Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Participation of selected carbonyl reductases in deactivation of anticancer drugs

Doctoral thesis

Supervisor: Prof. Ing. Vladimír Wsól, Ph.D.

Hradec Králové, 2010

Mgr. Romana Odiana (nee Novotná)

Acknowledgments

I would like to thank Prof. Wsól for the advice and help he rendered to me during my postgraduate studies, all Department of Biochemical Sciences staff and PhD students for friendship and support and last but not least my husband and family for endless patience and encouragement.

Contents

1. Introduction

Enzymes are predominantly protein structures that catalyze chemical reactions. Almost all processes in cells need enzymes to occur at significant rates. Some enzymes require a non-protein structure, a cofactor, to be bound for activity. These could be inorganic, e.g. metal ions, or organic structures, e.g. hem or flavin. Cofactors can be bound tightly to the enzyme, prosthetic groups, or released from the active site after reaction is complete, e.g. NADPH; NADH etc.

Enzymes greatly differ in the extent of substrate specificity. Shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Enzymes also show various levels of stereospecificity, regioselectivity and chemoselectivity.

Since enzymes are essential for homeostasis maintenance, any malfunction (mutation, overproduction, underproduction or deletion) of a single critical enzyme can lead to a disease.

Scientists have been trying to identify and characterize individual enzymes that take part in biological processes, specify the role of these enzymes in cells, find the optimal conditions for enzymes´ activity (pH, temperature, presence of cofactors, inhibitors and inductors) and also reveal the extent of enzymes´ influence on drug therapy. Medicines can become enzyme substrates, inhibitors or inductors, this fact can have a great impact on diseases and their treatment.

Unfortunately, many enzymes are very sensitive to even a slight change of the environment and can lose their activity (especially pH, used buffer, absence of phospholipids in membrane bound enzymes etc.). This makes a precise characterization of enzymes more complicated.

Protein purification is one of the ways how to characterize enzymes in greater detail. To find the right combination of purification steps which would not decrease or destroy enzyme´s activity is difficult and usually takes a long time.

Some anticancer agents used in practice, e.g. doxorubicin and daunorubicin, can be deactivated by reduction of carbonyl groups in their structure. This leads to a decrease in the therapy efficiency and increased occurrence of the side effects. That is why scientists focus not only on trying to develop new anticancer drugs (e.g. oracin) but also on the deactivation process as a whole. Their goal is to identify the enzymes participating in this metabolism. Some of these have been characterized, some still remain unknown. More detailed knowledge of the deactivation pathways would enable the use of specific enzyme inhibitors and therefore the increase of anticancer therapy impact.

2. Theoretical part

2.1. Biotransformation of xenobiotics

Xenobiotics metabolism is the set of metabolic pathways that modify the chemical structure of xenobiotics, which are compounds foreign to an organism's normal biochemistry, such as drugs, poisons, environmental pollutants, etc. These pathways are a form of biotransformation present in all major groups of organisms.

These reactions often act to detoxify xenobiotic compounds, however in some cases the intermediates in xenobiotic metabolism can themselves be the cause of toxic effects.

The extent of metabolism each xenobiotic undergoes depends on its chemical structure and physical properties. Since xenobiotics are in most cases hydrophobic molecules and therefore can diffuse across biological membranes, their biotransformation is carried out by enzymes with broad substrate specificities.

Xenobiotic metabolism can be divided into three phases. These reactions act in concert to detoxify xenobiotics and remove them from cells.

In *phase I*, a variety of enzymes acts to introduce reactive and polar groups into their substrates. The reactions in phase I include:

- Oxidation reactions have been extensively studied for a long time. The most common oxidations are carried out by cytochrome P-450-dependent mixed-function oxidase system.
- Reduction reactions will be described in more detail further in this thesis.
- Hydrolytic reactions occur mostly in plasma, erythrocytes and microsomal fraction of liver and kidneys.

In *phase II,* activated xenobiotic metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine (or other amino acids), glucuronic acid, or acetyl coenzyme A. These reactions are catalyzed by a large group of broad-specificity transferases, which in combination can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups.

After phase II reactions, the xenobiotic conjugates may be further metabolized. A common example is the processing of glutathione conjugates to acetylcysteine (mercapturic acid) conjugates.

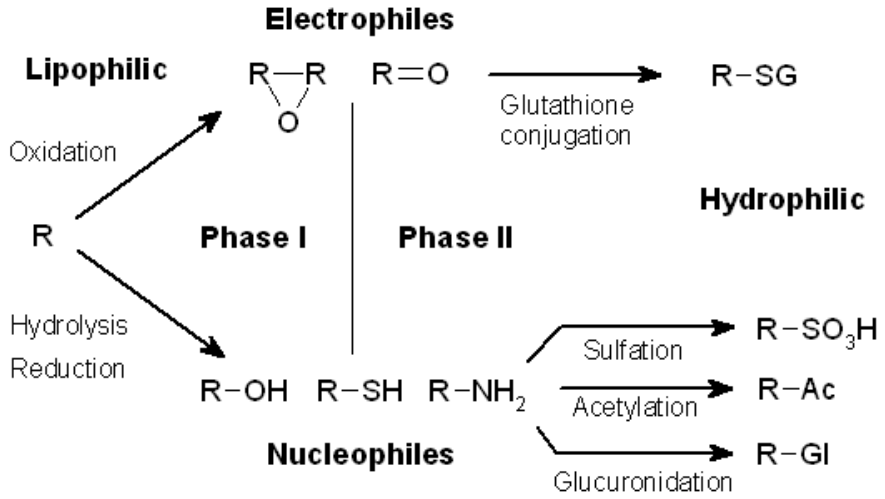


Fig 1. Some of the most common reactions of phase I and phase II xenobiotic metabolism

Conjugates and their metabolites can be excreted from cells in *phase III* of their metabolism. Transport mechanisms act to remove phase II products to the extracellular medium. In humans, transporters belonging to P-glycoprotein and multidrug resistance protein families can catalyze excretion of huge variety of hydrophobic compounds. The transport is ATP dependent and is induced by the presence of xenobiotics.

Reduction reactions have not been studied as extensively as oxidation reactions. That is one of the reasons why we decided to focus on these paths of biotransformation and enzymes which catalyze them.

2.2. Reduction reactions in xenobiochemistry

Reduction is the reverse of oxidation and therefore it can involve loss of oxygen atom or the addition of two hydrogen atoms. Many of enzymes involved in oxidation can also mediate reductions. For example, some drugs, especially those that are very electron deficient because of nitro groups, etc., can be reduced by cytochromes P450. Some other enzymes, such as alcohol dehydrogenase (ALD), are readily reversible and the same enzyme can also catalyze reduction.

Much reduction occurs in anaerobic bacteria in the gut because, being anaerobic, much of the metabolism of these organisms involve reductive pathways.

The nomenclature of reductases is yet to be clarified. There are several ways of reductase classification.

EC Number classification

This is based on the substrate utilized by the enzymes,

- **EC 1.1** Substrate contains the CH-OH group. These dehydrogenases act on alcohols and hemi-acetals.
- **EC 1.2** Substrate contains the aldehyde or oxo group. These dehydrogenases act on aldehydes.
- **EC 1.3** Substrate contains the CH-CH group. These dehydrogenases introduce double bond by direct dehydrogenation of a single bond.
- **EC 1.4** Substrate contains the CH-NH_2 group. Amino acid dehydrogenases.

- **EC 1.5** Substrate contains the CH-NH group. These enzymes converts secondary amines by introducing of double bond C=N.
- **EC 1.6** Substrates are NADH or NADPH. The catalytically important is reverse reaction - reduction of the substrate with NAD^+ or $NADP^+$ acceptors.
- **EC 1.7** Donor for other nitrogenous compounds.
- **EC 1.8** Substrate contains a sulphur group. Substrates usually inorganic or organic thiols.
- **EC 1.9** Substrate contains a heme group. Cytochrome oxidases and nitrate reductases are member of this group.
- **EC 1.10** Donor for diphenols and ascorbates.
- **EC 1.11** Acceptor for a peroxide. The only one sub-subclass - peroxidases.
- **EC 1.12** Donor for hydrogen. Reduction of H^+ to H_2.
- **EC 1.13** Incorporation of oxygen from O_2 to a single donor Second hydrogen donor is not required.
- **EC 1.14** Incorporation of oxygen from O_2 to a paired donors.
- **EC 1.15** Acceptor for superoxide. Only one subclass.
- **EC 1.16** Oxidizing metal ions to a higher valency state.
- **EC 1.17** Oxidizing of -CH- or -CH_2- groups to -CHOH- or -COH-.
- **EC 1.18** Acting on iron-sulfur proteins.
- **EC 1.19** Acting on reduced flavodoxin with dinitrogen as acceptor.
- **EC 1.20** Donors contains phosphorus or arsenic.
- **EC 1.21** Formation of X-Y bond from X-H and Y-H.
- **EC 1.97** Other oxidoreductases, which are not included into previous categories.
- **EC 1.98** Enzymes using H_2 as reductant
- **EC 1.99** Other enzymes using oxygen (O_2) as oxidant

According to Nomenclature recommendations form Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), the classical name is donor:acceptor oxidoreductase, but the name dehydrogenase is also acceptable. It is also possible to use name acceptor reductase, but all these classifications can be difficult in cases where many different acceptors can be utilize by the same enzyme (enzymes.me.uk).

Classification according to sequence similarity

Enzymes are grouped into superfamilies, families and subfamilies according to the amino acid sequence of protein chain, three-dimensional structure and function. Some superfamilies group a large number of enzymes; some would be formed by a single enzyme.

Some enzymes have common names which were used before the classification systems and naming rules were introduced (e.g. pepsin).

Enzymes involved in reduction reactions

Carbonyl reductases are the main topic of this thesis that is why more detailed information about them can be found in the following chapters. In part, let me briefly introduce examples of some other enzymes participating in reductive metabolism.

Cytochrome P450

Under certain conditions, especially anaerobic conditions or with certain substrates, cytochrome P450s can act as reductases. They can catalyze reductive dehalogenation; nitrogen-containing functional groups of various oxidation states can be reduced to corresponding saturated nitrogen-containing functional group, e.g. amine, hydrazine, amidine, etc.

Xantine oxidase and Aldehyde oxidase

These molybdenum-containing cytosolic enzymes are often the major contributors in the reduction of nitrogen-containing compounds.

NAD(P)H Quinone oxidoreductase

The major enzyme responsible for quinone reduction is NAD(P)H quinone reductase, termed NQO1 or QR1. A second form of the enzyme, NQO2 or QR2, was characterized almost 50 years ago. The two enzymes are

widely distributed throughout the body, but expression varies considerably with individual, organ and physiological state. Both enzymes are flavin dependent but utilize different reducing cofactors. QRs are direct two-electron reductants, i.e. quinone is reduced to hydroquinone in a single step. This reaction doesn't lead to a formation of reactive and toxic semiquinone radical (Uetrecht and Trager, 2007).

Other examples of reductases are: Dihydrofolate reductase; 5α reductase; methemoglobin reductase, HMG-CoA reductase etc.

2.2.1. Aldo-keto reductases

The Aldo-keto reductases (AKRs) represent a large superfamily of NAD(P)(H)-dependent soluble oxidoreductases (Jez et al., 1997a). They all possess the $(\alpha/\beta)_8$-barel motif characteristic of triose phosphate isomerase (TIM), a conserved cofactor binding site and a catalytic tetrad, variable loop structures that define substrate specificity and contain approximately 320 amino acids per monomer. They catalyze the reduction of aldehydes and ketones, monosaccharides, ketosteroids and prostaglandins; and also the oxidation of hydroxysteroids and *trans*-dihydrodiols of polycyclic aromatic hydrocarbons. Less is known about their drug substrates (Hyndman et al., 2003).

With 159 members and 15 families, AKRs can be found across biological kingdoms and phyla (in procaryotes and eucaryotes including yeast, plants, amphibia and mammals). This system is quite complex and that is why, to avoid confusion, a nomenclature system was proposed and accepted at the Eight International Symposium on Enzymology and Molecular Biology of Carbonyl Metabolism (Jez et al., 1997b). A Web site that provides updates to the AKR superfamily is available (www.med.upenn.edu/akr), where information on sequence, alignments, common names, structure and potential AKR members is available as well as its link to the Human Genome database (Hyndman et al., 2003).

The nomenclature system includes the root symbol “AKR” for aldo-keto reductase; an Arabic number indicating the family; a letter describing the subfamily and an Arabic number designating the unique protein sequence.

The criteria follow that members within a family have less than 40% amino acid sequence identity with other families and that members within a subfamily have greater than 60% sequence identity. Currently, there are 15 families with nine having multiple subfamilies. The largest of which is AKR1, which contains aldose reductases, aldehyde reductases, hydroxysteroid dehydrogenases and steroid 5β-reductases.

Proteins with greater than 97% amino acid sequence identity are considered alleles, unless they have different enzymatic activities, have distinct 3´ untranslated regions (UTRs), or are derived from genes of different structures and/or chromosomal localization (Hyndman et al., 2003).

The majority of known AKRs are monomeric, however new evidences suggest multimeric forms in the AKR2, AKR6 and AKR7 families (Kelly et al., 2000; Kavanagh et al., 2002; Kozma et al., 2002).

2.2.1.1 Human AKR1C isoenzymes

According to the Aldo-keto superfamily homepage, there are 26 members of this subfamily (www.med.upenn.edu/akr).

Let me concentrate on those isoforms present in human. These family members share greater than 86% sequence identity at the amino acid level but the individual enzymes show significantly different biological properties. Human AKR1C isoenzymes catalyze the oxidoreduction of different substrates to varying degrees, and exhibit different inhibition and expression patterns (Table 1) (Khanna et al., 1995; Matsuura et al., 1997; Penning et al., 2000).

Table 1. Nomenclature of human AKR1C subfamily members (Bauman et al., 2004)

Nomenclature	Other names	Tissue distribution	Sequence identity (%)*
AKR1C1	20α-HSD DD1	Lung > liver > testes > mammary gland	86.0
AKR1C2	Type III 3α-HSD DD2 Human bile-acid binding protein	Lung and liver > prostate, testis and mammary gland	87.9
AKR1C3	Type II 3α-HSD Type V 17β-HSD DDX	Mammary gland > prostate > liver and lung	99.4
AKR1C4	Type I 3α-HSD Chlordecone reductase DD4	Liver specific	84.1

*Sequence identity is compared to AKR1A1, its sequence is considered 100%

The multiple substrates utilized by human AKR1C members include steroids, prostaglandins, *trans*-dihydrodiols of polycyclic aromatic hydrocarbons, bile acids, and a number of endogenous and xenobiotic aldehydes and ketones (Khanna et al., 1995; Matsuura et al., 1998; Breyer-Pfaff and Nill, 2000; Atalla and Maser, 2001).

Human AKR1Cs are very important in pre-receptor regulation of steroid hormone action. Each isoform is able to catalyze the reduction of 3-, 17- and 20-ketosteroids and each can also catalyze the oxidation of 3α-, 17β- and 20α-hydroxysteroids to varying extents (Penning et al., 2000).

Table 2. Human AKR1C isoforms and steroid metabolism* (Bauman et al., 2004)

Isozyme	3α-HSD activity 5α-DHT reduction k_{cat}/K_m ($min^{-1}mM^{-1}$)	17β-HSD activity Androsterone reduction k_{cat}/K_m ($min^{-1}mM^{-1}$)	20α-HSD activity Progesterone reduction k_{cat}/K_m ($min^{-1}mM^{-1}$)
AKR1C1	+	+	+++
AKR1C2	++	-	-
AKR1C3	+	++	-
AKR1C4	+++	-	-

*Where (-) means 0-0.9; (+) means 1-10; (++) means 11-100; (+++) means 101-1000

Table 3. Manipulation of nuclear hormone action by the human aldo-keto reductase 1C (AKR1C) isoenzymes

Activation of the receptor		Inactivation of the receptor
AKR1C1 ↓	progesterone receptor	AKR1C1 ↑
AKR1C3 ↓		AKR1C3 ↑
AKR1C2 ↓	androgen receptor	AKR1C2 ↑
AKR1C3 ↑		AKR1C3 ↓
AKR1C3 ↑	estrogen receptor	AKR1C3 ↓

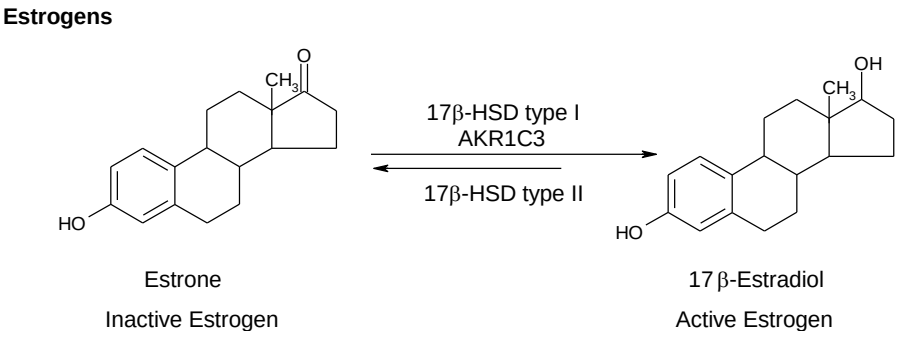


Fig 2. The metabolism of estrogens by hydroxysteroid dehydrogenases

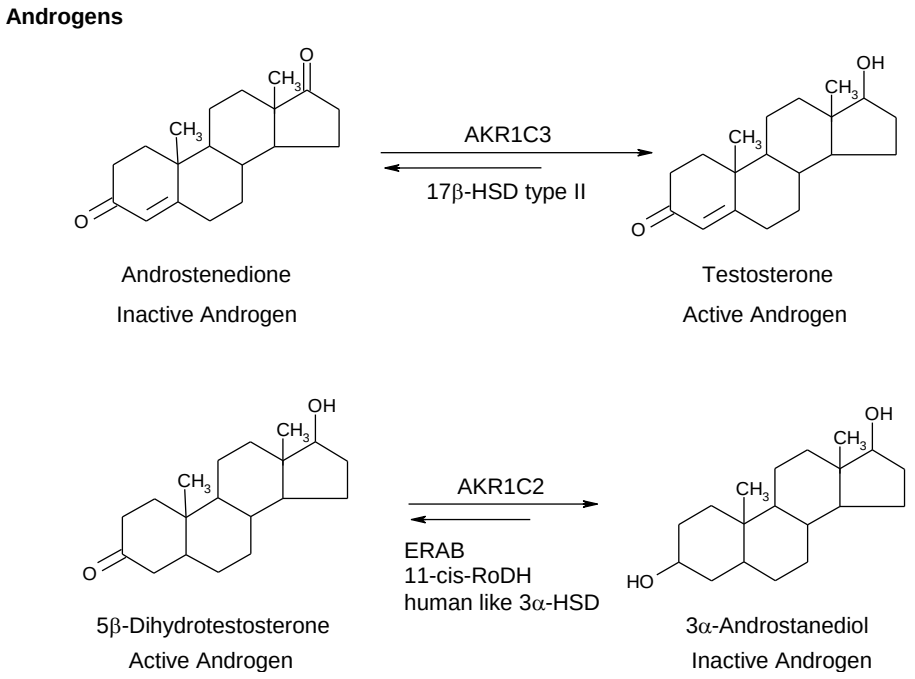


Fig 3. The interconversion of active and inactive androgens by hydroxysteroid dehydrogenases

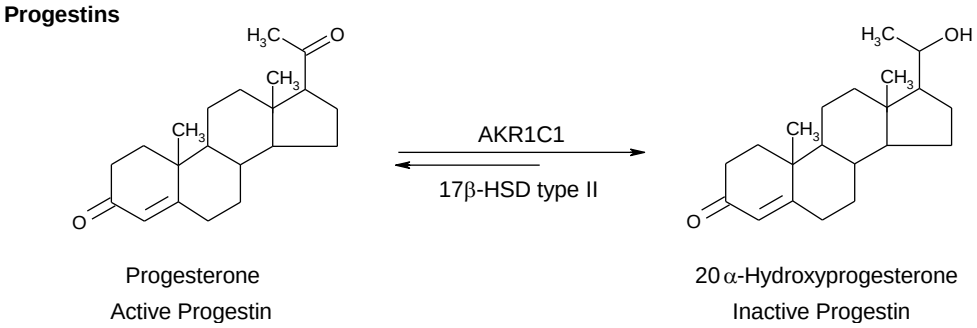


Fig 4. Metabolism of progestins by hydroxysteroid dehydrogenases

2.2.1.1.1. AKR1C1

AKR1C1 was for the first time cloned by Stoltz et al. (1993). Its mRNA expression is highest in the lung, followed by the liver, testis, mammary gland and endometrium (Penning et al., 2000). AKR1C1 catalyzes the reduction of progesterone to 20α-hydroxyprogesterone most efficiently. In rodents, 20α-HSD activity has a major role in terminating pregnancy (Ben-Zimra et al., 2002). In

humans, it is not clear if AKR1C1 plays a dominant role in the termination of pregnancy; however, it is the major 20α-HSD discovered up to date.

The enzyme exhibits additional 3α- and 3β-HSD activities depending on the steroid structure: It reduces 3-keto-5β-dihydrosteroids into the 3α-hydroxy derivatives, whereas 3β-hydroxy products are formed on the reduction of 3-keto-5α-dihydrosteroids (Steckelbroeck et al., 2004; Ishikura et al., 2005). The 5α-androstane-3β-17β-diol and 5α,3β-tetrahydroxypregnanes produced are ligands of estrogen receptor β (Weihua et al., 2001) and antagonists for the γ-aminobutyric acid type A ($GABA_A$) receptor (Wang et al., 2002; Turkmen et al., 2004), respectively. Thus, AKR1C1 plays important roles in steroid metabolism through the formation of steroids, as well as inactivation of progesterone, neuroactive 5α/β-pregnan-3α-ol-20-ones (positive modulators of $GABA_A$ receptor), and 5α-dihydrotestosterone (Bauman et al., 2004).

AKR1C1 differs from AKR1C2 (3α-HSD type 3) by only seven amino acids, of which the residue at the position 54 (Leu in AKR1C1 and Val in AKR1C2) has been demonstrated to be a determinant of the steroid specificity of the two enzymes in site-directed mutagenesis and crystallographic studies (Matsuura et al., 1997; Couture et al., 2003; Ishikura et al., 2005).

AKR1C1 reduces variety of non-steroid carbonyl compounds including prostaglandins, NNK, and xenobiotic carbonyl compounds. The drug substrates which are metabolized by AKR1C1 are: dolasetron, naloxon, naltrexon, oxycodone, oracin, befunolol, ketotifen, 10-oxonortriptyline, haloperidol, loxoprofen, acetohexamide, daunorubicin, ethacrynic acid (Ohara et al., 1995; Breyer-Pfaff and Nill, 2000; Breyer-Pfaff and Nill, 2004; Wsól et al., 2005a). Although, these drugs are not specific substrates for AKR1C1, catalytic efficiency comparison indicated that dolasetron, an antiemetic 5-HT_3 receptor antagonist, is most efficiently reduced by this enzyme (Breyer-Pfaff and Nill, 2004). AKR1C1 is selectively and potently inhibited by benzbromarone (Higaki

et al., 2003), suggesting a possible drug-drug interaction between this drug and the above substrates of the enzyme.

The enzyme is induced by ethacrynic acid, polycyclic aromatic hydrocarbons, other polycyclic aromatic compounds, electrophylic Michael acceptors, phenolic antioxidants, ROS, 4-hydroxynonenal, and dietary indoles and isothiocyanates (Ciaccio et al., 1994; Burczynski et al., 1999; Bonnesen et al., 2001; Burczynski et al., 2001).

2.2.1.1.2. AKR1C2

AKR1C2 was cloned for the first time by Qin et al. (1993) and, unlike the other human AKR1C isoforms, binds bile acids with a high affinity (nanomolar affinity). However, it does not catalyze the reduction or oxidation of the 3-keto/hydroxyl position on bile acids (Shiraishi et al., 1998).

AKR1C2 is also known as DD2 and type III 3α-HSD (Takikawa et al., 1990; Dufort et al., 1996). AKR1C2 mRNA is primarily expressed in the lung and liver, followed by the prostate, testis, mammary gland, uterus and brain (Shiraishi et al., 1998; Penning et al., 2000; Dufort et al., 2001).

AKR1C2 is the main bile acid binder in liver and intestine. Bile acids are crucial for the intestinal adsorption of fat, promotion of bile flow and regulation of hepatic cholesterol homeostasis (Stolz et al., 1993). In the liver, AKR1C2 acts as a bile acid transporter and does not function in steroid metabolism, as it is completely saturated with bile acids, which leads to a potent and complete inhibition of its enzyme activity (Deyashiki et al., 1992). On the other hand, in peripheral tissues the enzyme is involved in metabolizing steroids, since it efficiently metabolizes the reduction of DHT to 3α-diol while bile acids are not present.

Despite its structural similarity with AKR1C1, this enzyme mainly exhibits 3α-HSD activity. The enzyme is believed to play important roles in the

inactivation of 5α-dihydrotestosterone and the synthesis of neuroactive 5α/β-pregnan-3α-ol-20-ones from their precursors (Bauman et al., 2004). In addition to the roles of AKR1C2 in development and progression of prostate cancer, recent literature suggested possible involvement of the enzyme in the pathogenesis of glaucoma and obesity in women. Significantly high expression and activity of AKR1C2 are observed in human glaucomatous optic nerve head astrocytes (Agapova et al., 2003) and the expression and activities of AKR1C2 and AKR1C1 in omental adipose tissue are positive correlates with adiposity in women (Blouin et al., 2005).

The substrate specificity of AKR1C2 for non-steroid carbonyl compounds (Deyashiki et al., 1992; Nishizawa et al., 2000) is similar to that of AKR1C1 (dolasetron, naloxon, naltrexon, oxycodone, oracin, befunolol, ketoprofen, ketotifen, 10-oxonortriptiline, haloperidol, loxoprofen, acetohexamide, daunorubicin), but AKR1C2 is inactive towards ethacrynic acid and metyrapone (Ohara et al., 1995). The substrate specificity for drugs also overlaps with those of the other enzymes in the SDR and AKR superfamilies, and AKR1C2 may be the major enzyme in the reductive metabolism of ketotifen because of its extremely high reactivity towards both the *R*- and *S*-forms of the drug (Breyer-Pfaff and Nill, 2000).

