The latter was selected as an example of ABC transporter. Both $ONOO^-$ and 4HNE inhibited the efflux of BCRP/ABCG2 substrate with $ONOO^-$ exhibiting a stronger effect. The half maximal inhibitory concentrations for both reactive species obtained were in the micromolar range. These experiments have shown that BCRP/ABCG2 is susceptible to OS and further studies would be needed to investigate the severity of damage to other major efflux transporters such as MDR1/ABCB1, MRP/ABCC 1 to 3. Moreover, it is of great interest to investigate the efficacy of protective mechanism by antioxidants, synthesized in the body or digested with food, toward ABC transporters.

Finally, antioxidants are substances that are known to reduce the amount of ROS/RNS accumulated during OS. However, this research considered the use of antioxidants not as interceptors of ROS/RNS but rather as inhibitors of metabolic enzymes. Qc was selected for the study because of its abundance in natural products and strong antioxidant properties. Its five hydroxyl groups make this compound susceptible

to attack by UGTs and thus causes reduction in biotransformation to glucuronides of such compounds as 2Me-E2, a compound with potential to alleviate several medical conditions. The metabolism experiments were carried out with the LS180 cell line, which is reported to express a wide array of UGTs. The results indicate that Qc could easily inhibit the activity of majority of UGTs. However, in order to achieve higher intercellular concentrations of 2Me-E2, cells should be pre-incubated with Qc prior to the addition of 2Me-E2. The increase in cellular concentration of 2Me-E2 upon Qc treatment did not appear to be dose-dependent with Qc co-incubation between 15 – 50 μM. The mechanism of such a trend is not known and would require further investigation starting with the characterization of LS180 cells: UGTs as well as ABC efflux transporters that might be expressed in this cell line. It was concluded however, that under the present experimental conditions this effect/trend was not achieved via inhibition of 2Me-E2 active transport.

An attempt to characterize the role of the BCRP/ABCG2 transporter on the disposition of 2Me-E2 was also undertaken. Perhaps due to its high lipophilicity, no transport activity of BCRP/ABCG2 toward this compound was observed. Since it has been reported that E2 is a substrate/inhibitor of BCRP/ABCG2, additional experiments would need to be carried out at much lower concentrations of 2Me-E2 and with a more sensitive analytical technique to conclusively rule out the possibility of 2Me-E2 transport/efflux by BCRP/ABCG2.

This study revealed that 2Me-E2-3G was a major metabolite formed under the experimental conditions in LS180 cells. However, due to the complexity of the system, i.e. simultaneous presence of transport and metabolic activities, it was not possible to make firm conclusions about the effect of Qc and its glucuronides on 2Me-E2-3G efflux.

Therefore, further studies would be necessary to characterize the major transporters that are responsible for the efflux of both Qc and 2Me-E2 glucuronides independent of the metabolic processes.

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# APPENDIX I

A series of experiments with LS180 cells revealed that metabolism of 2Me-E2 exhibited substrate inhibition kinetics. To see if 2Me-E2 might also interfere with metabolism of quercetin (Qc), several experiments were carried out with 15 μM Qc in absence or in presence of 10 μM or 100 μM 2Me-E2. The results are depicted in Figure 20. As can be seen from the figure, within the concentration range of 0 to 100 μM 2Me-E2 did not interfere with Qc metabolism.

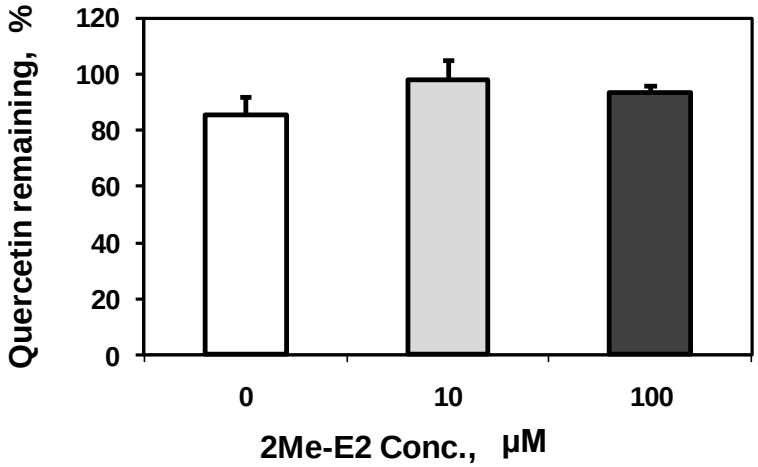


Figure 20: The effect of different 2Me-E2 concentrations on metabolism of Qc within 60 minutes of incubation. Data are expressed as mean ± SD (n= 3 – 4).

# VITA

Svetlana Verenich was born on August 17, 1976, in Tallinn, Estonia. She received her Master of Science in Chemical Engineering from Lappeenranta University of Technology, Lappeenranta, Finland in 1998. Subsequently she received a Ph.D. in Chemical Engineering from Lappeenranta University of Technology, Lappeenranta, Finland in 2003 and was a recipient of 'Best Ph.D. Thesis' award, Graduate School in Chemical Engineering (GSCE), Finland. From 2003 Svetlana was working as a post-doctoral researcher at Nonwovens Cooperative Research Center (NCRC), The Nonwovens Institute, North Carolina State University. She has published 19 research articles in peer reviewed journals, made 10 presentations in international conferences. She also has a bibliographical record in 'Who is Who in Science and Engineering', Marquis Who's Who Publication, 7th edition, 2003 – 2004. Svetlana was a recipient of 2010 VCU Graduate School Dissertation Assistantship.