There has been no report on the induction of AKR1C2 by xenobiotics.

2.2.1.1.3. AKR1C3

AKR1C3 was first cloned from human liver by Qin et al. (1993) and later from human prostate by Lin et al. (1997). Its mRNA expression was found primarily in the mammary gland and prostate, followed by the liver, lung, small intestines, adrenal, brain, uterus and testis (Khanna et al., 1995; Penning et al., 2000; Dufort et al., 2001).

Although its tissue expression can be detected by means of immunochemical methods (Lin et al., 2004) and amplification of the cDNA,

AKR1C3 has not been purified from human tissues, in contrast to the purification of other AKR1C enzymes (Hara et al., 1990; Deyashiki et al., 1992; Ohara et al., 1995; Breyer-Pfaff and Nill, 2000; Maser and Breyer-Pfaff, 2004). The inability to purify the enzyme from human tissues may result from the high liability of the enzyme in tissue homogenates and cell lysates (Dufort et al., 1999).

AKR1C3 was first named 3α-HSD type 2 (Khanna et al., 1995), but exhibits high 17β-HSD activity towards 4-androstene-3,17-dione, so it is also classified as 17β-HSD type 5 (Peltoketo et al., 1999). In addition, AKR1C3 is identical to PGF synthase that catalyzes both the formation of $PGF_{2\alpha}$ from PGH_2, and the interconversion between PGD_2 and 9α, 11β-PGF_2 (Matsuura et al., 1998; Suzuki-Yamamoto et al., 1999).

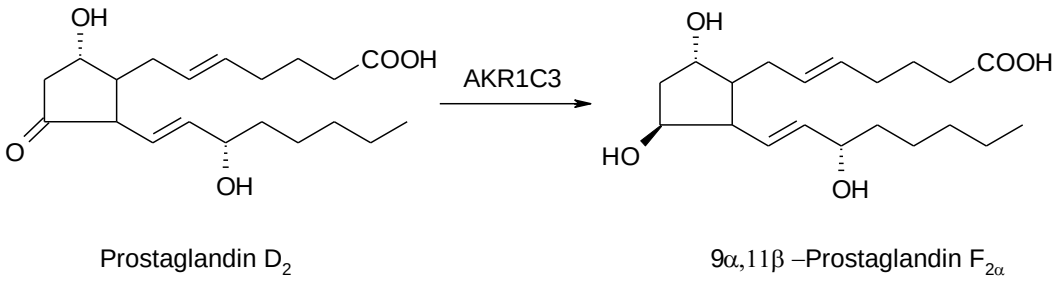


Fig 5. Interconversion of PGD_2 and 9α, 11β-$PGF_{2\alpha}$ by AKR1C3

It is the only isoform expressed in mammary gland, where it converts Δ^4-androstene-3,17-dione to testosterone which could be aromatized to estradiol, and also estrone to 17β-estradiol. Together these reactions may contribute to pro-estrogenic state in breast (Penning et al., 2004).

In prostate (where AKR1C2 and AKR1C3 can be found), it is capable of converting Δ^4-androstene-3,17-dione to testosterone, but it will also reduce 5α-DHT to 3α-diol (Penning et al., 2004).

Thus, the enzyme is involved in adrogene and prostaglandin metabolism, and is suggested to be involved in the development of prostate cancer by forming testosterone and the differentiation of leukemia cells caused by metabolizing PGD_2 (Desmond et al., 2003; Penning, 2004) AKR1C3 is significantly up-regulated during the differentiation of HL-60-cells by all-*trans*-retinoic acid and vitamin D_3 (Mills et al., 1998), and its mRNA expression in human fibroblasts is also induced by thyroid hormone (Moeller et al., 2005).

Only few xenobiotic substrates of AKR1C3 has been described: 9,10-phenantrenequinone (Nishizawa et al., 2000); 4-nitrobenzaldehyde (Suzuki-Yamamoto et al., 1999); naloxone and naltrexone (Matsunaga et al., 2006). We studied its ability to metabolize anticancer drugs doxorubicin and oracin.

The X-ray crystal structure of AKR1C3 reveals a substrate-binding site that consists mainly of hydrophobic aromatic amino acid side chains (Tyr24, Tyr55, Leu54, Trp227, and Phe306). The four conserved amino acids Asp50, Tyr55, Lys 84, and His117 have been proposed to form a catalytic tetrad involved in the oxidation of alcohol or reduction of ketone functional groups via a “push-pull” mechanism (Schlegel et al., 1998). An oxyanion hole, which is located at the bottom of the hydrophobic pocket, is formed by active site tyrosine (Tyr55), histidine (His117), and the coenzyme’s nicotinamide ring.

AKR1C3 (as well as AKR1C1) binds bile acids with micromolar affinity (Matsuura et al., 1997; 1998).

Inhibition of AKR1C3

Although AKR1C3 is a promising therapeutic target, only a few inhibitors were reported so far (Poirier, 2003). Development of inhibitors that consist of non-steroidal core, and thus devoid of residual steroidogenic activity, would be especially attractive. Dietary phytoestrogens (such as coumestrol, quercetrin, biochanin and 2′-hydroxyflavanone) and mycoestrogen zearalenon were reported to inhibit the enzyme in low micromolar concentrations (Krazeisen et

al., 2001; Skarydova et al, 2009), as well as some other small molecule compounds like benzodiazepines (Usami et al., 2002), benzofuranes, and phenolphthalein derivatives (Higaki et al., 2003). Indomethacin, flufenamic acid, and some related nonsteroidal anti-inflammatory drugs (NSAIDs) are also very potent inhibitors, as is the cyclooxygenase-2 selective inhibitor, celecoxib (Matsuura et al., 1998; Lovering et al., 2004; Bauman et al., 2005)

2.2.1.1.4. AKR1C4

AKR1C4 was first cloned by Winters et al. (1990), and because it reduced the insecticide chlordecone, it was first named hepatic chlordecone reductase (Winters et al., 1990, Qin et al., 1993). However, it is better known as DD4 and type 1 3α-HSD, and exhibits higher affinity and velocities for most 3-keto and 3α-hydroxysteroids than the other types of the enzyme do. It also has low 20α-HSD and 17β-HSD activities (Penning et al., 2000).

AKR1C4 is almost exclusively expressed in the liver, and it is involved in the catabolism of circulating steroid hormones (Khanna et al., 1995; Penning et al., 2000; Dufort et al., 2001), and the metabolism of bile acids, where it catalyzes the interconversion of the 3-keto/hydroxyl functional group (Russell DW, 2003). The liver-specific expression of AKR1C4 mRNA is regulated by transcription factors, hepatocyte nuclear factor (HNF)-4 α/γ, HNF-1α and variant HNF-1 (Ozeki et al., 2001; Ozeki et al., 2002).

The enzyme metabolizes various carbonyl compounds (dolasetron, naloxone, naltrexone, oxycodone, oracin, ketoprofen, loxoprofen, acetohexamide, ethacrinyc acid, metyrapone, chlordecone), although it is almost inactive towards befunolol, daunorubicin, haloperidol and ketotifen (Matsunaga et al., 2006).

Several drugs such as sulfobromophthalein (Matsuura et al., 1996), clofibric acid derivatives (Matsuura et al., 1998) and anti-inflammatory 2-arylpropionic acids (Yamamoto et al., 1998) enhance the enzyme activity of

AKR1C4. While there has been no report on the induction of AKR1C4 by xenobiotics so far, an interindividual difference in hepatic chlordecone reductase activity has been reported (Molowa et al., 1986). A large interindividual difference has also been noted in the hepatic expression of AKR1C4 mRNA, which is related to the differences in the amounts of HNF-1α, HNF-4α and HNF-4γ (Ozeki et al., 2003).

AKR1C4 is inhibited by phenolphthalein and steroidal anti-inflammatory agents (Higaki et al., 2003).

2.2.1.2. AKR1C isoformes in hormone dependent cancer

2.2.1.2.1. Breast cancer

Breast cancer is the most common malignant neoplastic disease in the female. The majority of breast carcinomas are invasive ductal or lobular carcinomas. Several endocrine and reproductive factors, such as early age menarche, nulliparity, or delayed first child birth, late age menopause, and obesity, are associated with its etiology (Vihko and Apter, 1989).

Estrogens are involved in breast cancer development. The local excess of 17β-estradiol, the most potent estrogen, is implicated in this disease (Suzuki et al., 2002). They play an important role in disease progression, since it does not develop in the absence of the ovaries (Russo and Russo, 1998).

Estrogens are formed in the ovaries from DHEA via 3β-HSD/KSI activity that leads to the formation of androstenedione, which is aromatized to estrone by subsequent cyp19-aromatase activity. The inactive estrogen, estrone, is activated by reductive 17β-HSD type I activity to the active estrogen 17β-estradiol, while the inactivation of 17β-estradiol is catalyzed by the oxidative 17β-HSD type II.

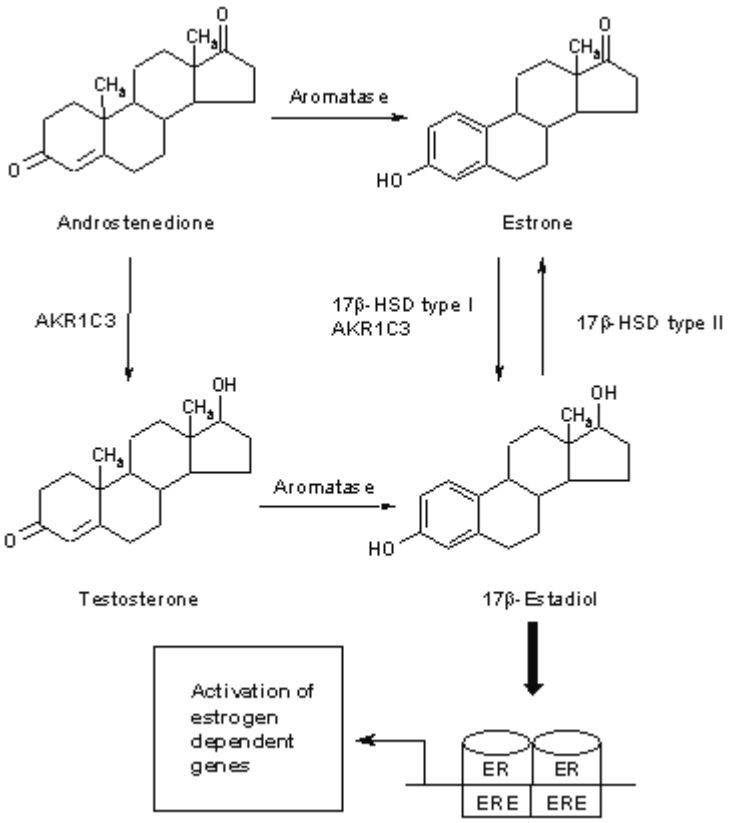


Fig 6. Intacrine regulation of ER ligand concentrations catalyzed by AKR1C3 in human breast The role of CYP19-aromatase is also indicated (Bauman et al., 2004).

The interconversion of estrone to 17β-estradiol can be performed by all AKR1C isoforms (Penning et al., 2000). AKR1C3 and AKR1C4 are most efficient at interconverting estrone to 17β-estradiol, but only AKR1C3 is present in estrogen target tissues like the mammary gland (Dufort et al., 1999; Penning et al., 2000). AKR1C3 not only reduces estrone to 17β-estradiol, but also androstenedione to testosterone. By working in concert with CYP19-aromatase, AKR1C3 may provide a source of estrogens via C19 steroids (Luu-The et al., 2001; Penning et al., 2001).

The local formation of estrogens is important in postmenopausal women, as the formation in the ovaries stops, and in breast tumors, since 17β-estradiol

levels are an order of magnitude higher inside the tumor than in the circulating plasma (Simpson et al., 2001).

Intratumoral estradiol levels are not significantly different between premenopausal and postmenopausal breast cancer patients, but the estradiol/estrone ratio is significantly higher in postmenopausal breast cancers (Miyoshi et al., 2001). The ratio of estradiol concentration in tumor tissue/plasma is 23 in postmenopausal breast carcinomas and only 5 in premenopausal breast cancers (Pasqualini et al., 1996; Pasqualini, 2004). In addition, the concentration of estradiol is 2.3-times higher in breast cancer tissues than in the areas considered as morphologically normal (Chetrite et al., 2000). The great majority of breast cancer occurs after the menopause and expresses ER (Suzuki et al., 2005).

mRNA expression of AKR1C3 was detected in 65-83% of breast cancer tissues (Ji et al., 2004; Vihko et al., 2005). In particular, Vihko et al. (2005) reported that AKR1C3 mRNA expression was significantly higher in breast tumor specimens than in normal tissues. They also demonstrated that a group of patients with AKR1C3 mRNA overexpression had a worse prognosis than other patients (Vihko et al., 2005).

AKR1C3 immunoreactivity was detected in normal mammary gland (Pelletier et al., 1999) and breast carcinoma in 53% of cases (Suzuki et al., 2001). AKR1C3 immunoreactivity was not significantly associated with other clinicopathological factors such as patient age, menopausal status, clinical stage, tumor size, lymph node status, histological grade, ER, PR, AR (Suzuki et al., 2005).

Not only estrogens are important in breast cancer development. The role of progestins has been also investigated. The actions of both, estrogens and progesterone, are required for normal growth and maturation of breast tissues, and progesterone is necessary for terminal duct formation needed for lactation

(Soyal et al., 2002). Estrogen can also regulate expression of the progesterone receptor (PR), linking the action of both these hormones and suggesting a complex interplay between estrogen and regulation of progesterone-dependent genes (Ji et al., 2004).

Ji et al. (2004) reported a substantial decrease (defined as >5 fold) in AKR1C1 expression in 13 out of 24 breast carcinoma cases, while AKR1C2 expression was absent in tumors or reduced in 6 of these 13 cases. These results correlate with Lewis et al. (2004). Their findings suggest 4-5-fold decrease in AKR1C1 and AKR1C2 gene expression. Surprisingly they also found 2.5-fold lower expression of AKR1C3, in contrast to Vihko et al. (2005).

AKR1C1 and AKR1C2 take part in progesterone catabolism, together with 5α-reductase (the expression of both isoforms is elevated in breast tumor).

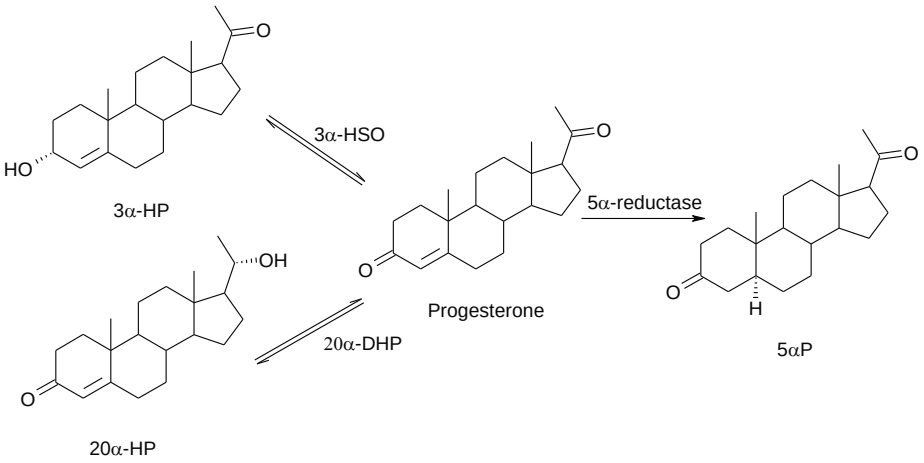


Fig 7. Progesterone metabolizing enzyme pathways and primary metabolites affected by altered gene expression in breast carcinoma tissues (Lewis et al., 2004)

Exposure of human breast cell lines (MCF-7, MCF-10A, and ZR-75-1) to 5α-pregnanes results in changes associated with neoplasia, including increased proliferation and decreased attachment (Wiebe et al., 2000), depolymerization of F-actin and decreases in adhesion plaque-associated vinculin (Wiebe and

Muzia, 2001). Exposure to 4-pregnenes results, in general, in opposite (Anti-cancer-like) effects (Wiebe et al., 2000; Wiebe and Muzia, 2001).

2.2.1.2.2. Prostate cancer

Androgens are involved in the development and progression of androgen-dependent diseases like prostate cancer and benign prostatic hyperplasia (BPH). Prostate cancer is the second-leading cause of cancer death in men, with approximately 184,000 new cases and 32,000 deaths each year. BPH affects approximately 50% of men by 50 years of age and increases with age (Weiss, 1992).

Androgens are essential for the development of the two diseases, as prepubescently castrated male beagles never develop prostate cancer or BPH (Walsh and Wilson, 1976). Consequently, maintenance of androgen homeostasis in the prostate is and important function that involves the human AKR1C isoforms.

The major circulating androgen is testosterone. It is produced in the testicular Leydig cells from dehydroepiandrosterone (DHEA) via 3β-HSD/KSI activity, which leads to the formation of androstenedione. This inactive androgen is reduced by type III 17β-HSD activity to the active androgen testosterone. In target tissues, it can be formed from circulating DHEA and androstenedione by local AKR1C3. Reduction of testosterone by two human 5α-reductase isoforms type I and type II leads to the formation of the most potent androgen, DHT, which is the predominant androgen in the prostate (Bauman et al., 2004).

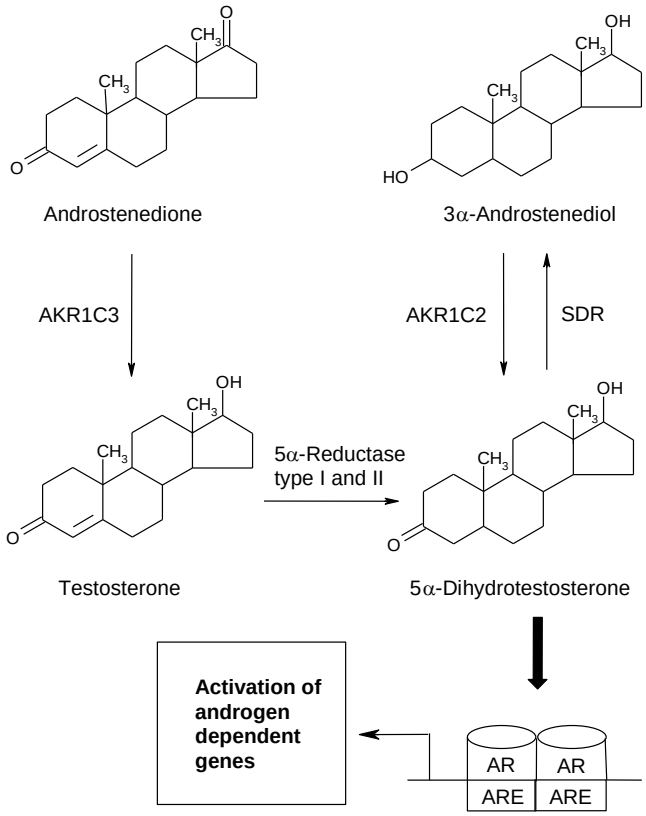


Fig 8. Intacrine regulation of AR ligand by aldo-keto reductase 1C2 and AKR1C3 in human prostate The role of 5α-reductase type II is also indicated (Bauman et al., 2004)

In the prostate, DHT can be also formed by oxidative reaction catalyzed by AKR1C2, which converts 5α-androstane-3α,17β-diol to DHT (Jin and Penning, 2001). A recent study showed, however, that in cultured prostate cells, AKR1C2 act as a reductase eliminating DHT (Lanisnik Rizner et al., 2003). Therefore, AKR1C2 protects the AR from androgen excess, while the oxidative 3α-HSD responsible for the reformation of DHT remains to be identified.

AKR1C3 is the only isoform capable of reducing androstenedione to testosterone (Dufort et al., 1999; Penning et al., 2000). It also catalyzes the reduction of DHT to 3α-diol by its 17β-HSD activity (Labrie et al., 2000). It has been assumed that 3α-diol doesn't have any androgenic effect until oxidized back to DHT first (Wilson et al., 2003). 3α-diol may stimulate prostate cell

proliferation through AR-independent, epidermal growth factor-like pathway(s) (Zimmerman et al., 2004).

Several studies reported elevated expression of AKR1C3 in prostate cancer.

- Harkonen et al (2003) found an increase in the expression of genes encoding AKR1C3.

- Stanbrough et al. (2006) reported increased expression of AR, up-regulated *AKR1C3* gene and also AKR1C3-related genes, AKR1C2 and AKR1C1. They also confirmed that AKR1C3 message was increased in prostate-independent cancer. The average increase of about 4-fold, with approximately one third of the androgen-independent prostate cancer showing particularly high levels, was found. Increased expression of AKR1C2 was similarly shown. But there was no evidence of correlation between increased expression of AKR1C3 and AR.

- Fung et al. (2006) concluded that AKR1C3 is up-regulated in prostate cancer, while increased immunoreactivity in adenocarcinoma cells is in correlation with the observation that elevated AKR1C3 (Lin et al., 1997) show increased transcript expression in primary cultures of prostate epithelial cells derived from cancerous sections of the prostate.

The mRNA expression of AKR1C2 has been also investigated by Ji et al. (2004), who reported its downregulation, and Lanisnik Rizner et al. (2003), who, on the contrary, found its up-regulation. The difference may be due to sample source.

The increased expression of enzymes mediating dihydrotestosterone synthesis (AKR1C3) and catabolism (AKR1C1 and AKR1C2) indicates that intracellular conversion of weak adrenal androgens to testosterone and dihydrotestosterone is enhances in androgen-independent prostate cancer cells

and may contribute to prostate cancer relapse after androgen deprivation therapy (Stanbrough et al., 2006).

Fung et al. (2006) also detected increased AKR1C3 immunoreactivity in non-neoplastic conditions including chronic inflammation, atrophic changes and urothelial (transitional) cell metaplasia.

2.2.1.2.3. Endometrial cancer

Endometriosis and endometrial cancer are common among women. Endometriosis is the leading cause of infertility and affects 5-10% of women of reproductive age (Kitawaki et al., 2003). Endometrial cancer is the most common cause of death from gynecological malignancy in the USA and Europe (Holloway, 2003).

Endometrial cancer is an estrogen-dependent disease localized in the endometrium, it is caused by the unopposed action of estrogens. Estrogen stimulates the growth and proliferation of the endometrium, while progesterone is antiestrogenic, protecting the endometrium from excess 17β-estradiol. The failure of endometrium to inactivate 17β-estradiol due to a progesterone deficiency is suggested, as progesterone induces the expression of 17β-HSD type II, which inactivates 17β-estradiol to estrone (Gurates and Bulun, 2003).

Current treatment includes removal of ovaries and uterus. However, endometrial cancer effects approximately 3-5% women under 40 of age, and this approach is undesirable (Utsunomiya et al., 2003).

Local estrogen synthesis is elevated in endometriosis (Simpson et al., 2001).

Progestins play pivotal role in the luteal stage of the menstruation cycle and pregnancy. Progesterone is synthesized from the precursor pregnenolone by 3β-HSD/KSI activity, and it is inactivated to 20α-hydroxyprogesterone by

AKR1C1. 20α-hydroxyprogesterone can be oxidized back to progesterone by type II 17β-HSD (Bauman et al., 2004).

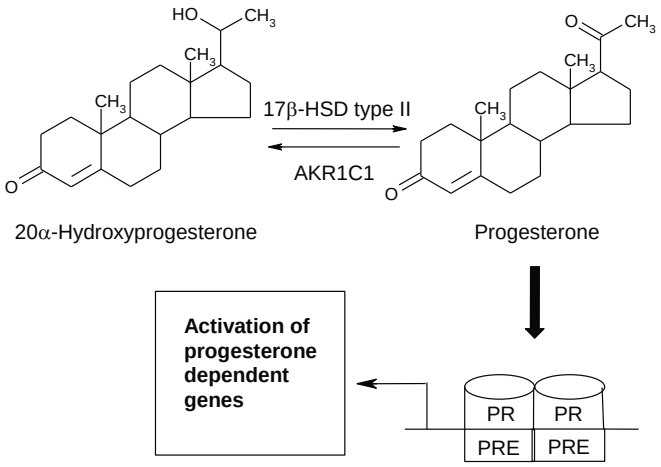


Fig 9. Intacrine regulation of PR ligand concentrations catalyzed by AKR1C1 in human endometrium The role of type II 17β-hydroxysteroid dehydrogenase is also indicated (Bauman et al., 2004)

AKR1C1 is expressed in the endometrium: consequently inhibition of AKR1C1 represents as approach to increasing local progesterone levels by decreasing the inactivation of progesterone to 20α-hydroxyprogesterone, providing a potential therapy for endometriosis and endometrial cancer.

Progesterone plays an important role during pregnancy, where women with a history of first-trimester abortion have decreased local concentrations of progesterone in the endometrium (Piccinni et al., 1998). Inhibition of AKR1C1 would act as a prevention of these unexplained recurrent abortions.

2.2.1.2.4. Lung cancer

Differential display has shown the overexpression of human AKR1C isoforms in non-small cell lung carcinoma. High expression was observed in 384 patients and was prognostic indicator of poor disease outcome (Hsu et al., 2001).

Palackal et al. (2002) reported the ability of AKR1Cs to metabolize polycyclic aromatic hydrocarbons (PAHs). PAHs are ubiquitous environmental pollutants and are tobacco carcinogens implicated in the causation of human lung cancer. PAHs are metabolically activated to exert their deleterious effect. There are three pathways of their activation. One of the pathways involves AKR1C isoforms. They divert PAH *trans*-dihydrodiols to the corresponding o-quinone (Smithgall et al., 1986).

The $NADP^+$-dependent oxidation of the *trans*-dihydrodiol initially results in a ketol that spontaneously rearranges to form a catechol. The catechol is unstable and undergoes autooxidation in air. The first one-electron oxidation results in the formation of an o-semiquinone anion radical and hydrogen peroxide. The second-electron oxidation produces the fully oxidized o-quinone and superoxide anion.

The resulting o-quinones are highly reactive Michael receptors, which can form stable and depurinating DNA adducts (Shou et al., 1993; McCoull et al., 1999).

The o-quinones can also undergo a two-electron nonenzymatic reduction to reform the catechol or a one-electrone enzymatic reaction to reform the o-semiquinone anion radical (Flowers-Geary et al., 1992; 1996).

These events establish futile redox cycles in which the generation of reactive oxygen species (ROS) can be amplified multiple times. Since ROS are the causative agents in radiation-induced carcinogenesis, the pathway of PAH metabolism initiated by AKRs may also explain how PAHs can act as complete carcinogens (Palackal et al., 2002).

The exposure to PAHs induces CYP1A1/CYP1B1 in human lung cells (Lang et al., 1998; Hukkanen et al., 2000), and AKR1C is induced by PAH and ROS (Burczynski et al., 1999). This concerted induction would increase the flux

of *trans*-dihydrodiols to redox-active o-quinones and enhance the production of ROS. This would lead to a further overexpression of AKR1C isoenzymes.

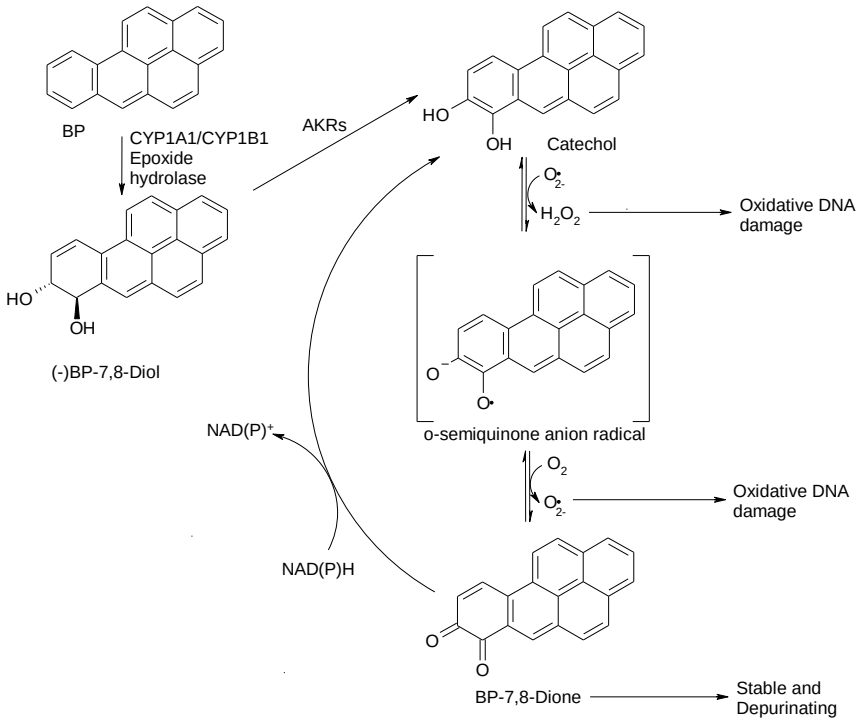


Fig 10. Metabolic pathway of PAH activation which involves AKRs (Palackal et al., 2002)

AKR1Cs will on the one hand provide a defense mechanism against the harmful effects of ROS, since they are efficient catalysts of 4-hydroxy-2-nonenal reduction, a decomposition product of lipid hydroperoxides (Burczynski et al., 2001).

At the same time, induced AKR1C izoenzymes can divert PAH *trans*-dihydrodiols from CYPs to the deleterious quinones.

If the o-quinones and the ROS, they generate, are not eliminated, they have the potential to cause covalent and oxidative DNA lesions, respectively, increasing the mutational load of PAH-exposed lung cells.

2.2.2. Short-chain dehydrogenases/reductases

The Schort-chain dehydrogenase/reductase (SDR) is one of the largest enzyme superfamilies with over 46, 000 members in sequence database. These enzymes are present in all domains of life, from simple organisms to higher eukaryotes, about 25% of all dehydrogenases belong to this superfamily (Oppermann et al., 2003; Kallberg and Persson, 2006).

A growing number of single-nucleotide polymorphisms have been identified in SDR genes, and a variety of inherited metabolic diseases have as their underlying cause genetic defects in SDR genes (Opperman et al, 2001).

The sequence identity of the members of this family is low, but the three-dimensional structures of many members exhibit highly similar α/β folding patterns with a central β-sheet, typical of the Rossmann-fold that participates in a cofactor binding. The catalytic tetrad of Asn-Ser-Tyr-Lys is conserved in the members of this superfamily (Persson et al., 1991; Jornvall et al., 1995; Oppermann et al., 1997), although variations of this general theme also exist (Persson et al., 2009).

The enzymatic reactions carried out by SDR enzymes can be grouped mainly as NAD(P)H-dependent oxidoreductions acting on a highly diverse set of substrates. However, also dehydratase and epimerase reactions are found, thus explaining that none of the residues found in the conserved motifs is completely conserved within the whole family (Jornvall et al., 1995).

2.2.2.1. SDR types

There are two types of SDR enzymes with many members, and at least four types with fewer members. The two major types are denoted “Classical”

and “Extended” and these are clearly distinguished by subunit size and sequence patternes at the coenzyme binding site and at segment N-terminally of the active site region. The current four minor SDR types are: “Intermediate”; “Divergent”; “Complex” and “Atypical” (Persson et al., 2009). Bray et al. (2009) have identified 54 human classical SDR members and 11 extended members within the human genome. In addition, they found 8 proteins that have significant sequence diversity or unusual predicted structural features. These members have been designated as ‘atypical’ SDRs in their analysis until more structural information is known.

The classical SDRs have a relatively small α-helical substrate binding site located between β strand 6 and 7 as shown on Fig 11.

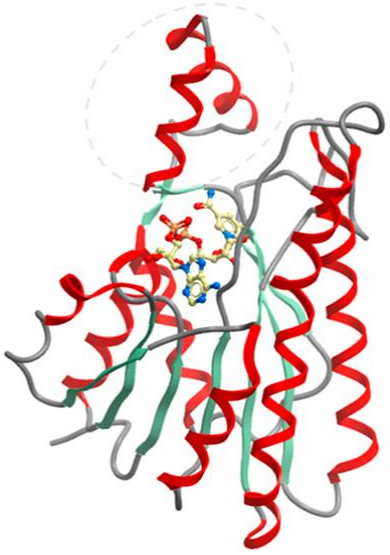


Fig 11. Structure of 3-hydroxybutyrate dehydrogenase, type 2 (BDH2) with a classical SDR fold (Bray et al, 2009)

Whereas the extended SDRs have a significantly larger substrate binding site including a two stranded parallel β-sheet and an α-helix bundle containing 3 helices (as shown on Fig 12).

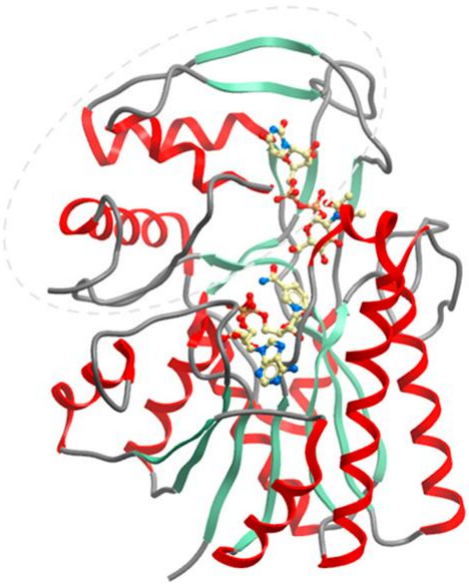


Fig 12. Structure of UDP-galactose-4-epimerase (GALE) with an extended SDR fold (Bray et al., 2009)

Sequence and predicted secondary structure analysis shows that the human classical SDRs can be divided into three main sequence clusters. Cluster C1 contains mostly non-membrane bound proteins (18 members, average length ~ 281 residues). Enzymes within this cluster act on a wide range of compounds including coenzyme A related compounds, prostaglandins, and quinone-like molecules. In contrast, enzymes in clusters C2 (19 members) and C3 (17members) are typically membrane-associated and generally process retinoid and steroid substrates (average length of C2 and C3 members is ~ 340 residues). The majority of these proteins differ from those in C1 due to the presence of a predicted N-terminal transmembrane (TM) helix and a C-terminal helix immediately after core β-strand 7 (Bray et al, 2009).

Based on their functional characteristics, human SDR forms can be grouped into three main categories (Oppermann et al., 2001):

1) Enzymes involved in intermediary metabolism, for example:
    - UDP-galactose epimerase – catalyses epimerization of UDP-glucose to UDP-galactose, and of UDP-N-acetylglucosamine to

UDP-N-acetylgalactosamine. Type III Galactosemia comprises several forms of reported UDP-galactose deficiencies, associated with allelic variants of the human gene, located on chromosome 1p36 (Maceratesi et al., 1998).

- Dihydropteridine dehydrogenase
- Sepiapterin reductase

Both are essential components of in the tetrahydrobiopterine biosynthesis pathway (Dianzani et al., 1998). Allelic variants in the dihydropteridine reductase gene on chromosome 4 code cause tape II phenylketonuria, emphasizing the essentiality of biopterin as cofactor in tyrosine hydroxylation.

- R-3-OH-butyrate dehydrogenase
- Dienoyl CoA reductase

Both are mitochondrial enzymes involved in β-oxidation and ketogenesis.

- *Trans*-enoyl CoA reductase – is a peroxisomal enzyme involved in β-oxidation of fatty acids.

2) Enzymes involved in hormone, mediator and xenobiotic metabolism

   This group represents the largest fraction of SDRs and is characterized by its involvement in lipid mediator and to some extent in xenobiotic carbonyl metabolism (Oppermann and Maser, 2000). Enzymatic activities of this group comprise various hydroxysteroid dehydrogenases (HSDs), prostaglandin dehydrogenases and retinoid metabolizing enzymes. Several of these enzymes display overlapping substrate specificities.

3) Enzymes identified as open reading frames (ORFs)

   - Orthologues of lower eukaryotic SDR enzymes of unknown function (Lai et al., 2000).
   - Members associated with malignant diseases (e.g. Hep27 with hepatocarcinogenesis) (Gabrielli et al., 1995).

2.2.2.2. Co-factor preference

SDR proteins use NAD(H) and NADP(H) co-factors to perform catalysis. These molecules bind to the Rossmann fold part of the SDR molecule. NAD(H) preferring enzymes usually contain an acidic residue at the C-terminus of the second core β-strand that interacts with 2' and 3'-hydroxyl groups of the adenine ribose part of the co-factor. Whereas NADP(H) preferring enzymes contain a basic residue one position further along the sequence (and no acidic residue) that compliments the additional phosphate group of NADP(H). NADP(H) preferring enzymes can also contain a basic residue in the glycine rich motif, just before the second glycine (Kallberg et al, 2002).

2.2.2.3. Numbering of individual family members

In the nomenclature scheme, the family number is followed by one letter designating the SDR type, thus making it clear from a quick glance at the family designation which type the SDR family belongs to, e.g., SDR1E represents an SDR of the extended type.

The nomenclature scheme is extended by adding a number after the type letter, so that each member of every SDR family is given an individual sequence of letters and numbers, *e.g.* SDR1E1. This is essential for tracking individual SDR members, since considerable confusion exists in the literature with multiple designations, aliases, names and abbreviations. Such individual numbering of enzyme members have since long been successfully implemented for other enzyme families.

Table 4. SDR Type and their designations (Persson et al., 2009)

Type	Designation
Classical	C
Extended	E
Atypical	A
Intermediate	I
Divergent	D
Complex	X
Unknown	U

The new SDR nomenclature is gene based. Thus, all splice variants derived from the same gene hold the same main number, but each splice variant is distinguished by a sub-number, separated from the main number by a dash, *e.g.* SDR15C1-1, SDR15C1-2. Similarly, polymorphic variants and SNPs are assigned using an asterisk, resulting in a nomenclature such as SDR11E1*1, SDR11E1*2, SDR11E1*3 (Persson et al, 2009).

Some SDR families consist of one enzyme only, some have more members.

Table 5. Examples of human SDR sub-familes and their nomenclature (Persson et al., 2009)

Sub-family	Sub-family name	Protein	Description
SDR1E	UDP-glucose 4-epi-merase	SDR1E1	UDP-glucose 4-epi-merase
SDR9C	Steroid and retinol dehydrogenase	SDR9C1	D-beta-hydroxybutyrate dehydrogenase
		SDR9C2	17-beta-hydroxysteroid dehydrogenase type
		SDR9C3	Corticosteroid 11beta-hydroxysteroid dehydrogenase
		SDR9C4	3-alpha-hydroxysteroid dehydrogenase
		SDR9C5	11-cis retinol dehy-drogenase
		SDR9C6	Oxidoreductase
		SDR9C7	Retinol dehydrogenase similar protein
		SDR9C8	Retinol dehydrogenase
SDR21C	Carbonyl reductase	SDR21C1	Carbonyl reductase 1
		SDR21C2	Carbonyl reductase 3
SDR22E	NADH dehydrogenase	SDR22E1	NADH dehydrogenase (Ubiquinone)

In the next two chapters, I will breafly introduce two types of enzymes belonging to SDR superfamily, which have been extensively studied. Carbonyl reductases represent soluble cytosolic enzymes, whilst 11β-HSDs are microsomal membrane-bound enzymes.

2.2.2.4. Carbonyl reductases

Carbonyl reductases are NADPH-dependent monomeric, cytosolic enzymes with broad substrate specificity for many endogenous and xenobiotic carbonyl compounds. The metabolism of substrates usually results in detoxification and inactivation of the reactive carbonyl groups. The ability to reduce aromatic ketones and quinones is characteristic for CBRs.

CBR can be distinguished from aldehyde reductases and alcohol dehydrogenases by using aromatic ketones for substrates and flavonoid inhibitors, such as rutin, quercetin or quercitrin (Ahmed et al., 1979; Sawada and Hara, 1979). Other inhibitors that have been used are indomethacin, furosemide and disulfiram (Iwata et al., 1989).

- *Carbonyl reductase 1*

This human enzyme was first cloned by Wermuth et al. (1988), who also characterized its properties using the enzyme purified from brain, and proposed its identity with xenobiotic ketone reductase and prostaglandin 9-ketoreductase (Wermuth et al., 1981). The enzyme is ubiquitously distributed in human tissues (Wirth and Wermuth, 1992).

Human CBR1 catalyzes the NADPH-dependent reduction of various carbonyl compounds, the best substrates being *p*- and *o*- quinones derived from polycyclic aromatic hydrocarbons (Wermuth et al., 1986). In human liver and placenta, CBR1 acts as a major quinone reductase, but the enzymatic reduction of several *o*-quinones results in redox cycling of the quinones, leading to the generation of semiquinones and the superoxide anion (Jarabak and Harvey, 1993). Thus, CBR1 seems to be involved in both the detoxification and intoxication of quinones.

The endogenous substrates of CBR1 are suggested to be prostaglandins, some 3-ketosteroids and isatin, of which isatin is the best one,

showing a high affinity and turnover number that are compatible with those of xenobiotic *o*-quinones (Usami et al, 2001). In addition, CBR1 catalyzes the reduction of the 4-keto aldehyde and a double bond of 4-oxonon-2-enyl, a product of lipid peroxidation (Doorn et al., 2004). CBR1 homozygous null mice are nonviable (Olson et al., 2003), suggesting that the enzyme plays a non-redundant role in cell signaling during embryogenesis and development. A study on the biological function of CBR1 in A549 adenocarcinoma cells suggested that the enzyme is involved in serum-free-induced apoptosis in the cells (Tanaka et al., 2005). On the other hand, several studies involving tumor tissue specimens have indicated that a decrease in CBR1 expression correlates with the degree of dedifferentiation in hepatocellular carcinomas (Suto et al., 1999), poor survival and lymph node metastasis in epithelial ovarian cancer (Umemoto et al., 2001), and tumor progression and angiogenesis in lung cancer (Takaneka et al., 2005). Therefore, elucidation of the mechanisms underlying the suggested roles of the enzyme in cells signaling, apoptosis and cancer progression in an important future goal.

CBR1 reduces several therapeutic agents and toxicologically important compounds. The drug substrates include daunorubicin and doxorubicin (Forrest and Gonzales, 2000); loxoprofen; metyrapone (Ohara et al., 1995); haloperidol (Breyer-Pfaff and Nill, 2000); etc. The enzyme also metabolizes 4-methyl-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), a tobacco specific potent carcinogen, into (S)-4-methylnitrosamino-1-(3-pyridyl)-1-butanol, which is more tumorigenic. The significance of NNK metabolism by CBR1 and other reductases was described in a review by Maser and Breyer-Pfaff (2004).

- *Carbonyl reductase 2*

This enzyme is not present in human tissues. CBR2 is highly expressed in the mitochondria of epithelial cells of mouse, guinea-pig and pig lungs (Nakayama et al, 1986; Matsuura et al, 1990; Oritani et al, 1992).

- *Carbonyl reductase 3*

The protein is composed of 277 amino acids, and its sequence identity with human CBR1 is 71%. The gene was first identified 62 kilobases apart from the CBR1 gene on the chromosome 21 (Watanabe et al, 1998). The mRNA for CBR3 is suggested to be expressed in various tissues (Terada et al., 2003), although the level is much lower than that of CBR1.

- *Carbonyl reductase 4*

The protein is composed of 237 amino acids, and exhibits low sequence identity (<25%) with other types of CBRs. The gene is present in laboratory animals and dog. Although it is named carbonyl reductase 4, its enzymatic properties and tissue distribution remain unknown (Matsunaga et al., 2006).

2.2.2.5. 11β-Hydroxysteroid dehydrogenase

11β-hydroxysteroid dehydrogenase (11β-HSD) catalyses the inter-conversion of receptor-active 11-hydroxy glucocorticoids (cortisol) and their receptor-inactive 11-oxo metabolites (cortisone). Regulating glucocorticoid access to the glucocorticoid and mineralocorticoid receptor, 11β-HSD plays an important role in maintaining the glucocorticoid homeostasis (Sandeep and Walker, 2001).

Two different isoforms have been identified, 11β-HSD 1 and 11β-HSD 2, which differ in their biological properties and tissue distribution (Albiston et al., 1994; Seckl, 1997).

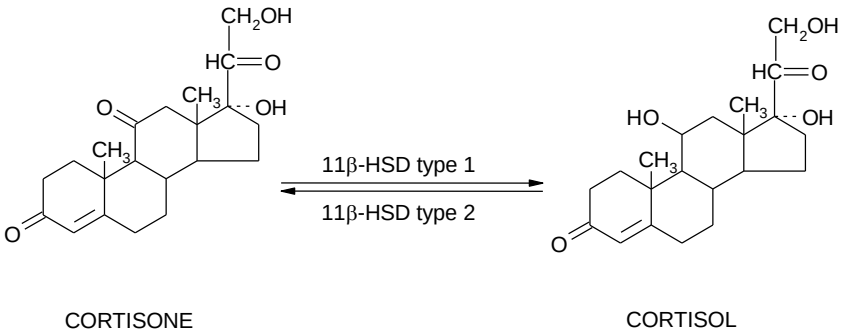


Fig 13. The interconversion of inactive 11-oxo glucocorticoid (cortisone) to its receptor-active 11β-hydroxy form (cortisol) by two isoforms of 11β-hydroxysteroid dehydrogenase

- *11β-HSD type 1*

11β-HSD type 1 is a glycosylated membrane-bound protein which exists as a dimer with a molecular mass 67.4 kDa (Maser et al., 2003).

It is a low affinity NADP(H) dependent dehydrogenase /oxoreductase, with an apparent K_m in the low micromolar range. The enzyme has an almost ubiquitous tissue distribution with the highest expression in liver. 11β-HSD type 1 can *in vitro* catalyze the reactions in both directions while *in vivo* functions primarily as a reductase, reactivating inert 11-oxo glucocorticoids to the corresponding receptor-active 11β-hydroxy forms. This activity is of a particular importance in tissues like liver, brain and adipose tissue, where it maintains high intracellular level of glucocorticoids (Low et al., 1994; Jamieson et al., 1995; Voice et al., 1996; Napolitano et al., 1998).

However, the physiological role of 11β-HSD 1 as a pre-receptor control device in regulating access of glucocorticoid hormones to the glucocorticoid receptor remains obscure in light of its low substrate affinity, which is in contrast to low glucocorticoid plasma levels and low K_d values of the receptor to cortisol. On the other hand, 11β-HSD 1 has been reported to play a role in the detoxification of non-steroidal carbonyl compounds, such as e.g. the tobacco-

specific carcinogen 4 methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), oracin, ketoprofen, metyropone, menadione, and some aromatic aldehydes and ketones (Maser and Oppermann, 1997; Hult et al., 2001; Wsól et al., 2004).

Increases in the activity and expression of 11β-HSD 1 have been implicated in the pathogeneses of many common conditions including obesity, insulin resistance, the metabolic syndrome, the polycystic ovarian syndrome, osteoporosis and glaucoma (Tomlinson, 2005). Therefore, its selective inhibition has been proposed a novel therapeutic strategy for many of these conditions.

Glycyrrhetinic acid, a constituent of licorice, is known as a classical inhibitor of 11β-HSD 1 (Steward et al., 1987).

- *11β-HSD type 2*

The type 2 11β-HSD is a high affinity, unidirectional, NAD^+-dependent dehydrogenase with an apparent K_m in nanomolar range for corticosterone and cortisol, and catalyzes their conversion to 11-dehydrocorticosterone and cortisone (Albiston et al., 1994; Agarwal et al., 1994).

The protein was for the first time purified from human placenta by Brown et al. (1996). It can be found principally in mineralocorticoid target tissues such as the kidney and colon, where it (by inactivating glucocorticoids) protects the mineralocorticoid receptor from cortisol excess (Edwards et al., 1988; Funder et al., 1988). 11β-HSD 2 can was also found in the placenta, where it protects the developing fetus from the effect of maternal glucocorticoids (Brown et al., 1993).

A defect in the 11β-HSD 2 gene results in a form of hypertension called apparent mineralocorticoid excess (Wilson et al., 1998).

11β-HSD type 2 can also be inhibited by licorice, this activity decrease can result in glucocorticoid dependent mineralocorticoid excess and hypertension (Sandeep et al., 2001).

Table 6. Typical xenobiotic substrates of human reductases (Matsunaga et al., 2006; Pilka et al., 2009)

Enzyme	Drugs	Others
CBR1	Daunorubicin, doxorubicin, haloperidol, bromperidol, metyrapone, loxoprofen, wortmannin, dolasetron	Quinones, aromatic aldehydes, aromatic ketones, NNK
CBR3	Oracin	Isatin, coniferyl aldehyde, acetohexamid, orthoquinones
11βHSD 1	Ketoprofen, metyrapone, insecticidal metyrapone analogues, oracin	NNK, menadione, aromatic aldehydes and ketones, 7-keto-cholesterol

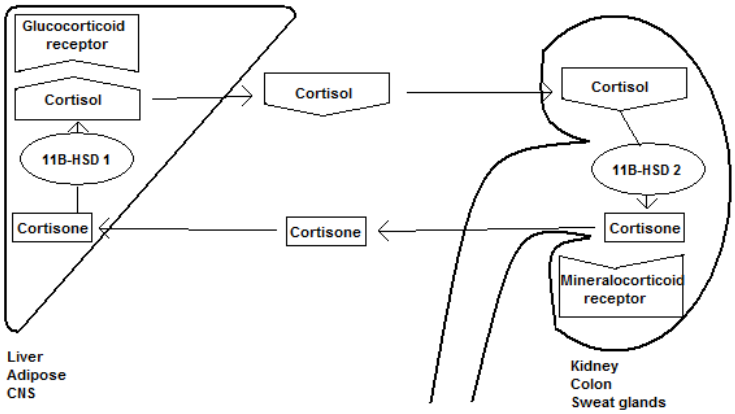


Fig 14. Contrasting functions of the isoenzymes of 11β-HSD. 11β-HSD 2 is an exclusive 11β-dehydrogenase which acts in classical aldosterone target tissues to exclude cortisol from otherwise nonselective mineralocorticoid receptors. Inactivation of cortisol also occurs in placenta. 11β-HSD 1 is a predominant 11β-reductase *in vivo* that acts in many tissues to increase local intracellular glucocorticoid concentrations and thereby maintain adequate exposure of relatively low affinity glucocorticoid receptors and their ligand (Seckl and Walker, 2001)

2.2.3. Medium-chain dehydrohenases/reductases

Medium-chain dehydrogenases/reductases (MDRs) constitute a large enzyme superfamily with (including species variants) close to 1000 members (Persson et al., 1994; Jornvall et al., 1999). The MDR enzymes represent many different enzyme activities, all of them utilize NAD(H) or NADP(H) as cofactor and several but not all of the members have one zinc ion with the catalytic function at the active site.

The MDR superfamily can be divided into 8 families. Half of the families are zinc-containing MDRs.

1) Dimeric alcohol dehydrogenases (ADHs) – present in animals and plants. ADHs are the most closely investigated enzymes in MDR superfamily. They participate in the oxidation of alcohols, detoxification of aldehydes/alcohols and the metabolism of bile acids (Jornvall et al., 2000; Marschall et al., 2000). ADHs also have a second zinc ion at a structural site, stabilizing an external loop present in those forms (Branden et al., 1975).
2) Yeast alcohol dehydrogenases (YADHs) – present in fungi.
3) Polyol dehydrogenases (PDHs) – their substrates are widespread in nature because of their derivation from glucose, fructose, and general metabolism. In some organisms these substrates can be accumulated at high concentrations constituting a protection against environmental stress, such as osmotic shock (Yancey et al., 1982), and reduced or elevated temperature (Czajka and Lee, 1990; Wolfe et al., 1999). Polyol accumulation can be harmful (Chakrabarti et al., 2000), suggesting a further protective role of these enzymes.
4) Cinnamyl alcohol dehydrogenases (CADs) – catalyse the last step of the biosynthesis of the monomeric precursor of lignin, the main constituent of plant cell walls (Boudet et al., 1995) Down-regulation or inhibition of CAD will reduce wood lignin content and yield in a pulp of high quality (Campbell and Sederoff, 1995).

The non-zinc-containing MDRs are:

5) Quinone oxidoreductases – one mammalian form functions as a lens protein (ζ-crystallin) (Gonzales et al., 1995), mutational loss of which may result in cataract formation at birth. This suggests that ζ-crystallin has a role in the protection of the lens against oxidative damage (Rao et al., 1997).
6) Mitochondrial respiratory function proteins (MFRs) – in yeast it has been shown to be essential for mitochondrial respiratory function (Yamazoe et al., 1994), the human orthologue might be of the same importance for the mitochondrial function.
7) Leukotriene B_4 dehydrogenases (LDTs) – in human, there are two isoforms (Nordling et al., 2002).
8) Acyl-CoA reductases (ACRs) – can be only found in humans, *D. melanogaster* and *C. elegans* (Nordling et al., 2002).

MDR is a very ancient protein superfamily that existed in the last universal common ancestor. It had at least two (probably three) different ancestral activities related to formaldehyde metabolism and alcoholic fermentation (Riveros-Rosas et al., 2003).

2.3. Anticancer drugs tested

2.3.1. Doxorubicin

Anthracyclines are well established as highly efficacious antineoplastic agents for various hematopoieic and solid tumors. A dose–response relationship has been demonstrated for anthracyclines and some tumors, with lower doses resulting in decreased survival and remission rates (Shan et al, 1996). On the other hand, dose escalation results in a dose-dependent cardiotoxicity (Von Hoff et al., 1979).

Two distinct types of anthracycline-induced cardiotoxicity ACT) have been defined:

- Acute ACT occurs during treatment, often immediately after the first dose, and manifests itself predominantly in the form of arrhythmias, hypotension, and rarely also as pericarditis-myocarditis or acute left ventricular (LV) failure.

- Chronic ACT presents within 1 year after anthracycline administration as congestive heart failure.

In addition, there are studies which postulate a distinct late-onset cardiotoxicity, which develops after years or even decades of asymptomatic survival (Shan et al., 1996).

Wojnowski et al. (2005) suggested that the genetic makeup of patients is also very important in their response to doxorubicin administration. For example, overexpression of the multiple drug resistance gene MDR1 protects the heart from the toxic effect of doxorubicin (Dell´Acqua et al., 1999).

The incidence of ACT depends on the cumulative dose of the drug. When doxorubicin is injected to patients in bolus, this value is 0.14% at doses < 400 mg/m^2 and rises from 7% at 550 mg/m^2 to 18% at 700 mg/m^2. The steep increase in cardiotoxicity at doses > 550 mg/m^2 has led to the commonly applied upper dosing limit of 550 mg/m^2. Switching from bolus to prolonged intravenous infusion (Legha et al., 1982a) has reduced the incidence of doxorubicin-induced cardiotoxicity (Zucchi and Danesi, 2003).

One-electron redox cycling of a quinone moiety in the tetracyclic ring of anthracyclines has been proposed to mediate cardiotoxicity induced by doxorubicin. In fact, several flavin-centered oxidoreductases (e.g. cytochrome P450 reductase, nitric oxide reductase, NADH dehydrogenase) catalyze one-electron reduction of this quinone to semiquinone which readily regenerates its parent compound by reducing molecular oxygen to superoxide anion ($O_2^{\bullet-}$) and its dismutation product hydrogen peroxide (H_2O_2). At the same time, the semiquinone reductively releases iron from ferritin by direct or $O_2^{\bullet-}$ mediated

electron transfer. All such processes increase the cellular level of H_2O_2 and Fe(II), and set the stage for formation of $^{\bullet}OH$, ferryl ions and other iron-oxygen complexes able to induce oxidative damage (Doroshow, 1983; Weiss, 1992; Singal and Iliskovic, 1998).

This mechanism possibly explains several aspects of acute ACT, but studies in laboratory animals have shown that chronic cardiomyopathy is not always accompanied by biochemical indices of oxidative damage (Olson and Mushlin, 1990). Moreover, antioxidants have not proven useful in delaying or preventing chronic cardiotoxicity in patients (Legha et al., 1982b; Myers et al., 1983).

Some investigators proposed that chronic cardiomyopathy develops after conversion of doxorubicin to the corresponding secondary alcohol metabolite, doxorubicinol (Olson et al., 1988; Olson and Mushlin, 1990; Forrest et al., 2000).

Doxorubicinol is formed after two-electron reduction of a side chain C-13 carbonyl group, mediated by poorly characterized cytoplasmic oxidoreductases sharing similarities with AKR and/or SDR multigene superfamilies (Jez and Penning, 2001; Oppermann et al., 2001).

The involvement of secondary alcohol metabolite is suggested by the following pharmacokinetic evidence:

- The cardiac function of anthracycline-treated rodents usually declines when alcohol metabolites reach their maximum levels in the heart (Olson and Mushlin, 1990).

- Transgenic mice bearing cardiac-restricted overexpression of human carbonyl reductase exhibit an accelerated course of development of cardiomyopathy (Forrest et al., 2000).

- Investigational anthracyclines with inherent resistance to carbonyl reduction induce less severe chronic cardiomyopathy in the rat (Cirillo et al., 2000).

Doxorubicinol, the primary circulating metabolite of doxorubicin, profoundly compromises both systolic and diastolic function of isolated rabbit papillary muscles. Relatively low (comparing to doxorubicin) muscle bath and cardiac tissue concentrations of doxorubicinol nearly abolish calcium loading and inhibit by > 90% of the ATPase activities of sarcoplasmic reticulum, mitochondria, and sarcolemma.

Conversely, doxorubicin, at concentrations as high as 700 μM, has little effect on systolic or diastolic function and doesn't substantially inhibit calcium loading by sarcoplasmic reticulum vesicles or ATPase activities of the three ion pumps.

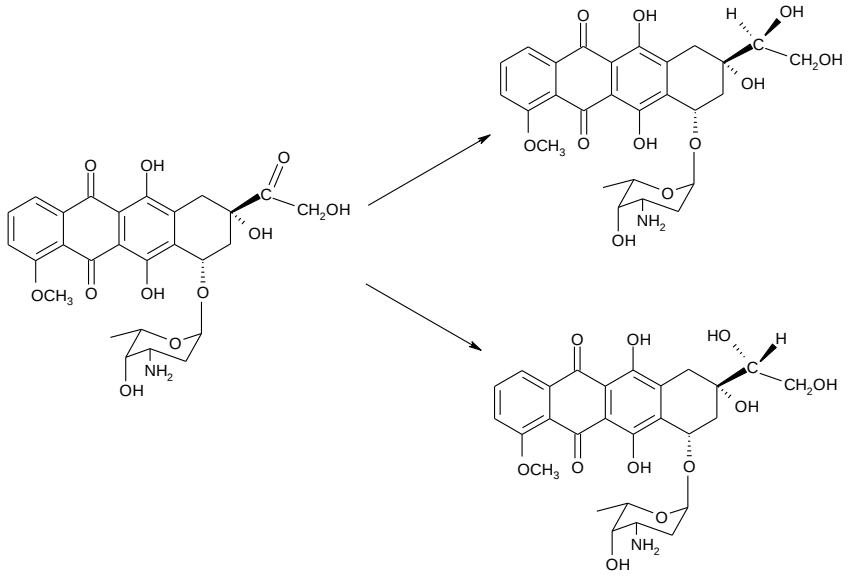


Fig 15. Metabolic conversion of pro-chiral drug doxorubicin to its main metabolite doxorubicinol

The heart can also metabolize doxorubicin to doxorubicinol, which could result in intramyocardial accumulation of this highly toxic metabolite.

Thus, doxorubicinol might be important in the chronic cumulative cardiac toxicity associated with doxorubicin chemotherapy.

Inhibition of the aldo-keto reductases that catalyze the C-13 carbonyl reduction of doxorubicin to doxorubicinol would result in more doxorubicin to kill cancer cells but less doxorubicinol to compromise cardiac function (Olson et al., 1988).

2.3.2. Oracin

Oracin is a promising potential cytostatic drug for peroral use. From its chemical structure, a DNA intercalation mode of action can be inferred, similar to that of anti-tumor antibiotics from the anthracycline group. However, the wide spectrum of tumors, which are sensitive to oracin does not only result from this single mechanism of action, which manifests itself by an inhibition of DNA and RNA synthesis, followed by a decrease in protein content in tumor cells (Melka, 1993).

Several other additional mechanisms affecting tumor cell gowth have been demonstrated. They involve the inhibition of topoisomeraze II, which was isolated from nuclei of Ehrlich ascites carcinoma cells (Miko et al., 2002), the stimulation of aerobic consumption of glucose and, to a lower extent, of formation of lactate in tumor cells, as well as induction of apoptosis (Melka, 1993).

Studies with Burkitt lymphoma cell lines suggest that oracin acts as an inhibitor of mitosis, probably by interfering with the G2 phase of the cell cycle (Klucar and Al-Rubeai, 1997).

The main advantages of this novel chemotherapeutics are:

- the possibility of peroral administration
- the above-mentioned combination of different anti-tumor mechanisms

- the absence of cardiotoxicity (Gersl et al., 1996), which is the main dose-restricting factor in clinical use of doxorubicin

- the negative Ames test on mutagenicity (Marhan, 1995)

- very low hepatotoxicity

- favourable pharmacokinetics

The principal metabolite of oracin is 11-dihydrooracin (DHO). This chiral metabolite is formed by reduction of the pro-chiral carbonyl group at the 11-position of the oracin molecule.

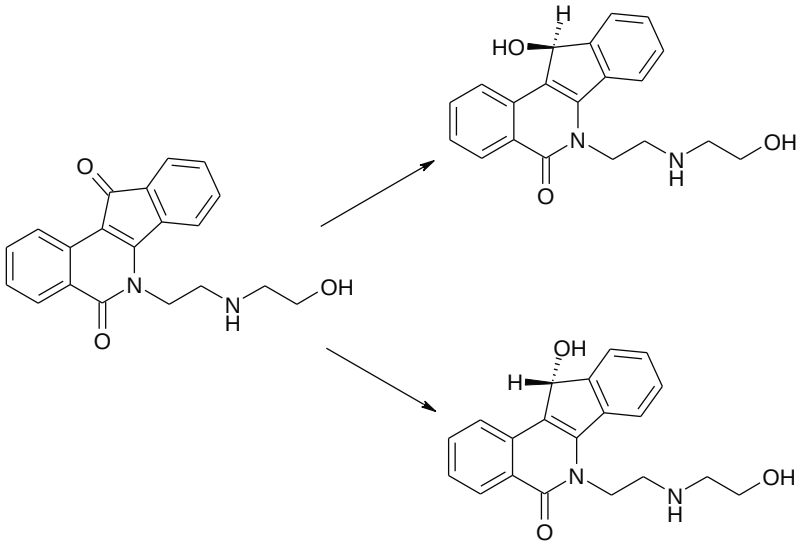


Fig 16. Metabolite conversion of pro-chiral drug oracin to its main metabolite 11-dihydrooracin

It was found that DHO is formed both in microsomal and cytosolic fraction of all species studied. The stereospecificity of reduction greatly differs in individual animal species, in that (+)-DHO predominates in mouse, rat, dog, and mini-pig, while (-)-DHO predominates in guinea pig and rabbit (Wsól et al., 1998). In addition, stereospecificity is also affected by gender of the laboratory

animals (Wsól et al., 1999). In man, almost the same amounts of both enantiomers are formed (Wsól et al., 2000).

In human liver, 11β-HSD type I (Wsól et al., 2004) and AKR1C1, AKR1C2 and AKR1C4 (Wsól et al., 2005b) have been found to catalyze the reuction of oracin to 11-dihydrooracin.

2.4. Protein purification

The purification is in itself seldom the final goal, but rather a necessary phase on the way to the final research goal to gain information about the biochemical mechanisms and structures of the biomolecules involved.

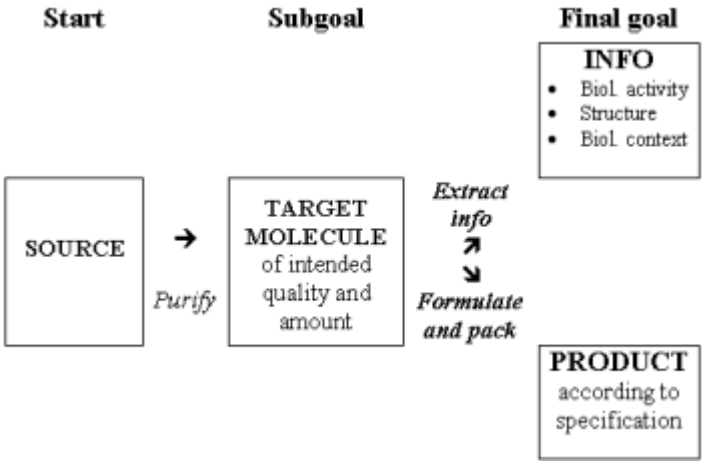


Fig 17. The purpose of purification (www6.gelifesciences.com)

The purification simply aims at obtaining the target molecule pure enough in the shortest possible time. A structured way of developing a purification protocol is therefore the quickest and most reliable way to acceptable results.

The purification of crude biological samples can be divided into three distinct phases (Fig 18.).

The capture (C) step aims at concentrating the sample and removing the bulk of the contaminants. Emphasis is primarily on speed and load capacity. Media with high load capacities and good flow properties are used often under step elution conditions.

The intermediate purification (iP) step(s) aims at separating the components of the now concentrated partially purified sample. Emphasis here is on maximum resolution since the remaining contaminants have rather closely related chromatographic properties. This is done by combining techniques of independent selectivities run in high resolution mode (gradient elution, smaller bead sizes etc.). Flow rates and load capacities have to be restricted or resolution will suffer.

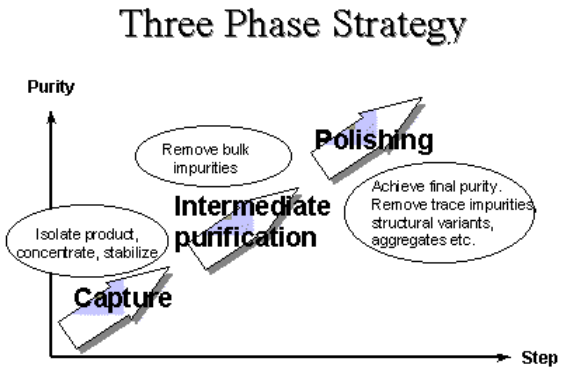


Fig 18. The three-stage strategy applied when purifying crude samples (www6.gelifesciences.com)

The polishing (P) step(s) serves to achieve the final purity and to eliminate contaminants such as polymers etc. and is also often used to condition the final product, e.g. to remove salts, when the final product is to be lyophilised.

No chromatographic technique will provide 100% yield of active material and the overall yield will therefore depend on the number of steps included in the purification protocol.

Minimizing the number of steps (including inter-step conditioning) is therefore important to arrive at a high overall yield. To minimize the number of steps, method development should aim at optimizing each step for the intended purpose and arranging them in an order that minimizes inter-step treatments.

Purification by removing the target molecule from the contaminants

Affinity chromatography techniques are very specific for the target molecule or for a group of molecules with closely related biological properties. This makes them capable of “fishing out” the target molecule (or the group), leaving all contaminants behind. When applicable these techniques are to be preferred, since they drastically simplify the purification protocol.

Purification by removing the contaminants from the target molecule

When a suitable affinity chromatography technique is not at hand, one has to rely on a sequence of general chromatography techniques to remove the contaminants. A typical purification protocol when nothing is known about the target protein employs the IEX- HIC- GF sequence of purification steps (www6.gelifesciences.com).

2.4.1. Extraction of membrane proteins

Membrane proteins are categorized as either peripheral or integral.

Because peripheral membrane proteins are extracted by relatively mild treatments, care must be taken not to partially release these membrane proteins accidentally during subcellular fractionation procedures prior to purification. Isolation of a soluble form of an extrinsic membrane protein is usually achieved by a single technique or combinations of mild extraction procedures depending on the particular properties of the protein under investigation and the starting material (Ohlendieck, 2003).

Integral membrane proteins represent a much greater problem. Since they are embedded within and often right across the membrane, it is normally necessary to solubilize the membrane itself in order to release the protein. And once in solution, it will be necessary to retain a lipophilic component (usually a detergent) together with the solubilized protein to prevent it from aggregating (Scopes, 1994).

Detergents are amphipatic molecules and the preferred form of aggregation in water is the formation of micelles. Detergents are characterized by a unique critical micelle concentration (cmc), and below the specific cmc value, individual detergent molecules predominate in solution. Cmc values differ quite significantly between individual classes of detergents (Ohlendieck, 2003).

2.4.2. Protein purification techniques

2.4.2.1. Ion-exchange chromatography

One of the most precise methods for the fractionation of biological substances, ion exchange chromatography (IEX) separates biomolecules based on differences in their anionic or cationic charge characteristics. Ion exchange chromatography is generally used as a batch step in initial purification or a selective step in the latter stages of a purification scheme.

Adsorption

Proteins carry charged amino acids on their surfaces and can thus be adsorbed to ion exchangers. Proteins with net negative charges (excess of negative charges) adsorb to anion exchangers, while those with net positive charges (excess of positive charges) adsorb to cation exchangers. The strength of the adsorption increases with increased net charge.

Charged amino acids contain either weak acid or amino groups, whose degree of dissociation depends on pH. Consequently the net charge will vary with pH in a way that is fairly specific for each individual protein.

Desorption

Essentially two possibilities exist to desorb sample molecules from the ion exchanger:

- Reducing the net charge by changing pH - a powerful way of influencing the net charges of the sample molecules; it is therefore normally used to control the selectivity (elution order and distance between eluted peaks).

- Adding a competing ion to "block" the charges on the ion exchanger – it will not influence the selectivity, but provide a means of desorbing the sample molecules in order of increasing net charges.

The elution is performed stepwise or with a continuous gradient where gradient mode is used more often.

Most IEX experiments use a neutral monovalent salt such as NaCl as the desorbing agent, mainly because NaCl has little or no effect on the running pH.

The higher the net charge, the stronger the adsorption and the higher the salt concentration needed to desorb the sample molecule.

Ion exchangers

Ion exchange (IEX) ligands can either be negatively or positively charged. Negatively charged ion exchangers adsorb cations (cation exchangers -CIEX) while positively charged ones adsorb anions (anion exchangers -AIEX).

Depending on the pKa value of the charged ligand, the ion exchangers are further divided into strong and weak (acids or bases).

Strong ion exchangers are fully charged over the total pH range normally applicable to proteins and peptides. With weak ion exchangers, on the other hand, the charge displayed is a function of the eluent pH.

Table 7. Ion-exchangers used in protein purification (www6.gelifesciences.com)

Anion exchangers	Strength	Structure
Diethylaminoethyl (DEAE)	weak	- O - CH_2 - CH_2 - $N^+H(CH_2 - CH_3)_2$
Quaternary aminoethyl (QAE)	weak	- O - CH_2 - CH_2 - $N^+(C_2H_5)_2$ - CH_2 - CHOH - CH_3
Quaternary ammonium (Q)	strong	- O - CH_2 - CHOH - CH_2 - O-CH_2 – CHOH- CH_2 – $N^+(CH_3)$
Cation exchangers	**Strength**	**Structure**
Carboxymethyl (CM)	weak	- O - CH_2 – COO^-
Sulfopropyl (SP)	strong	- O - CH_2 – CHOH - CH_2 – O - CH_2 - CH_2 - CH_2 SO_3^-
Methyl sulfonate (S)	strong	- O - CH_2 – CHOH - CH_2 - CH_2 – CHOH - CH_2.SO_3^-

For protein and peptide purification, the weak ion exchangers are nowadays less frequently used since they provide no essential advantage over the strong ones.

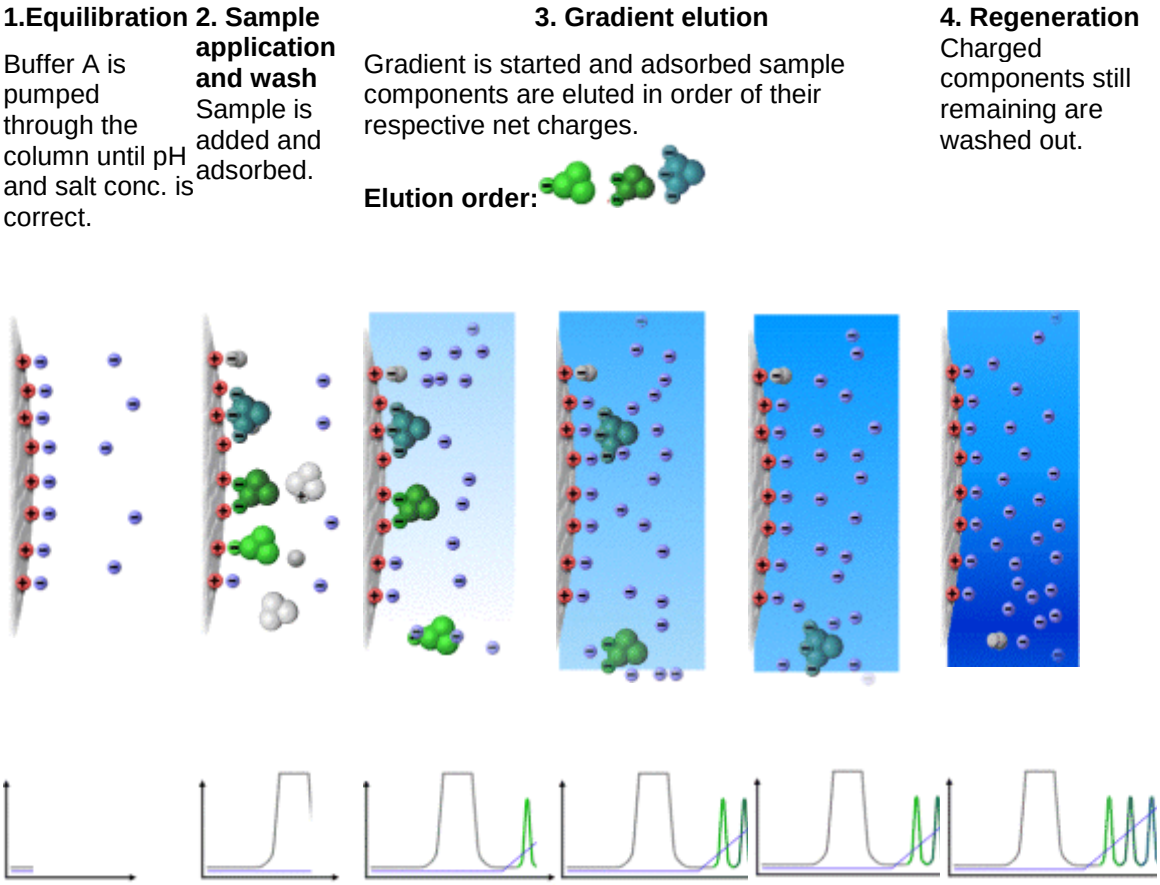


Fig 19. Four phases of IEX experiment (www6.gelifesciences.com)

2.4.2.2. Gel Filtration (GF) and Desalting

Gel filtration is a simple and reliable chromatographic method for separating molecules according to size. Its versatility makes it generally applicable to the purification of all classes of biological substances, including macromolecules not readily fractionated by other techniques. Biological molecules vary greatly in size and gel filtration techniques need to be adapted accordingly.

The ability of gel filtration to separate molecules according to size resides with the highly porous structure of gel filtration media and is basically a question of accessible volumes.

Molecules excluded from the pores of the stationary phase (100% in the mobile phase) move down the column at the same speed as the mobile phase. They will consequently leave the column after one void volume (V_0) of mobile phase has passed through the column.

Molecules with partial access to the pores will be retarded in relation to their respective degree of access to the pores: in other words they will elute from the column in order of decreasing sizes.

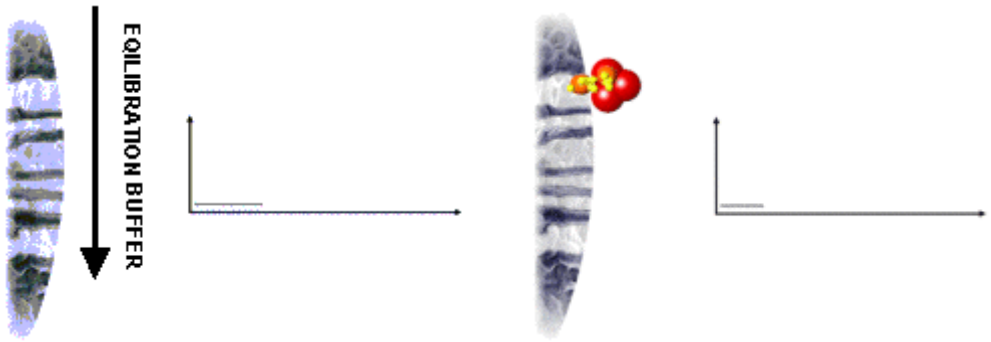
Molecules with full access to the pores will all move down the column at the same speed and remain unseparated from each other. Using a GF medium with a backbone volume of ~5% they leave the column after slightly less than one column volume of mobile phase has passed through the column.

1. Equilibration.

The column is equilibrated with**.**
the buffer intended for the separation.

2. Sample application

The sample is added as a sharp narrow zone at the top of the column. For good resolution the sample volume should be restricted to 0.5 - 5% of the column volume.



3. Elution.

Elution buffer (normally the same type buffer as that used for equilibration) is pumped through the column to make the sample components separate by continuous re-partitioning.

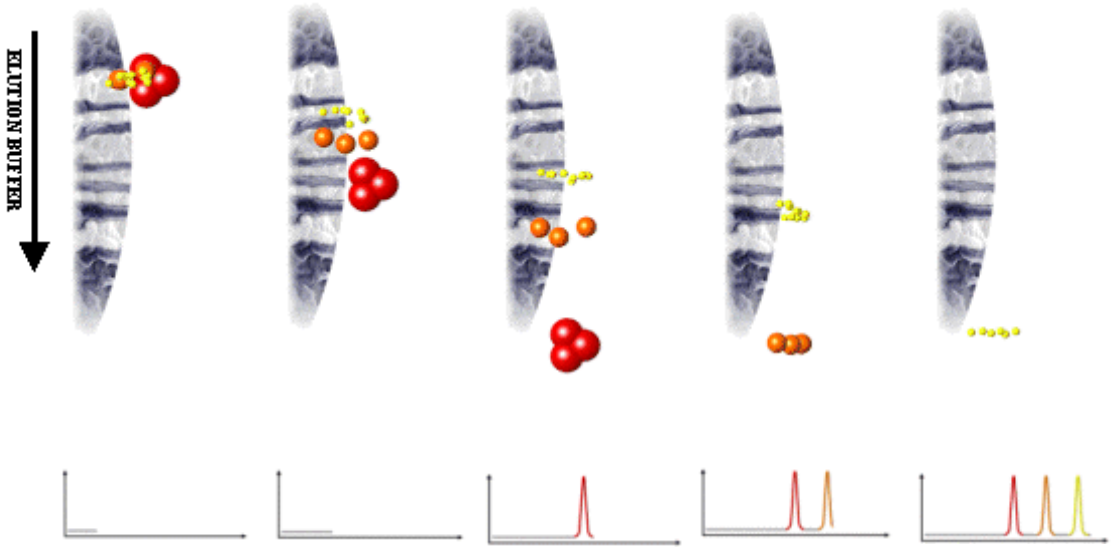


Fig 20. The typical gel filtration experiment (www6.gelifesciences.com)

High resolution mode is used to separate individual macromolecules. This can only be achieved for molecules with partial access to the pores of the gel filtration medium.

Group separation mode is used to remove small molecules from macromolecules. The fractionation range is chosen to elute the macromolecules in the void volume and the small molecules as late as possible. Being completely excluded from the pores, macromolecules will not separate from each other but elute as a group at an elution volume equal to the void volume (www6.gelifesciences.com).

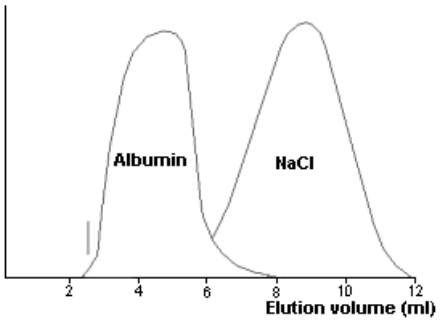


Fig 21. Group separation mode-desalting (www6.gelifesciences.com)

2.4.2.3. Hydrophobic Interaction Chromatography (HIC)

HIC separates proteins with different hydrophobicity. The method is based on the reversible interactions between a protein and the hydrophobic surface of a chromatographic medium. This interaction in enhanced by high ionic strength buffer which makes HIS an ideal next step after precipitation with ammonium sulphate or elution in high salt during IEX.

Elution is usually performed by decreased salt concentration (stepwise or continuous gradient). Other elution procedures include eluent polarity, adding chaotropic species or detergents, changing pH or temperature (Protein Purification Handbook, 2001).

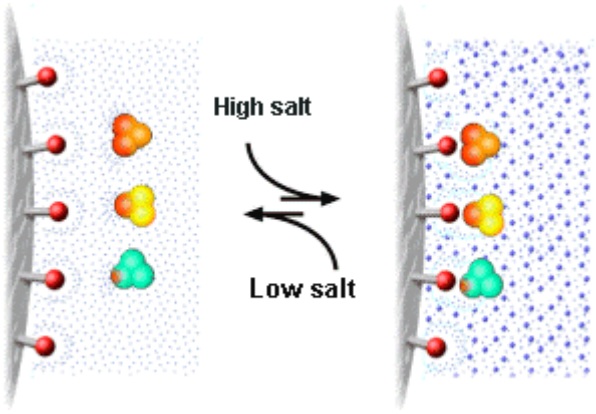


Fig 22. Effect of salt concentration in HIC (www6.gelifesciences.com)

2.4.2.4. Reversed Phase Chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interactions with the medium. Due to the nature of the reversed phase matrices, the binding is usually very strong and requires the use of organic solvents (usually acetonitrile) and other additives (ion pairing agents) for elution (Protein Purification Handbook, 2001).

2.4.2.5. Affinity Chromatography (AC)

AC separates proteins on the basis of reversible interaction between a protein (or group of proteins) and specific ligand attached to a chromatographic matrix. This technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest.

The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed out and the target protein is recovered by changing conditions (competitive ligand, changing pH, ionic strength or polarity) (Protein Purification Handbook, 2001).

1. Equilibration

The column is conditioned to promote adsorption of the target molecule by equilibrating it with *binding buffer.*

2. Sample application and wash

The sample is applied under binding conditions.
The target molecule binds specifically to the affinity ligands, while all other sample components are washed through.

3. Elution

The target molecule is desorbed and eluted by switching to *elution buffer*.

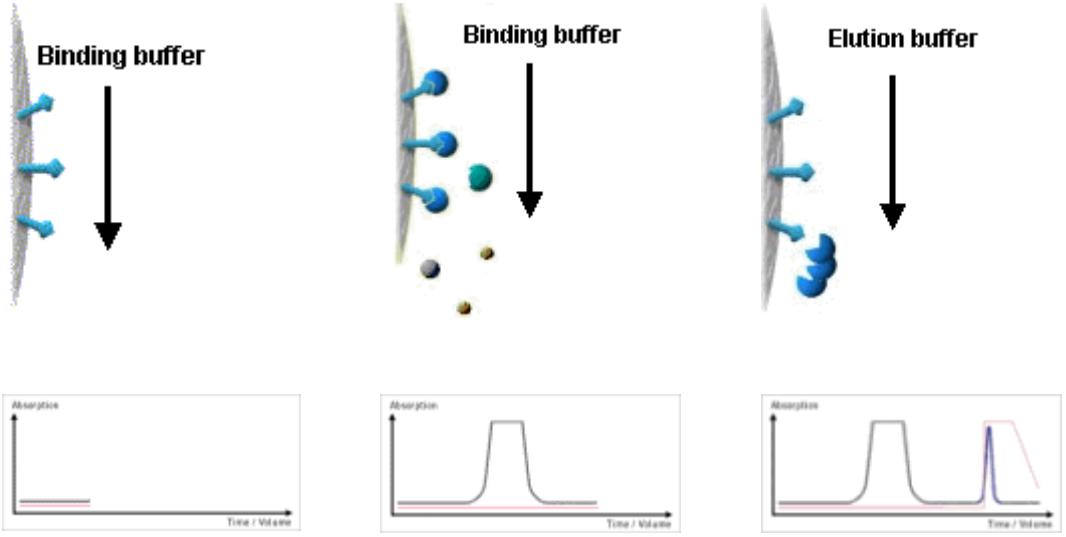


Fig 23. The mechanism of AC protein separation (www6.gelifesciences.com)

3. The Aims

The aim of our work was to study selected carbonyl reducates which can influence anticancer therapy. These were: AKR1C3, soluble cytosolic enzyme, present in several human tissues; and an unknown human liver microsomal reductase. Doxorubicin and oracin, both anticancer agents, were used as substrates in this study.

The aims can be formulated in four points

- To determine the extent of oracin deactivation by human recombinant AKR1C3 by its reduction to dihydrooracin
- To determine the extent of doxorubicin reduction to doxorubicinol by human recombinant AKR1C3
- To introduce, test and optimize several protein purification techniques
- To use the optimized separation conditions in purification of a new human liver membrane-bound reductase

4. Materials and methods

4.1. Materials

4.1.1. Chemicals

Doxorubicin and doxorubicinol – Toronto Research Chemicals (Toronto, Canada)

Oracin, DHO and its enantiomers – Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic)

Glucose-6-phosphate dehydrogenase - Boehringer Mannheim (Mannheim, Germany)

ECL plus Western Blotting Reagent System (Lumigen PS-3) solution A + B – Amersham Biosciences, USA.

Blotting grade Blocker Not-fat Dry Milk – Bio-Rad (Prague, Czech Republic)

Fluka (Prague, Czech Republic)

- Bovine serum albumin (BSA)
- Sodium perchlorate monohydrate (APS)
- Lubrol PX (Octaethylene glycol monododecyl ether)

Sigma Aldrich (Prague, Czech Republic)

- NADPH and $NADP^+$
- Glucose-6-phosphate
- Acetonitrile HPLC grade
- Ethyl acetate HPLC grade
- Bicinchoninic acid (BCA) protein assay reagents
- Sodium dodecylsulphate (SDS)
- Bradford assay reagent
- Iodoacetamide crystalline
- Coomassie Brilliant Blue G-250

All other chemicals used were of HPLC or analytical grade commertially available.

4.1.2. Apparatus

ÄKTA design chromatography system: ÄKTA purifier (Amersham Biosciences, USA)

Fig 24. ÄKTA purifier

Columns: HiTrap Desalting 5 ml
HiTrap Q Fast Flow 1 ml
HiTrap SP Fast Flow 1 ml
Superdex 75 10/300
Ni^{2+} HisTrap Fast Flow 1ml
GSTrap 1ml
Mono P 5/50

Centrifuges: Heraeus Biofuge stratos
Sorvall Ultracentrifuge ODT combi (Du Pont)
Beckman Coulter

High-performance liquid chromatography:

Agilent 1100 Series chromatographic system consisting of gradient pump, autosampler, degasser, thermostated column department and fluorescence detector (Agilent Technologies, USA)

Potter S Homogeniser, B. Braun Bitech International

Gel Doc XR System, Bio-Rad

Mini Protean 3 System, Bio-Rad

Microrotofor isoelectric focusing cell

UV-VIS Spectrophotometer Helios β

Other common laboratory equipment (pipettes, tips, shakers, concentrator, thermoblock, etc.)

4.1.3. Biological Material

Human liver tissue was obtained from Cadaver Donor Programme of Transplant Centre of the Faculty of Medicine (Charles University, Hradec Králové, Czech Republic).

4.1.4. Recombinant AKR1C3

Preparation of recombinant AKR1C3 was performed according to standard techniques by Prof. Wsól.

4.2. Methods

4.2.1. Preparation of subcellular fractions

The 5 g samples of human liver were homogenized in 30 ml of a 0.1 M Na-phosphate buffer (pH 7.4) using sonicator Sonopuls. Microsomal and cytosolic fractions were isolated by centrifugation at 20, 000 g for 20 min and fractional ultracentrifugation at 105, 000 g for 60 min. The microsomal pellets were resuspended and centrifuged twice to get rid of cytosol traces. The final microsomal pellets were resuspended in a homogenization buffer with 20% glycerol. All preparations were carried out at 4°C. Both fractions were preserved in aliquots at -80°C.

4.2.2. Incubations with doxorubicin

The kinetics of doxorubicin was determined by incubating 0.05 to 1.00 mM substrate with recombinant AKR1C3 (8.8 µg in one assay) in 0.15 ml of a 50 mM sodium phosphate buffer, pH 7.4, containing NADPH generating system (final concentrations: $NADP^+$, 3 mM; glucose-6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 1.8 units/0.1 ml; $MgCl_2$, 10 mM).

Reaction was started by adding the substrate solution after 5 minutes preincubation of NADPH generating system in the buffer. Reaction mixtures were incubated for 1 hour.

Reaction was stopped by cooling of the reaction mixture on ice and 0.15 ml 0.2 M Na_2HPO_4, pH 8.4, was added. Extraction was carried out by shaking the reaction mixture with 1.2 ml of chloroform and 1-heptanol mixture 9:1 (v/v) for 15 minutes. Samples were centrifuged for 5 minutes at 5, 000 rpm and organic phases were transported to new tubes. After adding 0.15 ml of 0.1 M *ortho*-phosphoric acid to each tube, the mixtures were shaken intensively for 1 minute. Aqueous (upper) phases were collected and analyzed by HPLC.

4.2.3. Incubations with oracin

Kinetics of oracin was studied using incubation of 0.025 to 0.800 mM substrate with recombinant AKR1C3 (3.52 µg in one assay) in 0.1 ml reaction mixture containing a 0.1 M sodium phosphate, pH 7.4, with NADPH generating system (final concentrations: $NADP^+$, 3 mM; glucose-6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 1.8 units/0.1 ml; $MgCl_2$, 10 mM).

Reaction was started by adding the substrate solution to preincubated (5 minutes) NADPH generating system in the buffer.

Reaction was stopped after 60 minutes by adding 40 µl of 30 % ammonia and cooling on ice. Samples were extracted by 10 seconds of very intensive shaking with 0.3 ml of ethyl acetate, centrifuged for 3 minutes at 13, 000 rpm, the organic phases were evaporated under vacuum, residues were dissolved in 250 µl mobile phase and analyzed by use of HPLC.

4.2.4. HPLC analysis

4.2.4.1. Doxorubicinol

For HPLC determination and quantification of doxorubicinol, the main metabolite of doxorubicin, modified method by Fogli et al. (1999) was used (mobile phase: 50 mM sodium phosphate buffer/ACN (75/25, v/v), pH 4.0; flow rate: 1.5 ml/min; chromatographic column: Supelco Discovery C_{18}, 150×4 mm, 5 µm, Supelco, USA, with precolumn). Doxorubicinol was measured by fluorescence detection using an excitation wavelength 480 nm and emission wavelength 560 nm. 25µl of each sample was injected into the system. Doxorubicinol was identified by the retention time of the reference standard. Retention time of doxorubicinol varied between 2.2 and 2.4 minutes.

4.2.4.2. Dihydrooracin

4.2.4.2.1. Achiral HPLC

For HPLC assessment of dihydrooracin, method of Wsól et al. (1996) was used (mobile phase: 10 mM hexane suphonate buffer with 7.1 ml triethyl amine per liter/ ACN (75/25, v/v); pH 3.27; flow rate: 1.5 ml/min; chromatographic column: BDS Hypersil C_{18}, 250×4 mm, 5 µm, Thermo Electron Corporation, UK). DHO was determined with the fluorescence detection at excitation wavelength 340 nm and emission wavelength 418 nm. 100 µl of each sample was injected into the system. Dihydrooracine was identified by retention times of the reference standard. The retention time of DHO was 2.6 min.

4.2.4.2.2. Chiral HPLC

Separation of DHO enantiomers was carried out according to Wsól et al. (2003) with Chiralcel ODR (250 x 4.6 mm, 5 µm) column. Using a mobile phase of 0.3 M sodium perchlorate with acetonitrile (69:31 v/v) and flow rate of 0.5 ml/min, the following retention times were achieved: (+)-DHO enantiomer 9.0 –

9.2 min and (-)-DHO enantiomer 12.4 - 12.9 min. The wavelengths of fluorescence detection were the same as indicated above.

4.2.5. Solubilization and desalting of microsomes

Microsome suspension was gently mixed with the same volume of a solubilization buffer (10 mM NaH_2PO_4; 1 mM EDTA; 1 M NaCl; 40 % (w/v) glycerole; 0.4 % (w/v) Lubrol PX; pH 7.4) on ice for 45 minutes. The mixture was ultracentrifuged at 100, 000 g for 60 minutes. 0.4 % (w/v) of sodium cholate was added to the supernatant.

Desalting was carried out with help of Äkta purifier on Desalting HiTrap 5ml column and a buffer used in the next purification step (flow rate 2 ml/min). Protein fraction was captured manually. Wave length 280 nm and conductivity monitoring were used for detection of proteins and salts, respectively.

Protein fractions from individual desalting runs were pooled and concentrated by centrifugation at 4, 000 g in centrifugation tubes Amicon.

4.2.6. Ion-exchange chromatography

4.2.6.1. Anion-exchange chromatography

HiTrap Q Fast Flow 1 ml column was used for separation of proteins present in desalted solubilized microsomes. Elution was carried out by gradient elution mode with two buffers using flow rate 0.5 ml/min. Before every experiment the column needed to be washed with 15 column volumes of 20 % ethanol, purified water, buffer B and finally with buffer A until the base line was steady.

BUFFER A (Equilibration)	20 mM Tris HCl
	10 % glycerol
	0.2 mM mercaptoethanol
	pH 8.0 (adjusted with HCl)

BUFFER B (Elution) - Buffer A with 1 M NaCl

Gradient pattern:	1st section	1 ml (flow through fraction)	0 %B
	2nd section	8 or 6 ml	0 % B
	3rd section	12 ml	0 – 50 % B
	4th section	4 ml	50 -100 %
	5th section	6 ml	100 % B

1 ml of desalted solubilized microsomes was injected onto the column. Fraction size was set to be 1 ml.

4.2.6.2. Cation-exchange chromatography

The second procedure used for treating of desalted solubilized microsomes was separation with use of HiTrap SP Fast Flow 1 ml column and system of two buffers with flow rate 0.5 ml/ml.

BUFFER A (Equilibration)	50 mM sodium acetate
	10 % glycerol
	0.2 mM mercaptoethanol
	pH 6.0 (adjusted with acetic acid)

BUFFER B - Buffer A with 1 M NaCl

Gradent pattern:	1st section	1 ml (flow through fraction)	0 %B
	2nd section	8 ml	0 % B
	3rd section	4 ml	0 – 20 % B
	4th section	3 ml	20 -100 %
	5th section	6 ml	100 % B

1 ml of desalted solubilized microsomes was injected onto the column. Fraction size was set to be 1 ml.

4.2.7. Gel filtration

Superdex 75 10/300 GL column was washed by 30ml of 20 % ethanol, and the same volume of purified water before stabilizing the system in

separation buffer. Active fractions from ion-exchange chromatography (injection volume 0.2-0.5 ml) were injected onto the column.

BUFFER	20 mM Tris HCl
	10 % glycerol
	0.2 mM mercaptoethanol
	0.1 % Lubrol PX
	pH 8.0 (adjusted with HCl)

4.2.8. Affinity chromatography (to capture GST)

0.25 ml of active fraction from ion-exchange chromatography was injected onto GSTrap 1ml column with flow rate 0.2 ml/min.

BUFFER A - PBS pH 7.3

BUFFER B	50 mM Tris HCl		
	40 mM GSH		
	0.1 % Lubrol PX		
Gradient pattern:	1st section	1ml (wash out of the unbound sample)	0 %B
	2nd section	8ml	0 %B
	3rd section	14ml	100 %B
	4th section	6ml (gradient delay)	100 %

4.2.9. Electrophoresis methods

4.2.9.1. SDS-PAGE

Bio-Rad Mini Protean 3 system was assembled according to manufacturer instructions.

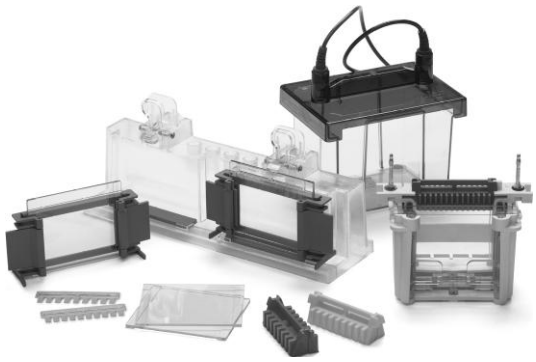


Fig 25. Mini Protean 3 system components (www.bio-rad.com)

Discontinuous polyacrylamide gel system was prepared using a glass plates with 1.5 mm spacers.

Resolving gel (12.5%): 3.2 ml redestilled water
2.5 ml 1.5 M Tris-HCl buffer, pH 8.8
0.1 ml 10 % SDS
4.2 ml acrylamide (30 %) and bis-acrylamide (0.8 %)

Polymerization initiators:	TEMED	4 µl
	Amonium persulphate	58 µl

The gel monomer mixture was poured in between the glass plates, overlayed with 50 % isobutanol and allowed to polymerize for 45 minutes. Immediatelly after polymerization the alcohol layer was removed with distilled water and stacking gel was poured in until the top of the short plate was reached. The comb with desired number of wells was inserted.

Stacking gel: 3.13 ml redistilled water
1.25 ml 0.5 M Tris-HCl buffer, pH 6.8
0.05 ml 10 % SDS
0.5 ml acrylamide (30 %) and bis-acrylamide (0.8 %)

Polymerization initiators:	TEMED	2.5 µl
	Amonium persulphate	30 µl

Stacking gel was allowed to polymerize for 2 hours. After the time was up, the comb was removed and wells washed with distilled water.

The gel cassette sandwiches were places in the electrode assembly and slided into the clamping frame. This way the inner chamber was formed. This chamber was lowered into the Mini tank and filled with an electrode buffer.

Electrode buffer: 70 ml of stock electrode buffer mixed with 280 ml of distilled water.

Stock electrode buffer: 7.2 % glycine
1.5 % Tris-HCl
0.5 % SDS
pH 8.3

The samples were mixed with a sample buffer (1:1) and boiled for 3 minutes.

Sample buffer: 1.3 ml redistilled water
1.0 ml 0.5 M Tris-HCl buffer, pH 6.8
2.0 ml glycerol
3.0 ml 10 % SDS
0.6 ml 0.5 % bromphenol blue
50 µl/ml mercaptoethanol

Samples and protein marker were loaded into the wells with help of sample loading guide. The system was connected to power source and the electrophoresis was run at 100 V until the resolving gel was reached and then increased to 200 V. Electrophoresis process run in ice bath.

After electrophoresis was complete, the glasses were pryed apart and gels were placed into a container with a solution used for staining.

4.2.9.2. Native electrophoresis

The apparatus and process including the gels´ composition were the same as used in SDS-PAGE. There were only a few differences:

- SDS was not used in order to avoid denaturalization of proteins;

- The resolving gel was less concentrated (8 %);
- The samples were not boiled;
- The separation ran at 185 V.

4.2.9.3. Westen blotting

The transfer apparatus was assembled according to manufacturer's instructions. The proteins were transferred from the developed electrophoresis gel onto nitrocellulose membrane with electric current. The electroblotting took 90 minutes at 100 V.

Blot buffer: 25 mM Tris HCl
192 mM glycine
20 % methanol

After the transfer was complete, the membrane was washed, placed in 8 % low fat milk solution in TBST (Tris-Buffered-Saline-Tween) and shaked overnight. This necessary step was made to prevent a direct primary antibody binding to the membrane.

Several washing steps had to be performed to enable the protein detection:

- Washing in TBST twice;
- Washing in the first antibody solution for 90 minutes (anti 11β-HSD 1 or CBR1 in TBST);
- Washing in TBST for 15 and three times 5 minutes;
- Washing in the second antibody solution for 90-120 minutes (anti-rabbit antibody in TBST);
- The same washing like after the incubation with the first antibody solution.

In developing room, Western Blotting Detection System Lumigen solutions A and B were mixed in the ratio 40:1 and pipetted onto the membrane. After 5 minutes, the excess of the solution was blotted away with a paper. Membrane was placed in a foil and air bubbles were smoothed out.

A film was placed against the membrane in a developing cassette and allowed to develop. Fixing solutions were used.

4.2.10. Gel staining

4.2.10.1. Coomassie Blue stain

This staining was performed with both, SDS-PAGE and Native electrophoresis gels. 0.25 % Coomassie Brilliant Blue G-250 in 10 % acetic acid was used as a staining solution. In order to speed up the staining, gels in the staining solution were incubated for 20 minutes at 50°C. The excess of CBBG was washed away with 10 % acetic acid.

4.2.10.2. Silver stain

This staining is more sensitive than Coomassie Blue stain and was performed only with SDS-PAGE.

The process of staining consisted of ten washing steps:

- 20 minutes in 2 % $CuCl_2$; 12 % trichloroacetic acid; 50 % methanol
- 10 minutes in 10 % ethanol; 5 % acetic acid
- 10 minutes in 0.01 % $KMnO_4$
- 10 minutes in 10 % ethanol; 5 % acetic acid
- 10 minutes in 10 % ethanol
- 10 minutes in redistilled water
- 10 minutes in 0.1 % $AgNO_3$
- The gels were rinsed with distilled water
- 2-5 minutes in 10 % K_2CO_3
- The gels were washed in 2 % K_2CO_3; 0.01 % formaldehyde untill bands appeared.
- The reaction was stopped by transfering the gels into a solution of 10 % ethanol and 5 % acetic acid.

4.2.10.3. Native electrophoresis gel staining

The developed gel was incubated for 1 hour at 37°C with a solution of 1.0 mM NADPH and 0.5 mM oracin in a 10 mM Na-phosphate, pH 7.4. Washed gel was stained overnight with 0.05 mM Meldola Blue and 0.31 mM p-iodonitrotetrazolium in a 10 mM Na-phosphate buffer, pH 7.4.

This method stains NADPH, enzymes which use NADPH as a coenzyme for oracine reduction appear on the gels as transparent bands on a purple background.

4.2.11. Protein assay

4.2.11.1. Bicinchoninic acid method

This method is compatible with detergents and most of buffers but is not compatible with reducing agents (e.g. mercaptoethanol).

Proteins in buffers without reducing agents were determined according to Smith et al (1995). The method was modified for buffers with reducing agents. Adding 2 mM iodoacetamide into the reaction mixture solved the problem of interactions of copper present in BCA reagents and mercaptoethanol.

4.2.11.2. Bradford assay

Fractions obtained from Q Sepharose and SP Sepharose separations were assayed for proteins with Bradford assay according to manufacturers´ instructions. Bovine serum albumine was used as a standard in both methods.

4.2.12. Kinetic parameters estimation

The apparent kinetic parameters and their statistics were calculated using the GraphPad Prism 5.0 kinetic computer software. This program was also used for drawing the enzyme kinetics figures.

4.2.13. GST activity determination

Glutathione S-transferase catalyses the formation of S-2,4-dinitro-phenylglutathione in a solution containing 1-chloro-2,4-dinitrobenzene and glutatione in phosphate buffer pH 6.5. The formation is followed spectrophotometrically as a change of absorbance at 340 nm in time (five measurements 60 seconds apart).

The reaction mixture of 1 ml contains: 970 µl of 0.1 M phosphate buffer pH 6.5; 10 µl of 0.1 M glutathione and 10 µl of the sample. After mixing the last ingredient is added, 10 µl of 0.1 M 1-chloro-2,4-dinitrobenzene. The absorbance is measured immediately and then in four 1 minute intervals (Habig and Jakoby, 1981).

4.2.14. Lactate dehydrogenase activity determination

Activity is measured spectrophotometrically at 340 nm, the increase of absorbance is caused by LDH catalyzing reaction of lactate and NAD^+ to form pyruvate and NADH.

There is a kit commercially available for this method from Sigma, the method used is Sigma Diagnostics procedure No 228-UV

The LDH content was calculated:

$$LDH = \Delta A * (TV * 1000) / (6.22 * SV * CS)$$

Where ΔA is the average difference in absorbance; TV total volume of the reaction mixture (0.7ml); SV sample volume (0.2ml); CS cuvette size in cm.

5. Results and discussion

5.1. AKR1C3

5.1.1. Doxorubicin

Doxorubicin is a well-established and highly effective antineoplastic agent for various hematopoietic and solid tumors. A dose-response relationship has been demonstrated for anthracyclines and some tumors, with lower dose resulting in decreased survival and remission rates. Its main metabolite in human is a corresponding secondary alcohol, doxorubicinol. Since doxorubicinol is an inactive metabolite, there is a constant effort to understand the process of doxorubicin deactivation on cellular level. Human carbonyl reductase 1, one of the main reductases in human tissues, catalyses reduction of doxorubicin to doxorubicinol.

We tested the extent of doxorubicin reduction catalyzed by AKR1C3. In our study we demonstrated that carbonyl reductase 1 is therefore not the only enzyme contributing to doxorubicin deactivation.

5.1.1.1. Incubation conditions optimization

The incubation conditions needed to be optimized.

Incubation time optimization

To find a suitable length of incubation was crucial to avoid wasting of time as well as to enable the enzyme to convert a substantial part of the substrate provided to reaction product easily detectable by HPLC.

We tested three incubation times (30min, 45min and 60min) using 10µl of enzyme solution and substrate concentration 0.5 mM in the reaction mixture in two parallel samples. Results are shown in Table 8 and Fig 26.

Table 8. Incubation time optimization

Incubation time [min]	Peak area – average of two parallel samples
30	184.88
45	290.41
60	540.55

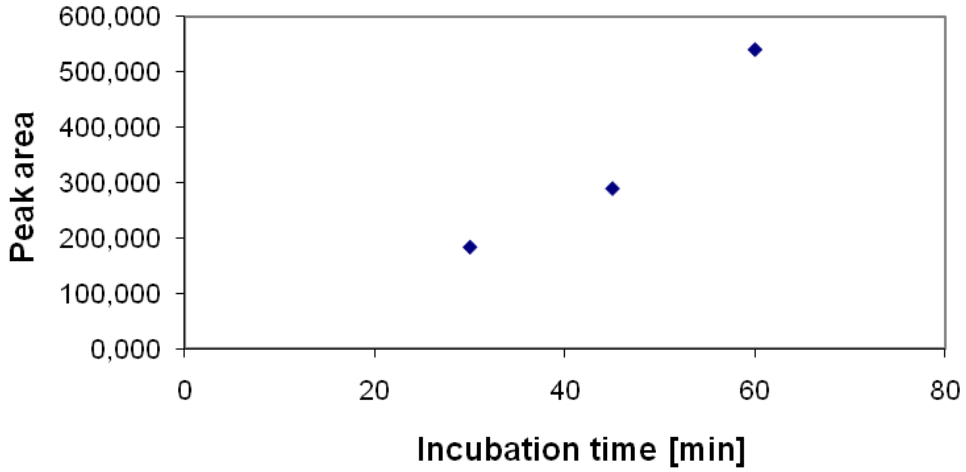


Fig 26. Incubation time optimization results

According to the optimization results, 60 minutes incubation time was chosen to obtain the best results.

Amount of the enzyme optimization

We wanted to find out what amount of enzyme in the reaction mixture would be sufficient for the reaction to run at a substantial velocity. AKR1C3 is a NADPH dependent enzyme therefore NADPH generating system was added to reaction mixture.

Two distinct enzyme amounts were tested in one experiment, 5 µl and 10 µl of enzyme solution in reaction mixture (containing 8.8 µg and 17.6 µg of recombinant AKR1C3, respectively). Incubation time was set to 60 minutes and

0.5 mM concentration of substrate was used in two parallel samples for each enzyme amount.

Table 9. Optimization of enzyme amount in the reaction mixture

Enzyme amount [µl]	**Peak area – average of two parallel samples**
5	357.04
10	679.23

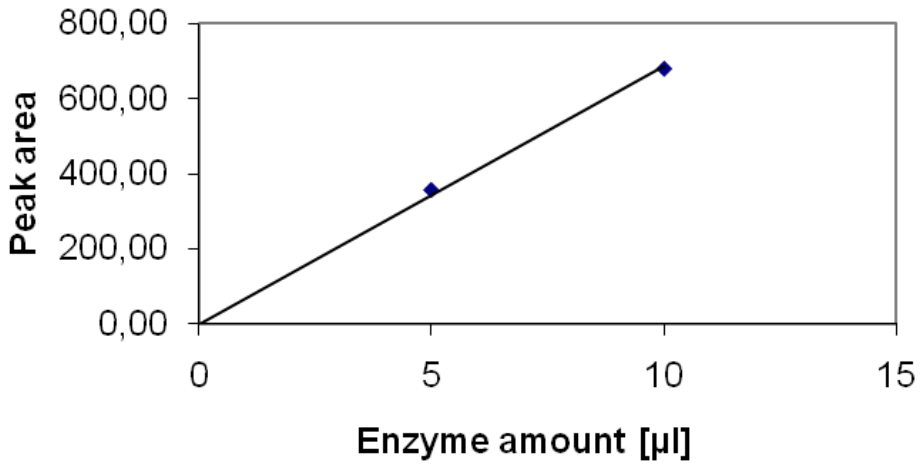


Fig 27. Enzyme amount optimization results

In the end, we decided to use 5 µl of enzyme solution to preserve recombinant AKR1C3, considering that use of lower amount of enzyme still resulted into satisfactory and easily detectable amount of reaction product.

Optimization of substrate concentration

To establish the substrate concentration range suitable for the enzyme kinetics determination, we carried out an experiment using substrate concentrations from 0.05 mM to 0.5 mM.

Using the previously optimized incubation conditions, the results showed the necessity of including higher substrate concentrations to obtain the whole enzyme kinetics of the reaction.

5.1.1.2. The reaction kinetics

The kinetics of doxorubicin carbonyl reduction by AKR1C3 was then studied using substrate concentrations: 0.05 mM; 0.1 mM; 0.2 mM; 0.3 mM; 0.5 mM; 0.75 mM and 1.0 mM. And the amount of product doxorubicinol was determined by HPLC.

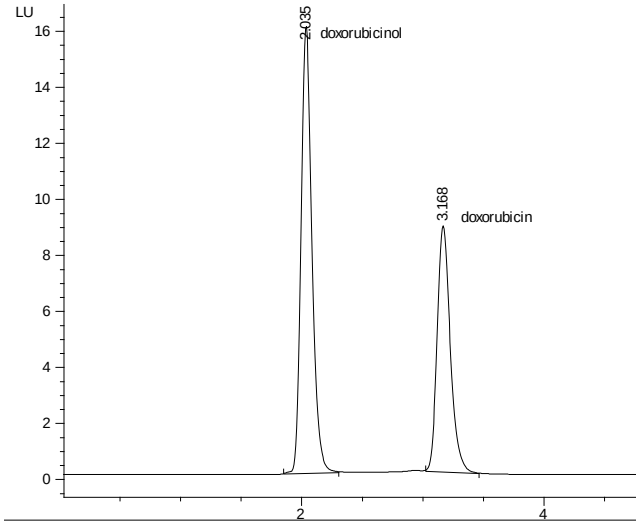


Fig 28. HPLC separation chromatogram of doxorubicinol and doxorubicin after incubation with AKR1C3 Concentration of doxorubicin in reaction mixture was 0.05 mM.

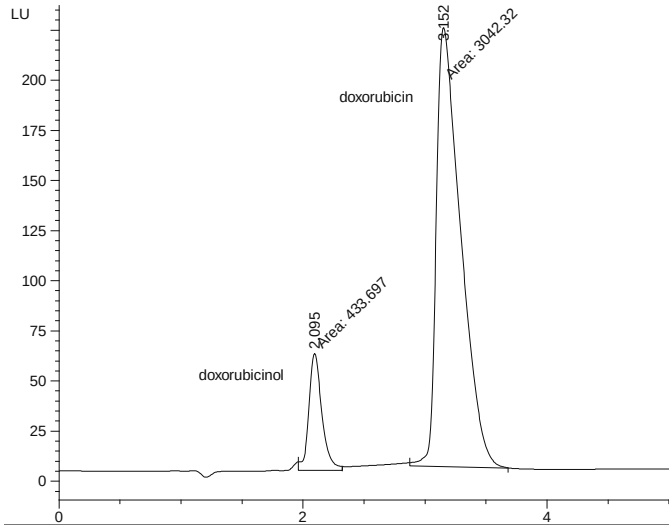


Fig 29. HPLC separation of doxorubicinol and doxorubicin after incubation with AKR1C3 Concentration of substrate was 1.0 mM.

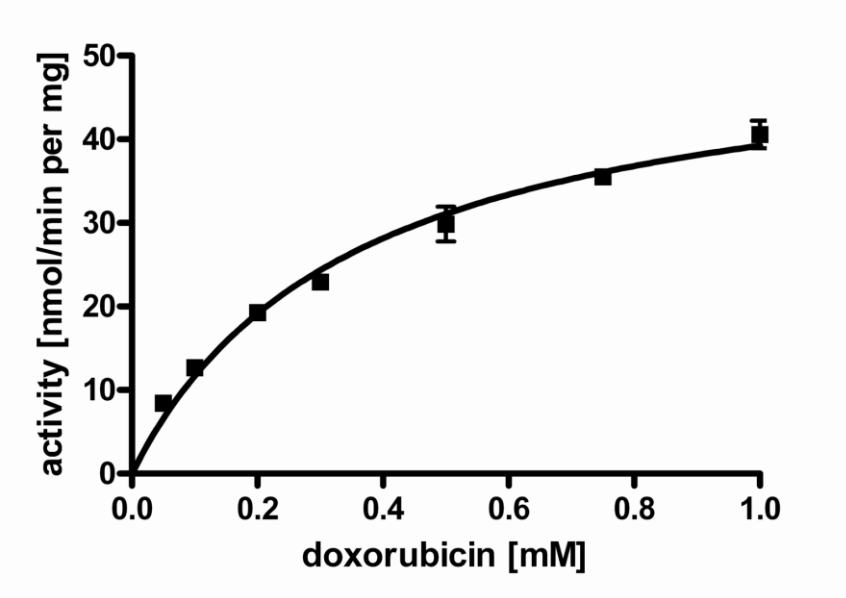


Fig 30. Kinetic profile of doxorubicinol formation by recombinant AKR1C3

Michaelis-Menten constant (K_m), maximum velocity (V_{max}) and clearance (CL_{int}) were calculated (Table 12.) to characterize the reaction more.

Our experiments proved the ability of AKR1C3 to metabolize doxorubicin through a two-electron reduction of its C-13 keto group which leads to formation of doxorubicinol and therefore to the drugs deactivation.

Recombinant AKR1C3 was stored at -80°C and only removed from the freezer when needed to preserve its activity.

In the prostate, AKR1C3 is responsible for conversion of inactive androgens to active 5-DHT and testosterone.

Fung et al. (2006) demonstrated that AKR1C3 is overexpressed in prostate carcinoma cells as well as in benign glands in areas with chronic inflammation, atrophic changes and urothelial cell metaplasia. Results of Stanbrough et al. (2006) prove the same fact on gene expression level and show that there was an average AKR1C3 message increase of about 4-fold, with approximately one third of the androgen-independent prostate cancer showing particularly high expression levels.

In the breast, AKR1C3 metabolizes conversion of inactive estrone to active estrogen, 17β-estradiol.

Vihko et al. (2005) detected AKR1C3 in epithelial cells of normal and malignant breast tissue specimens from both pre- and postmenopausal women. However, the expression of AKR1C3 was significantly higher in breast cancer specimens than in normal breast tissue. Altogether 65% of the breast cancer specimens were positive for AKR1C3 expression. This group had a worse survival than the groups with lower or no expression. These results show that there are interindividual differences between patients with breast cancer.

Table 10. Final enzyme kinetics of doxorubicin reduction by recombinant AKR1C3 – experiment results

substrate concentration [mM]	product concentration [μM]	reaction velocity [nmol/l/s]	activity [nmol/min per mg of protein]	average	standard deviation
0.05	27.58	7.66	7.83		
	31.46	8.74	8.94		
	29.99	8.33	8.52	8.43	0.908
0.10	45.93	12.76	13.05		
	43.21	12.00	12.28		
	44.91	12.47	12.76	12.69	0.319
0.20	67.57	18.77	19.20		
	66.94	18.59	19.02		
	68.94	19.15	19.58	19.27	0.237
0.30	81.69	22.69	23.21		
	77.94	21.65	22.14		
	82.58	22.94	23.46	22.94	0.571
0.50	83.02	23.06	23.58		
	97.75	27.15	27.77		
	112.37	31.21	31.92	27.76	3.404
0.75	124.06	34.46	35.24		
	124.95	34.71	35.50		
	125.97	34.99	35.79	35.51	0.222
1.00	131.29	36.47	37.30		
	148.30	41.19	42.13		
	148.90	41.36	34.84	38.09	3.029

On the other hand, Wiebe and Lewis (2003) demonstrated that AKR1C3 activity was significantly lower in tumorous cell cultures than in nontumorous cultures and Lewis and coworkers (2004) confirmed these results in breast tissue specimens. Ji et al. (2004) found a significant decrease in AKR1C3

expression in breast cancer samples only in one case of 13 and did not note any substantial changes in AKR1C3 expression in breast cancer cell lines.

These discrepancies between particular works indicate that the level of AKR1C3 expression differs from patient to patient and could significantly influence their prognosis.

The importance of AKR1C3 has been studied in relation to endogenous metabolism, where it contributes to maintenance of steroid hormone homeostasis, but the impact of AKR1C3 action on anticancer drugs was not evaluated. It was not known whether a drug stays active or is metabolized, how effective the metabolism is. We described that the enzyme, when overexpressed, can significantly contribute to doxorubicin deactivation. This fact, together with the ability of AKR1C3 to create pro-estrogen or pro-androgen environment in diseased breast, or prostate respectively, could notably influence the anticancer treatment.

5.1.1.3. Method development for chiral separation of doxorubicinol

As mentioned before, doxorubicin is a pro-chiral molecule and its metabolite expresses chiral properties. That is one of the reasons why we attempted to develop a HPLC method for separation of doxorubicinol.

We tested a number of chiral HPLC columns and separation conditions. During this process we used manufacturers' instructions and recommended modifications according to our separation results.

We did not achive a total separation of enantiomers of doxorubicinol but we suggested the further steps and ruled out the columns and conditions which were not suitable.

The best separations results were achived with the use of Chirobiotic V column (flow rate 0.5 ml/min, 1mM NH_4OAc pH 4.4: ACN/55:45) and Ultron ES-

OMV column (flow rate 0.3 ml/min, 1 mM NaOAc) as shown on Fig 31 and 32, respectively.

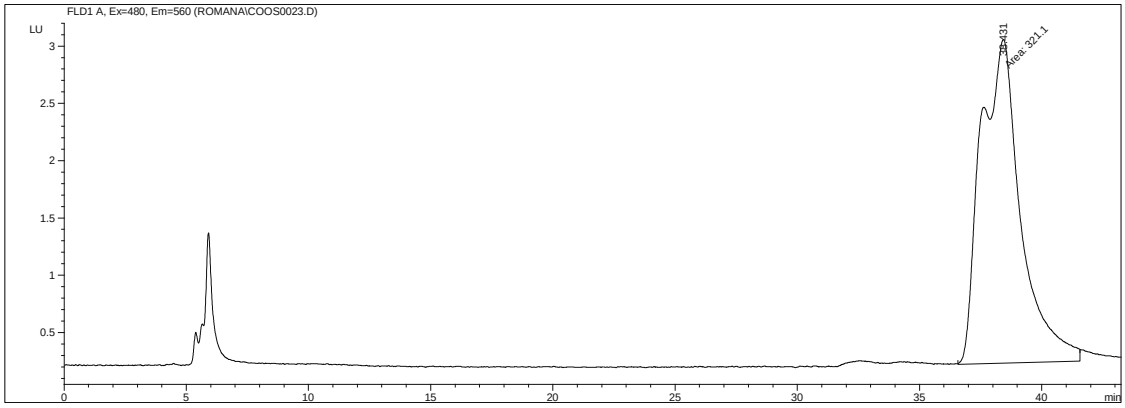


Fig 31. Chiral separation of doxorubicinol on Chirobiotic V column

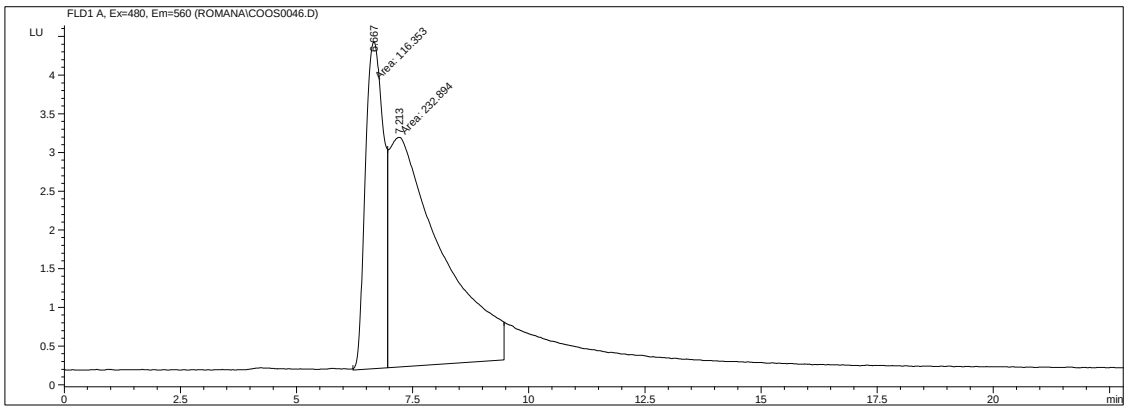


Fig 32. Chiral separation of doxorubicinol on Ultron ES-OMV column

5.1.2. Oracin

Oracin is a promising potential cytostatic drug for peroral administration. A DNA intercalation mode of action can be inferred, similar to that of anthracyclines. However, the wide spectrum of tumors that are sensitive to oracin does not only result from this single mechanism of action, several other mechanisms effecting tumor cell growth have been demonstrated. The main advantages of this drug are the possibility of peroral administration, the combination of different antitumor mechanisms, the absence of cardiotoxicity,

the negative results in the Ames test on mutagenicity, very low hepatotoxicity, and its favorable pharmacokinetics. The principal metabolite is dihydrooracin (DHO). This chiral metabolite is formed by reduction of the pro-chiral carbonyl group at the 11-position of the oracin molecule.

We found that oracin is deactivated to DHO by AKR1C3.

5.1.2.1. Incubation conditions optimization

Similarly to doxorubicin, the incubation conditions needed to be optimized before carrying out the final experiments. We performed similar series of experiments and the final incubation conditions were set to 60 min incubation time, 2µl of enzyme solution in the reaction mixture, the range of substrate concentration from 0.025 mM to 0.800 mM.

5.1.2.2. The reaction kinetics

Kinetic studies were performed with oracin as a substrate using substrate concentrations 0.025 mM; 0.050 mM; 0.100 mM; 0.150 mM; 0.200 mM; 0.300 mM; 0.500 mM and 0.800 mM.

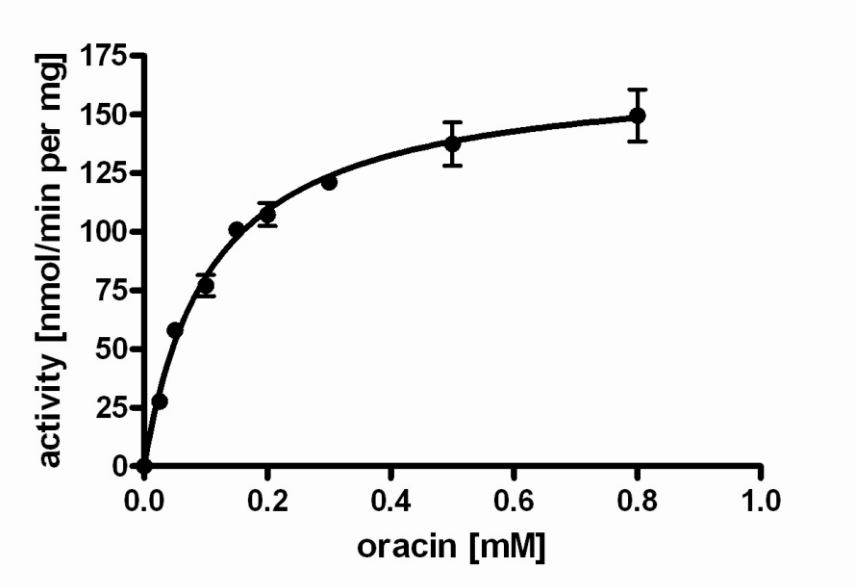


Fig 33. Kinetics of DHO formation by recombinant AKR1C3

Table 11. Final reaction kinetics for oracin reduction by recombinant AKR1C3 – experiment results

substrate concentration [mM]	product concentration [µM]	reaction velocity [nmol/l/s]	activity [nmol/min per mg of protein]	standard deviation
0.025	58.45	16.24	27.68	2.766
0.050	122.34	33.98	57.92	2.518
0.100	162.70	45.19	77.03	6.361
0.015	212.97	59.16	100.84	4.235
0.200	226.67	62.96	107.32	4.828
0.300	255.74	71.04	121.09	2.808
0.500	290.15	80.60	137.38	9.198
0.800	315.97	87.70	149.61	15.680

Michaelis-Menten constant, maximum velocity and clearance were also calculated (Table 12.).

Table 12. Kinetic parameters for carbonyl reduction of doxorubicin and oracin by recombinant AKR1C3

Substrate	K_m [µM]	V_{max} [nmol/min per mg]	CL_{int} [ml/min per mg]
doxorubicin	355 ± 36	53 ± 2	1.54
oracin	110 ± 11	169 ± 6	0.15

Among the AKR1C subfamily members, AKR1C1 has the highest catalytic efficiency in oracin inactivation (Wsól et al., 2007), directly being followed by AKR1C3 (our study). AKR1C2 and especially AKR1C4 have a much lower catalytic efficiency (Wsól et al., 2007). However, considering the tissue specific expression of AKR1C3 in prostate and breast tissue, as well as its overexpression in prostate and breast cancers, AKR1C3 may become an important target for chemotherapy supporting strategies. In other words, specific

inhibition of AKR1C3 upon chemotherapy may prevent metabolic inactivation of anticancer drugs bearing a carbonyl group, thereby improving the clinical outcome of chemotherapy. Moreover, as far as doxorubicin is concerned, inhibition of doxorubicinol formation may prevent the incidence of cardiomyopathy upon cancer treatment.

5.2. Protein purifications

Protein purification usually consists of several steps. In our experiments, all purification steps, assays and measurements were carried out as fast as possible and possibly also at 4°C in order to preserve as much activity of the protein of interest as possible.

Purification of membrane-bound proteins has always been a more difficult procedure than purification of soluble proteins. The membrane has to be solubilized first and this step often leads to inactivation of the protein of interest since the presence of membrane components is often crucial for proteins native state.

5.2.1. Preparation and solubilization of microsomes

Microsomes are prepared from human liver samples by a series of ultrasound homogenization in a phosphate buffer, pH 7.4, and subsequent centrifugation. In the third set microsomes are separated from cytosol. To make sure prepared microsomes were free of any residual traces of cytosolic content, we repeated the last separation step twice more to wash away cytosol completely.

Cytosol, centrifugation supernatant from last two separation steps and final microsomes were tested for presence of lactate dehydrogenase. This enzyme is strictly present only in cytosol and since its activity assay is easy to perform, it is used to detect cytosol traces.

The assay is based on LDHs ability to convert lactate to pyruvate in presence of NAD^+.

$$\text{L-lactate} + NAD^+ \rightarrow \text{pyruvate} + NADH + H^+$$

The absorption maximum of NADH is 340nm. The activity of LDH in a sample is then measured as the increase of samples' absorbance at this wavelength.

The results of LHD determination experiment were very satisfactory and assured us of the complete absence of cytosol traces in the microsomes obtained.

For membrane solubilization, we modified a method which was successfully used by Maser et al. (2002) for 11β-HSD type 1 purification.

Solubilization of microsomal membrane is very crucial for the success of the whole experiment. It is necessary to carry out all steps quickly and at low temperatures. Solubilization takes 45 minutes and therefore must be done on ice and in a cold room, if possible. The total volume of microsomes after solubilization and centrifugation (which removes any remnants of not solubilized membrane) is usually about 180 % of the initial volume of microsomes before solubilization.

5.2.2. Desalting

Microsomes needed to be desalted because our chosen first purification step was an ion-exchange chromatography which requires a low salt content in the sample and protein bound to the column matrix is then washed out by salt gradient in the elution buffer.

Hi Trap Desalting column was used with flow rate set to 2 ml/min, the solubilized microsomes were injected in 1ml sets to avoid loss of protein and excessive dilution. Samples were captured manually according to the detection respose. UV detector was set to 280 nm.

The advantage of the desalting step is a transfer of the sample into the next purification step buffer; the disadvantage is that the sample volume increases more than twice. That makes a direct use of the product of desalting impossible. We concentrated the solution to 8-10 times smaller volume by centrifugation.

Table 13. An example of sample volume changes during microsomes solubilization and desalting

Experiment stage	Volume	Notes
Microsomes	2.1ml	Equal amount of solubilization buffer added
Solubilized microsomes	4.2ml	Sample centrifuged for 60 min at 32000 rpm
Solubilized microsomes after centrifugation	3.6ml	Sample desalted on HiTrap Desalting column with flow 2ml/min, injected onto the column in 4 runs
Desalted microsomes	8.35ml	Sample centrifuged at 4000g
Final volume of prepared microsomes	1.12ml	

5.2.3. Purification steps

We tested several purification patterns, including ion-exchange, affinity and hydrophobic interaction chromatography and gel filtration.

Some of the experiments showed to be a no through road. But we managed to introduce a large number of methods and solve some problems for the future work.

5.2.3.1. Purification pattern 1

We started our protein purifications in cooperation with Prof. Maser´s team from Institute of Toxicology and Pharmacology for Natural Scientists, Kiel, Germany.

Anion-exchange chromatography

We decided to use anion-exchange chromatography as the first purification step. The sample was transferred into buffer A in the previous step, desalting.

The method was programmed on ÄKTA purifier - Fraction size 1ml, flow 0.5ml/min, gradient: 7 column volumes 0 % B buffer, 12 CV 0-50 % B buffer, 4 CV 50-100 % B buffer, 4 CV 100 % B buffer + 2 CV delay. Buffer B was almost identical to buffer A, the only difference was 1M NaCl added. The pressure in the system stabilized at 0.3 MPa.

In our experiments, we injected 0.5-1 ml of concentrated desalted solubilized microsomes and detected the protein content in the eluted mobile phase at 280 nm.

We achieved very satisfactory ability to repeat this experiment with identical results with the highest reductive activity in flow through fractions and fractions A11, A12, and B12.

Oracin was used as a model substrate for enzyme´s reductive activity assessment.

100 µl of each fraction obtained from the anion-exchange chromatography experiment was preincubated with NADPH generating system in a phosphate buffer, pH 7.4, at 37°C for 5 minutes and subsequently incubated with oracin (final concentration in reaction mixture 0.5 mM) for 30 minutes,

reaction was stopped by adding of 40 µl of 25% ammonia. We used HPLC to analyze the content of reaction product, dihydrooracin.

Unfortunately, the activity of biological material decreases during manipulation and carrying out the experiments. The average yield of IEX experiment is usually about 50%.

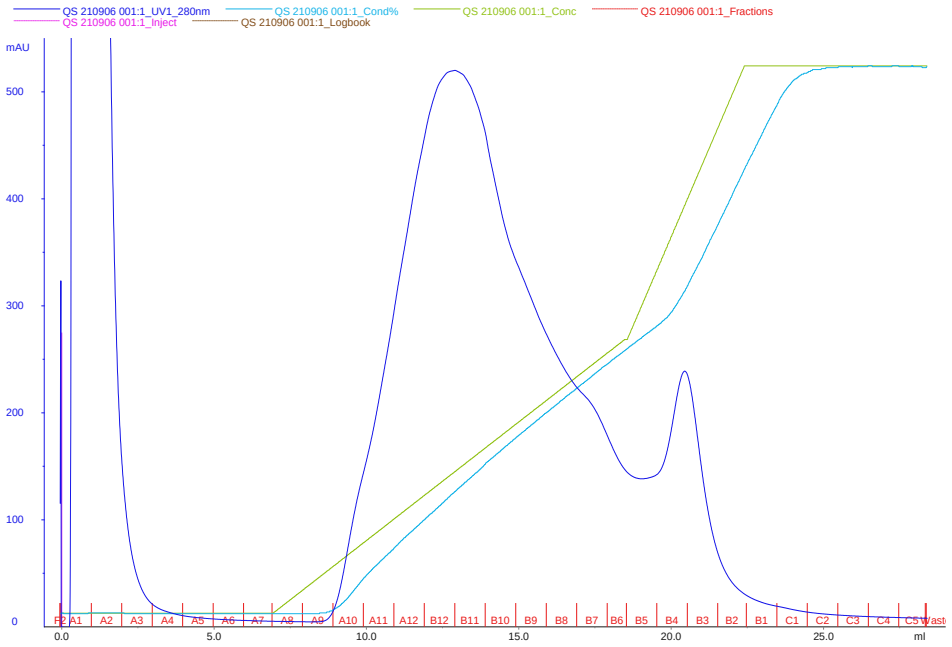


Fig 34. The anion- exchange experiment carried out using HiTrap Q Fast Flow 1ml column, dark blue line shows protein content, light blue line salt content in buffer, green line method programmed.

There is a significantly high activity in the first two fractions as well. These fractions contain enzymes which were not able to bind to the column matrix at the experiment conditions and enzymes which were not bound to the column because the binding capacity of the matrix has been exceeded.

IEX chromatography fractions were injected onto electrophoresis gel together with a molecular marker to establish the molecular size profile of proteins present in individual fractions.

Since the protein content in experiment fractions was low, the Coomassie blue staining was not able to reveal the whole spectrum of proteins present; therefore we had to use more sensitive staining method, Silver stain. This method is more sensitive but unlike Coomassie blue staining method does not show quantitative differences between protein bands.

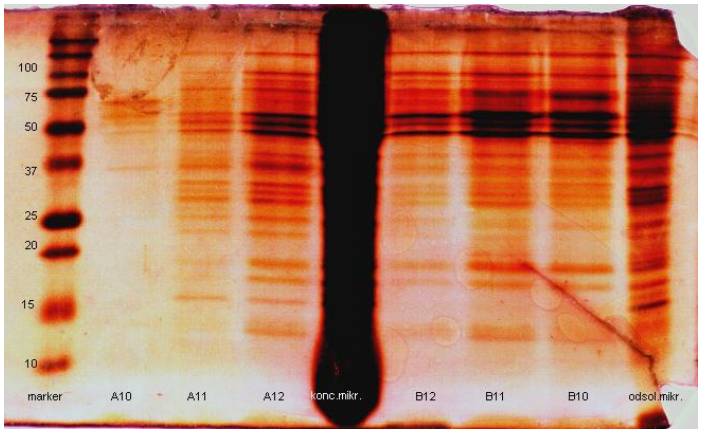


Fig 35. Silver stained electrophoresis gel of active Anion-exchange chromatography fractions with concentrated microsomes in the middle, molecular marker on the left and desalted solubilized microsomes on the right side of the gel

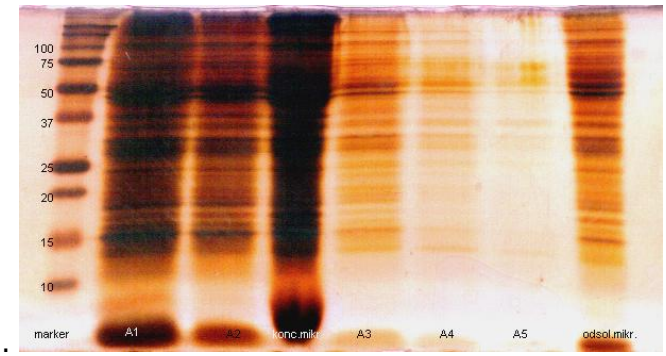


Fig 36. Silver stained gel with other fractions from Anion-exchange chromatography experiment

Table 14. HPLC results of ion-exchange chromatography separation fractions reductive activity assay

Fraction	Injected volume [µl]	DHO/inj [ng]	Incubated volume [µl]	DHO/ incubation [ng]	Total volume [ml]	Total DHO [ng]
microsomes	100	99.92	5	249.81	0,50	**24981.25**
A1+FT	100	46.06	50	115.14	1,25	2878.56
A2	100	20.67	50	51.67	1,00	1033.35
A3	100	3.16	50	7.90	1,00	158.10
A4	100	1.61	50	4.02	1,00	80.30
A5	100	1.04	50	2.61	1,00	52.20
A6	100	0.87	50	2.17	1,00	43.45
A7	100	0.86	50	2.15	1,00	43.05
A8	100	0.67	50	1.68	1,00	33.70
A9	100	0.68	50	1.70	1,00	34.00
A10	100	1.48	50	3.70	1,00	74.05
A11	100	17.19	50	42.98	1,00	859.60
A12	100	53.79	50	134.47	1,00	2689.35
B12	100	32.87	50	82.18	1,00	1643.70
B11	100	13.90	50	34.76	1,00	695.15
B10	100	5.84	50	14.61	1,00	292.20
B9	100	3.79	50	9.47	1,00	189.40
B8	100	3.86	50	9.64	1,00	192.80
B7	100	3.46	50	8.66	1,00	173.25
B6	100	2.29	50	5.72	1,00	114.50
B5	100	1.93	50	4.83	1,00	96.70
B4	100	1.61	50	4.01	1,00	80.30
B3	100	1.35	50	3.39	1,00	67.75
B2	100	1.04	50	2.61	1,00	52.15
B1	100	0.78	50	1.95	1,00	38.95
C1	100	0.70	50	1.75	1,00	35.00
C2	100	0.50	50	1.25	1,00	25.05
C3	100	0.41	50	1.01	1,00	20.25
C4	100	0.32	50	0.79	1,00	15.90
C5	100	0.30	50	0.74	1,00	14.90
					TOTAL	**11727.66**
						46.95%

In Table 14: Microsomes – concentrated desalted solubilized microsomes injected onto the IEX column; injected volume – volume of the treated sample injected onto the HPLC column; incubated volume – sample volume used for incubation; total volume – total volume of the fraction or microsomes injected onto the IEX column; total DHO – DHO content in the total volume.

As mentioned above, the enzyme activity decreased during manipulation with the biological material. To increase the protein of interest concentration in the tested sample, we always performed two or more runs of Mono Q Sepharose separation and pooled the most active fractions, which were used in the next steps after concentration by centrifugation.

Gel filtration with the flow through fraction

Gel filtration, size exclusion chromatography, separates proteins according to the size of their molecules. It is a time consuming technique because the columns used for this chromatography are very sensitive to high pressure and have quite large matrix volume comparing to other techniques used at our department.

Since the proteins do not bind to the column matrix there is no need for elution buffer of buffer gradient. Flow rate 0.5 ml/min generated pressure of max 1.9 MPa, 1ml fractions were collected after injecting 0.25 to 0.5 ml of the sample.

We got the first results from Gel filtration as a second step after Q sepharose separation during our research stay in Germany where we tested the active Flow through fraction.

After incubation with oracin, fraction 11 showed to be highly active. The activity was almost completely concentrated in this fraction.

We performed SDS-PAGE with the active fraction. The result is shown on Fig 38. The elipsed band of approximate molecular weight 27 kDa was sent for MALDI-TOF analysis.

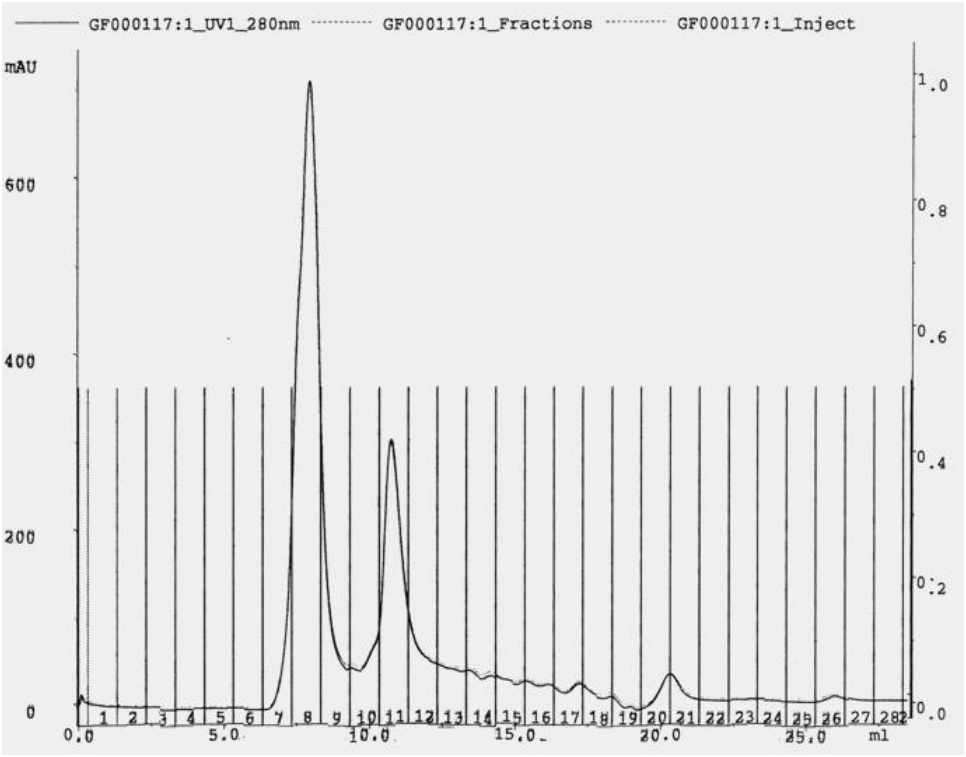


Fig 37. Gel filtration experiment carried out with the anion-exchange chromatography flow through fraction

The results suggested that the active enzyme in gel filtration fraction 11 was with the highest probability Glutathione S-transferase. The partial amino acid sequence was similar to cytosolic GSTs but a contamination of microsomes by cytosol was very unlikely since the purity of microsomes was confirmed by the LDH asay and the absence of signals in western-blots with antibodies against cytosolic enzymes.

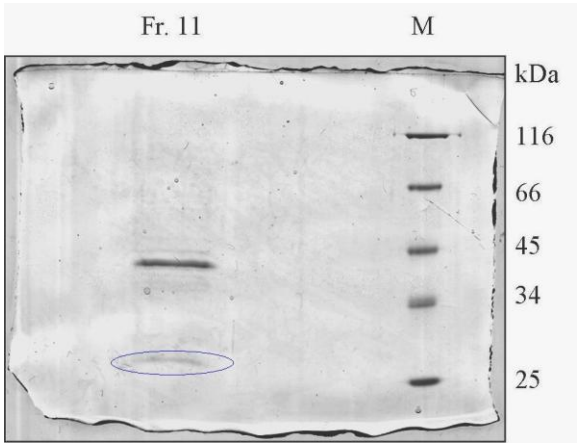


Fig 38. Coomassie blue stained 10% SDS-PAGE of the active fraction of gel filtration experiment with molecular marker on the right side

Table 15. MALDI-TOF analysis results of the elipsed band from Fig 38.

MALDI-TOF ANALYSIS			
proteins matching used set of peptides			
GTA2 HUMAN	Mass: 25531	Total score: 131	Peptides matched: 9
Glutathione S-transferase A2 (EC 2.5.1.18); GST - gamma			
GSTA1 HUMAN	Mass: 25484	Total score: 129	Peptides matched: 9
Glutathione S-transferase A1 (EC 2.5.1.18); GST - epsilon			
GSTA3 HUMAN	Mass: 25285	Total score: 129	Peptides matched:9
Glutathione S-transferase A3-3 (EC 2.5.1.18); GST class-alpha			

The molecular weight of enzyme tested was approximately 27 kDa as shown on the SDS-PAGE. The molecular weight differs from other known microsomal GSTs (ca 17 kDa). Prabhu et al. (2004) isolated a microsomal GST of a similar molecular weight of 25.5 kDa.

These results led to the logical set of subsequent experiments when we tried to confirm the MALDI-TOF analysis outcome with use of Affinity chromatography.

Affinity chromatography (binding GST)

Affinity chromatography is based on temporary binding of the protein of interest on the column matrix and its subsequent elution by a gradient or step change of buffer where elution buffer contains a substance with higher affinity to the protein than the matrix.

In our case we needed to capture Glutathione S-transferase on the column. For that purpose we used GSTrap column specifically developed to bind GSTs.

We tested several separations conditions starting with the manufacturer recommendations and modifying the conditions according to our results:

- Separation conditions 1 – buffer A PBS pH 7.3, buffer B Tris HCl pH 8.0 with 10 mM GSH. 0.25 ml of flow through fraction from Q sepharose separation injected. Gradient pattern – 1ml wash out unbound sample 0 %B; 10ml 0 %B, 10ml 100 %B. Flow rate 1ml/ml. There was a peak washed out during washing of the column with 20 % Ethanol. After activity assay using oracin as a substrate, the results rvealed the same activity in all separation fractions except high activity in flow through fractions.

Considering the results of previous experiments, we decreased the flow rate and increased the GSH concentration.

- Separation conditions 2 – buffer A PBS pH 7.3, buffer B Tris HCl pH 8.0 with 20 mM GSH, flow rate 0.2 ml/min. Gradient pattern the same like separation 1 as well as injection volume. The highest reductive activity was detected in flow through fractions, a slight increase of activity was observed in fraction A11. In comparison to other fractions, this activity was not considered significant.

- Separation conditions 3 – buffer A PBS pH 7.3, buffer B Tris HCl pH 7.4 with 40 mM GSH and 0.1 % Lubrol PX, other conditions the same like separation 2.

Table 16. HPLC experiment results of separation on GSTrap using separation conditions 3.

fraction	incubation [µl]	DHO/ inj [ng]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
B2	50	1.32	3.31	1.00	66.25
B1	50	10.55	26.36	0.25	131.83
C1	50	6.97	17.43	1.00	**348.55**
C2	50	2.16	5.40	1.00	107.95
C3	50	1.01	2.52	1.00	50.35
C4	50	0.90	2.24	1.00	44.90
C6	50	0.90	2.26	1.00	45.20
C7	50	0.96	2.40	1.00	47.95
C8	50	0.90	2.25	1.00	44.95
C9	50	0.91	2.28	1.00	45.70
C10	50	1.05	2.62	1.00	52.40
C11	50	1.14	2.85	1.00	57.05
C12	50	1.98	4.94	1.00	**98.90**
D12	50	2.62	6.54	1.00	**130.80**
D10	50	1.16	2.90	1.00	58.10
D9	50	0.99	2.48	1.00	49.65
D7	50	1.03	2.56	1.00	51.30
D6	50	0.88	2.21	1.00	44.25
D5	50	1.10	2.74	1.00	54.90

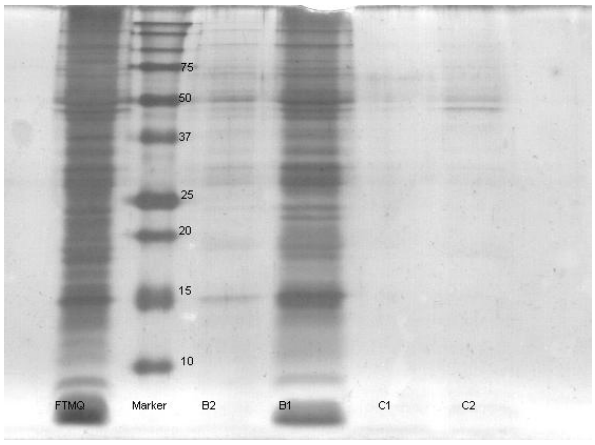


Fig 39. Silver stained SDS-PAGE of GSTrap experiment, from the left: Q sepharose flow through fraction, marker, GSTrap fractions

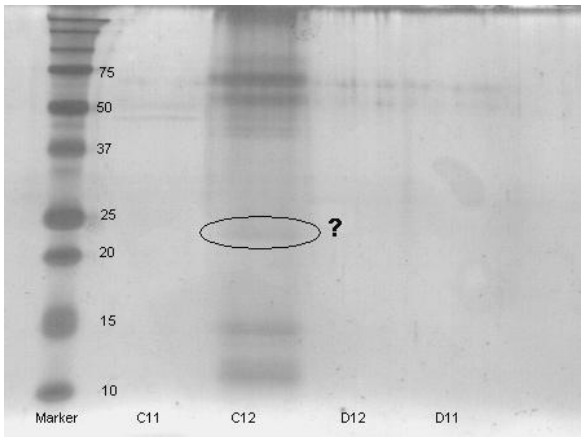


Fig 40. Silver stained gel. Elipsed band was considered as possible band of GST.

- Separation conditions 4 – buffers the same as separation 3 with gradient pattern – 1 ml wash out unbound sample, 8 ml 0 % B, 12 ml 100 % B and 4 ml gradient delay with 100 %B.

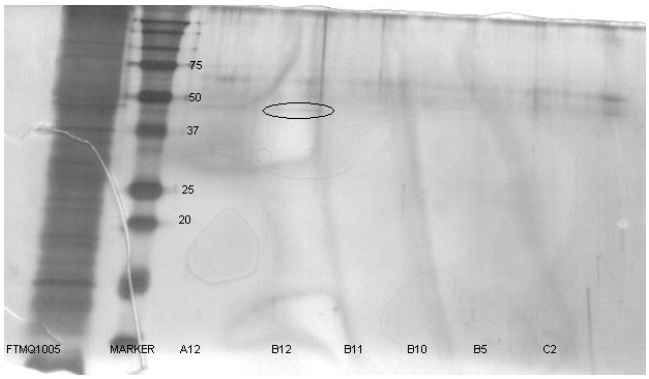


Fig 41. Silver stained gel with the most active fractions, GSTrap experiment, separation conditions 4, from the left: Q sepharose flow through fraction, molecular marker, separation fractions

High activity in fraction C2 was the reason to prolong the elution phase of the experiment.

Table 17. HPLC results of GSTrap experiment using separation conditions 4

fraction	DHO/inj [ng]	incubation [μl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
FTMQ	102.99	50	**257.48**	0.25	**1287.39**
A1	0.39	50	0.97	1.00	19.45
A2	9.78	50	24.45	0.25	122.26
A3	14.57	50	36.43	1.00	728.60
A4	1.24	50	3.09	1.00	61.90
A5	0.26	50	0.64	1.00	12.85
A6	0.18	50	0.44	1.00	8.80
A7	0.11	50	0.28	1.00	5.65
A8	0.10	50	0.26	1.00	5.20
A9	0.04	50	0.11	1.00	2.15
A10	0.16	50	0.39	1.00	7.80
A11	0.19	50	0.48	1.00	9.65
A12	0.14	50	0.35	1.00	7.00
B12	0.36	50	0.89	1.00	17.85
B11	0.44	50	1.09	1.00	21.90
B10	0.12	50	0.31	1.00	6.25
B9	0.19	50	0.46	1.00	9.25
B8	0.21	50	0.52	1.00	10.35
B7	0.11	50	0.26	1.00	5.25
B6	0.52	50	1.30	1.00	26.00
B5	0.97	50	2.43	1.00	48.50
B4	0.22	50	0.543	1.00	10.90
B3	0.13	50	0.32	1.00	6.45
B2	0.28	50	0.69	1.00	13.80
B1	0.10	50	0.26	1.00	5.15
C1	0.15	50	0.37	1.00	7.35
C2	6.03	50	**15.06**	1.00	**301.30**

- Separation conditions 5 – buffers the same, gradient pattern – 1 ml wash out unbound sample, 8 ml 0 % B, 14 ml 100 % B and 6 ml gradient delay with 100 % B.

  Unfortunatelly, the activity in C2 fraction was not confirmed.

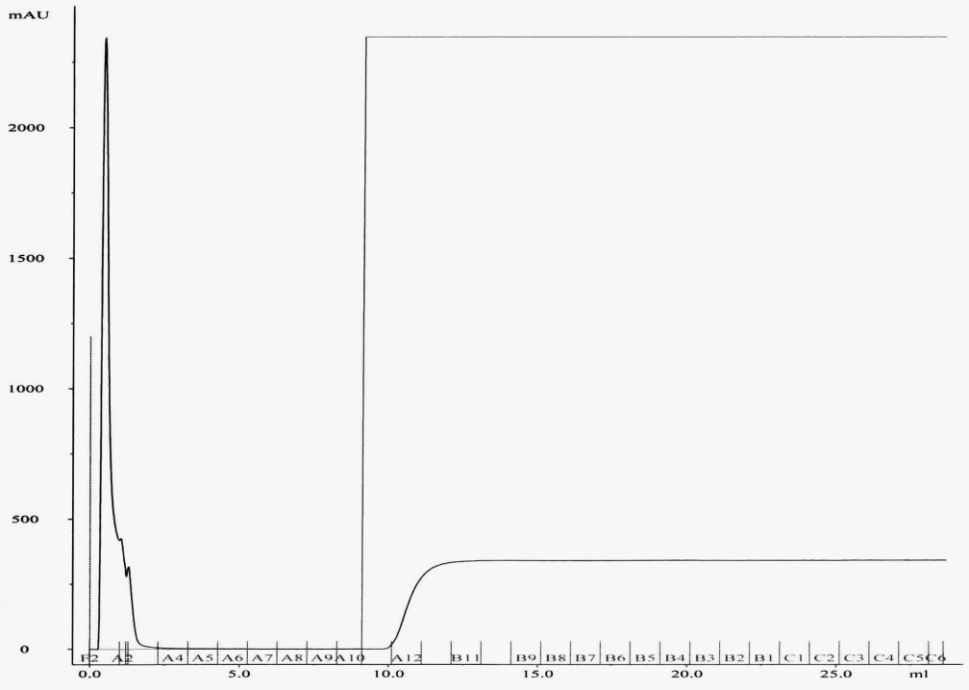


Fig 42. GSTrap experiment chromatogram

We performed spectrophotometric assay of GST activity as well. Activity of GST in active fractions was proved by spectrofotometric determination of its activity with measuring the increase of absorbance in solution containing 1-chloro-2,4-dinitrobenzene, glutathione and particular fraction in phosphate buffer pH 6.5.

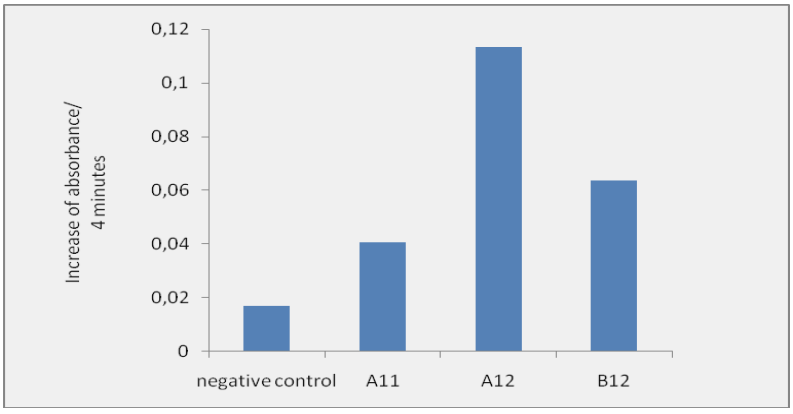


Fig 43. Spectrophotometric determination of Glutathione S-transferase activity – formation of S-2,4-dinitrophenylglutathione in 4 minutes

Western Blott of active fractions and Q sepharose flow through fraction with anti-GST antibody did not reveal any GST band in fractions but did show a significant band in cytosol.

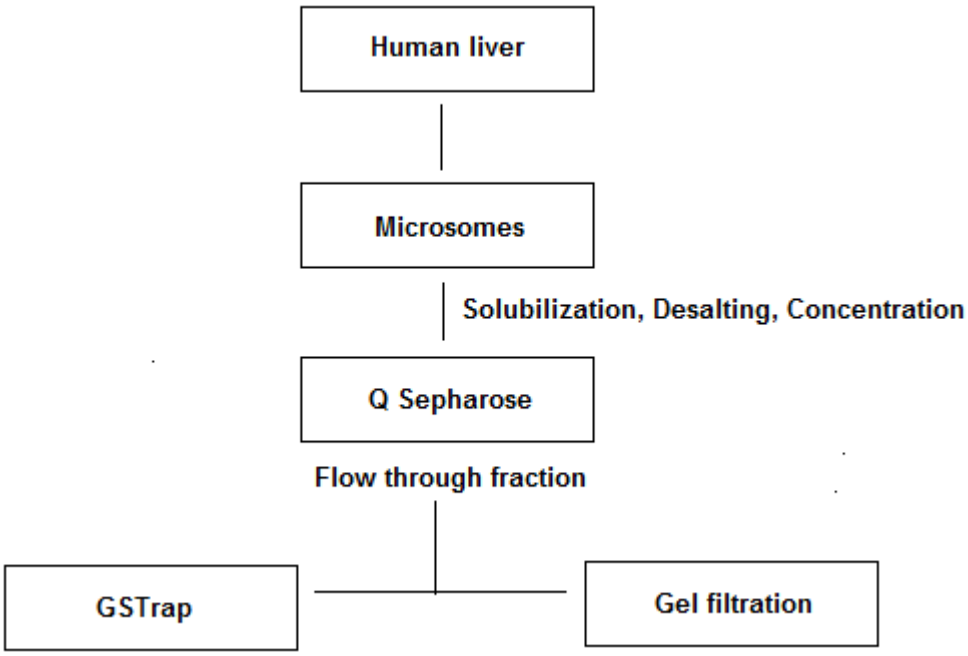


Fig 44. Scheme of purification pattern 1

Unfortunatelly, we did not succeed in proving that the enzyme showing reductive activity in our fractions is really Glutathione S-transferase. MALDI-TOF analysis was quite specific about the type of proteins present but even though we tested several purification conditions we did not manage to achive GST´ s binding to the affinity chromatography column.

The results can be explained by GST´s presence in the Gel filtration fraction which was sent for MALDI-TOF analysis but GST not being the active enzyme in the fraction. As seen on Fig 38, the analyzed band is not the only band present in the fraction. Therefore we can assume that the active enzyme is not in the band tested.

5.2.3.2. Purification pattern 2

In our other experiments, we turned out attention to active fractions of Q sepharose separation which contained enzymes able to bind to column matrix under experiment conditions. Afer concentrating the most active fractions A11-B12 (usually from two Q sepharose separation runs) we injected them onto gel filtration column.

Gel filtration separation conditions: 20 mM Tris HCl, pH 8.0; 10 % glycerol; 0.2 mM mercaptoethanol and 0.1 % Lubrol PX, flow rate 0.5 ml/min.

We achieved the ability to repeat our experiments with expected results. The most active fractions after Gel filtration were A10, A11 and A12.

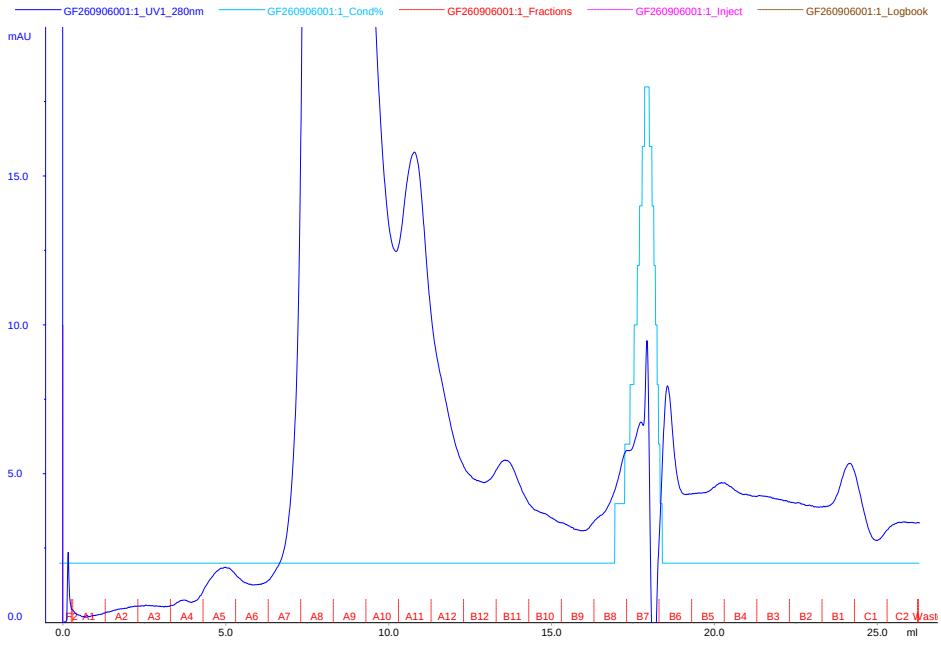


Fig 45. Chromatogram of gel filtration separation of Q sepharose active fractions

Gel filtration SDS-PAGE bands were sent for MALDI-TOF analysis.

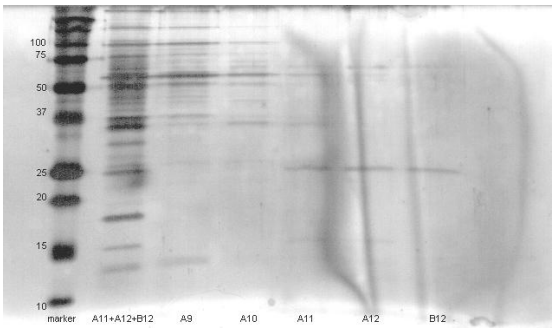


Fig 46. Silver stained SDS-PAGE of GF experiment active fractions, from the left: molecular marker, pooled active Q sepharose fractions, GF fractions

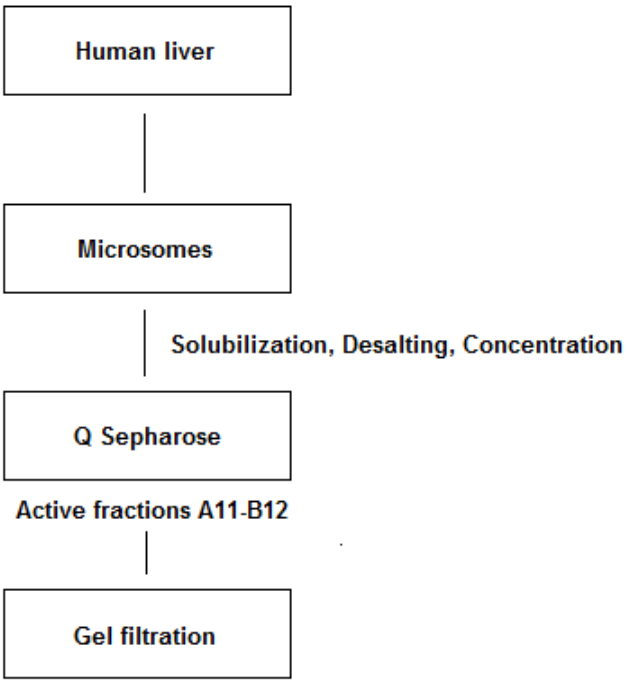


Fig 47. Scheme of purification pattern 2

Table 18. HPLC experiment results of GF fractions, pooled concentrated fractions from two Q sepharose runs (180 µl) were injected in this experiment

fraction	injection [µl]	DHO/ inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
A1	100	0.06	50	0.14	1.0	2.81
A2	100	0.02	50	0.06	1.0	1.21
A3	100	0.04	50	0.11	1.0	2.22
A4	100	0.04	50	0.10	1.0	2.04
A5	100	0.03	50	0.08	1.0	1.61
A6	100	0.06	50	0.16	1.0	3.22
A7	100	0.07	50	0.18	1.0	3.64
A8	100	0.08	50	0.21	1.0	4.23
A9	100	3.91	50	9.77	1.0	195.40
A10	100	6.08	50	15.19	1.0	303.80
A11	100	14.31	50	35.78	1.0	715.61
A12	100	12.28	50	30.71	1.0	614.20
B12	100	3.30	50	8.25	1.0	165.03
B11	100	0.81	50	2.03	1.0	40.62
B10	100	0.31	50	0.77	1.0	15.40
B9	100	0.26	50	0.66	1.0	13.21
B8	100	0.24	50	0.60	1.0	12.02
B7	100	1.18	50	2.95	1.0	59.02
B6	100	1.65	50	4.13	1.0	82.60
B5	100	0.48	50	1.21	1.0	24.24
B4	100	0.30	50	0.75	1.0	15.04
B3	100	0.24	50	0.60	1.0	12.03
B2	100	0.27	50	0.68	1.0	13.62
B1	100	0.25	50	0.63	1.0	12.60
C1	100	0.24	50	0.61	1.0	12.20
C2	100	0.22	50	0.55	1.0	11.01
					Total	2338.64
						44.35%

The MALDI-TOF results showed presence of two types of Glutathione S-transferase and peroxiredoxin 6.

Unfortunatelly, these results did not give us any different leads to follow than the previous experiments performed as part of purification pattern 1.

Gel filtration experiments lead to diluting the sample and losing large percentage of activity. That is the reason for almost no bands showing on

Coomassie blue stained SDS-PAGE of gel filtration fractions. Only Coomasie stained gels are suitable for MALDI-TOF analysis since chemicals used in silver staining unable performing the analysis. Silver staining of gels is more sensitive and shows more present proteins but is not quantitative so can not be used to estimate the content of protein present in each band. This problem leads to inability to analyze other than visible bands on Coomassie gels and can be a reason for the confusing results of the MALDI-TOF analysis.

Table 19. MALDI-TOF analysis results of GF experiment active fractions

database	AC	ID	score	peptide	cvg %	description
ipi.HUMAN	IPI00220301	IPI00220301.4	39,5	5	21%	PEROXIREDOXIN-6
ipi.HUMAN	IPI00009865	IPI00009865.1	27,5	4	7%	KERATIN, TYPE I CYTOSKELETAL 10
ipi.HUMAN	IPI00657682	IPI00657682.1	27,2	4	15%	GLUTATHIONE S-TRANSFERASE A1
ipi.HUMAN	IPI00220327	IPI00220327.2	26,5	3	5%	KERATIN, TYPE II CYTOSKELETAL 1
ipi.HUMAN	IPI00744630	IPI00744630.1	22,8	4	14%	ISOFORM 2 OF PROTEIN-L-ISOASPARTATE (D-ASPARTATE) O-METHYLTRANSFERASE
ipi.HUMAN	IPI00027547	IPI00027547.2	15,8	2	20%	DERMCIDIN PRECURSOR
ipi.HUMAN	IPI00006592	IPI00006592.1	15,4	2	11%	PEPTIDE METHIONINE SULFOXIDE REDUCTASE
ipi.HUMAN	IPI00021304	IPI00021304.1	14,8	2	3%	KERATIN, TYPE II CYTOSKELETAL 2 EPIDERMAL
ipi.HUMAN	IPI00383046	IPI00383046.2	14,6	4	9%	HYPOTHETICAL PROTEIN LOC134147
ipi.HUMAN	IPI00019359	IPI00019359.3	8,7	1	2%	KERATIN, TYPE I CYTOSKELETAL 9
ipi.HUMAN	IPI00419235	IPI00419235.4	7,3	1	7%	GLUTATHIONE S-TRANSFERASE MU 5

5.2.3.3. Purification pattern 3

For the next set experiments we chose Cation-exchange chromatography as a first step.

Cation-exchange chromatography

Purification separation was performed on SP sepharose column Hi Trap SP Fast Flow 1 ml. We tested several separation conditions.

The microsomes were transferred into SP sepharose buffer during desalting step and concentrated.

- Separation conditions 1

We used manufacturer's recommendations for our first experiments with cation-exchange chromatography and used our gradient scheme from anion-exchange chromatography experiment.

Buffer A – 50 mM sodium phosphate buffer pH 7.4; 10 % glycerol; 0.2 mM mercaptoethanol

Buffer B – buffer A with 1 M NaCl

Gradient pattern – 9 ml 0 % B, 12 ml 0-50 % B, 4 ml 50-100 % B, 4 ml 100 % B and 2 ml gradient delay with 100 % B, flow rate 0.5 ml/min

The HPLC results after incubation of the fractions with oracin did not show any substantial activity in the fractions, the higest activity was present in flow through fraction.

We did not repeat this separation or used the obtained fractions for any further experiments. The separation conditions were not suitable for binding of protein of interest on the column matrix.

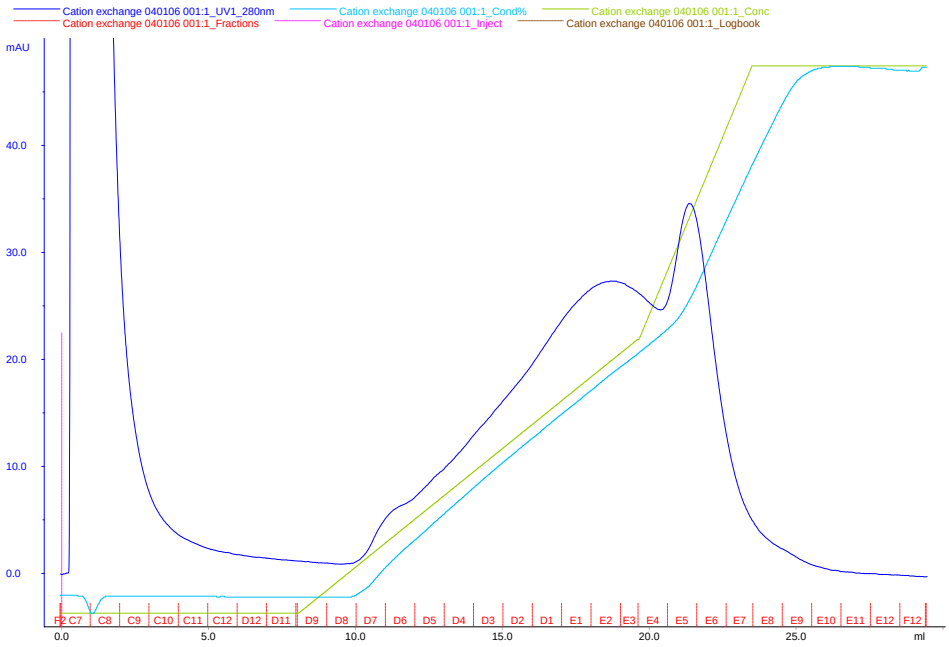


Fig 48. Chromatogram of SP sepharose experiment using separation conditions 1

- Separation conditions 2

We used the same buffers but adjusted the gradient pattern to 8 ml 0 % B, 4 ml 0-20 % B, 3 ml 20-100 % B and 6 ml 100 % B.

Even though the change of gradient pattern resulted into an improved elution, the HPLC results did not show any significant change.

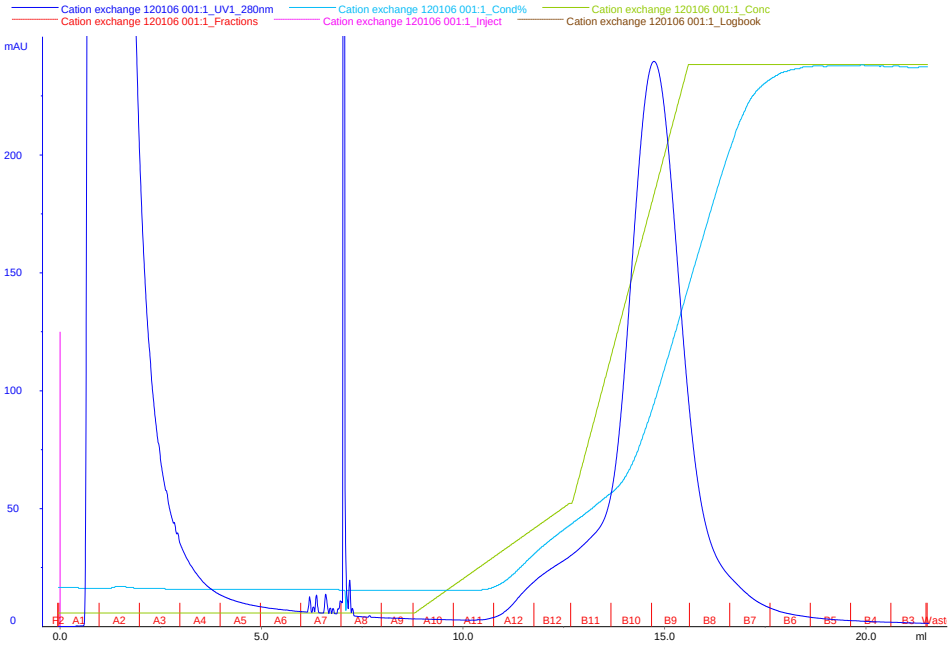


Fig 49. Chromatogram of SP sepharose separation using conditions 2

- Separation conditions 3

We decided to change the buffer system completely but keep the gradient pattern and flow rate.

Buffer A – 50 mM sodium acetate buffer pH 5.0 (adjusted with acetic acid); 10 % glycerol and 0.2 mM mercaptoethanol

Buffer B – buffer 1 with 1 M NaCl

The solubilized microsomes were transferred to buffer A during the desalting step and subsequently concentrated (in this case from 8.75 ml to 1.23 ml). 0.5 ml of these microsomes was injected onto the SP sepharose column.

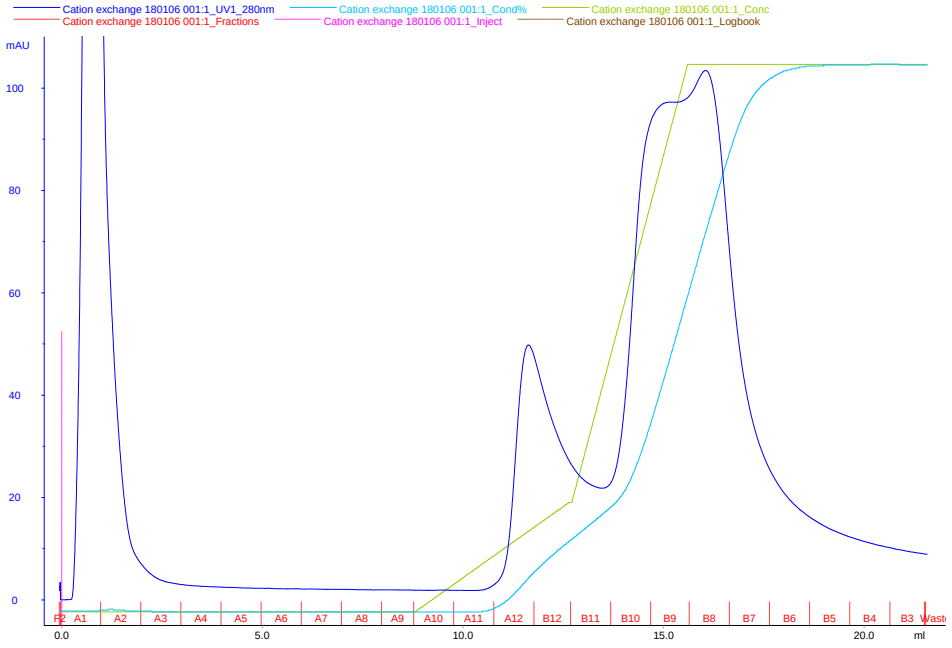


Fig 50. SP sepharose experiment chromatogram using separation conditions 3

As shown in Table 20, there the higest activity was found in fractions B9 and B8.

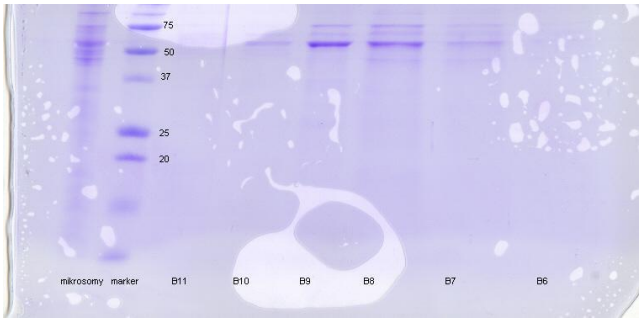


Fig 51. Coomassie Blue stained gel with SP sepharose active fractions (using sepharation conditions 3), from the left: microsomes, molecular marker, SP sepharose separation fractions

Table 20. HPLC results of activity assay using separation conditions 3

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
conc microsomes	30.99	5	77.48	0.5	**7747.75**
A1	0.19	50	0.47	1.0	9.40
A2	13.46	50	33.65	1.0	673.00
A3	4.73	50	11.83	1.0	236.65
A4	0.57	50	1.42	1.0	28.45
A5	0.17	50	0.42	1.0	8.45
A6	0.13	50	0.33	1.0	6.70
A7	0.14	50	0.34	1.0	6.80
A8	0.08	50	0.21	1.0	4.15
A9	0.74	50	1.85	1.0	37.05
A10	0.18	50	0.44	1.0	8.80
A11	0.11	50	0.28	1.0	5.70
A12	0.18	50	0.45	1.0	9.10
B12	0.55	50	1.37	1.0	27.50
B11	0.69	50	1.72	1.0	34.45
B10	3.79	50	9.48	1.0	189.70
B9	24.51	50	61.28	1.0	**1225.55**
B8	27.78	50	69.45	1.0	**1389.10**
B7	6.18	50	15.44	1.0	308.90
B6	0.96	50	2.39	1.0	47.85
B5	0.22	50	0.56	1.0	11.15
B4	0.17	50	0.41	1.0	8.30
B3	0.18	50	0.45	1.0	9.10

Total	4285.85
	55.32%

Since we achieved satisfactory activity restricted to two fractions, these fractions were concentrated (1.88 ml to 0.45 ml) and injected onto gel filtration column.

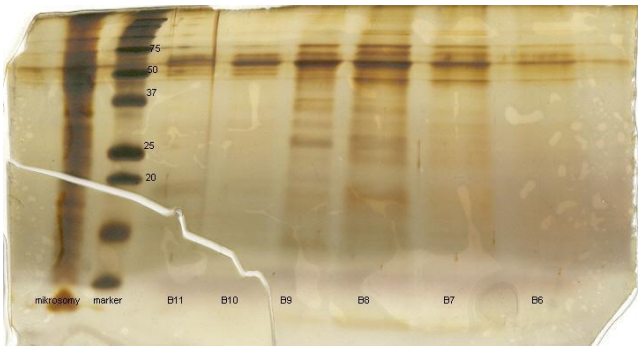


Fig 52. Silver stained SDS-PAGE of SP sepharose active fractions (using sepharation conditions 3), from the left: microsomes, molecular marker, SP sepharose separation fractions

Gel Filtration

The experiment conditions were the same as in our previous GF separations. 0.25 ml concentrated active SP sepharose fractions was injected.

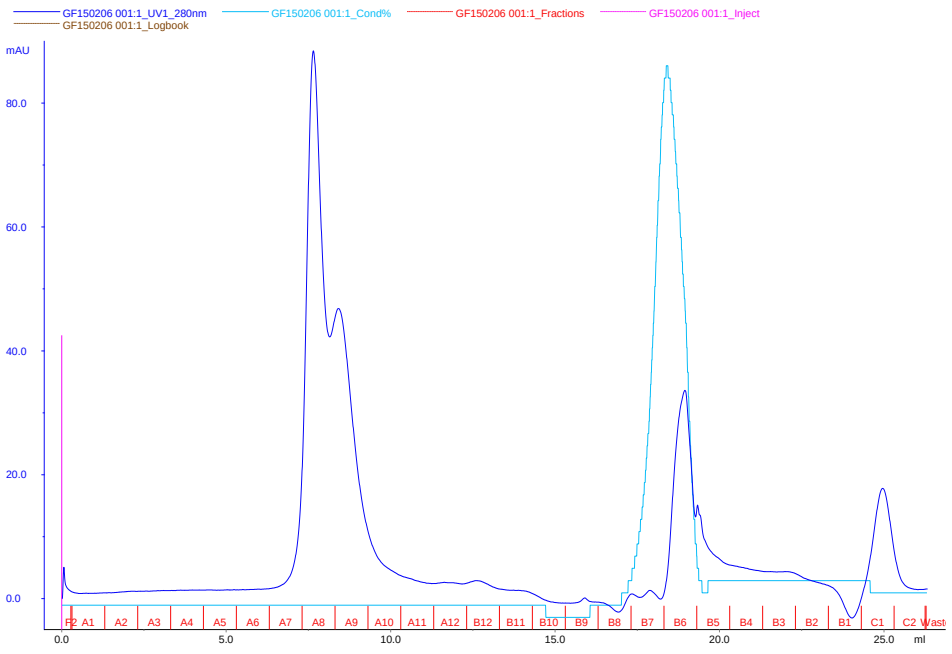


Fig 53. Chromatogram of gel filtration experiment as a second step after SP sepharose separation (separation conditions 3)

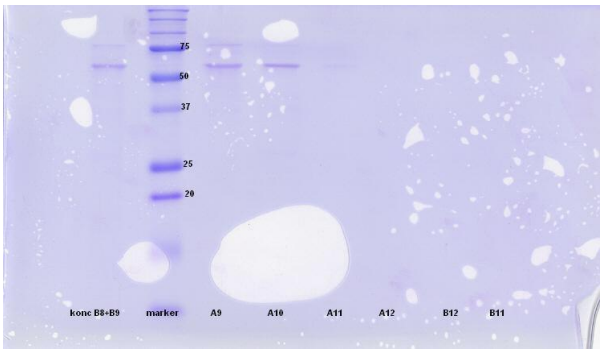


Fig 54. Coomassie blue stained SDS-PAGE with active fractions of GF experiment

Table 21. Reductive activity determination via use of HPLC after incubation of GF fractions with oracin

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
conc B8+B9 SP	4.820	5	12.0500	0.25	**602.50**
A1	0.151	50	0.3775	1.00	7.55
A2	0.101	50	0.2525	1.00	5.05
A3	0.169	50	0.4225	1.00	8.45
A4	0.106	50	0.2650	1.00	5.30
A5	0.113	50	0.2825	1.00	5.65
A6	0.133	50	0.3325	1.00	6.65
A7	0.133	50	0.3325	1.00	6.65
A8	0.172	50	0.4300	1.00	8.60
A9	0.260	50	0.6500	1.00	13.00
A10	2.470	50	6.1750	1.00	**123.50**
A11	3.265	50	8.1625	1.00	**163.25**
A12	1.657	50	4.1425	1.00	**82.85**
B12	0.832	50	2.0800	1.00	41.60
B11	0.628	50	1.5700	1.00	31.40
B10	0.551	50	1.3775	1.00	27.55
B9	0.293	50	0.7325	1.00	14.65
B8	0.284	50	0.7100	1.00	14.20
B7	0.290	50	0.7250	1.00	14.50
B6	0.234	50	0.5850	1.00	11.70
B5	0.168	50	0.4200	1.00	8.40
B4	0.103	50	0.2575	1.00	5.15
B3	0.141	50	0.3525	1.00	7.05
B2	0.244	50	0.6100	1.00	12.20
B1	0.174	50	0.4350	1.00	8.70
C1	0.146	50	0.3650	1.00	7.30
C2	0.357	50	0.8925	1.00	17.85

The activity of active SP sepharose fractions decreased drastically probably due to quite low pH of buffer B they were stored in since the experiment till the GF separation.

We decided to perform an experiment to investigate this decrease by adjusting the fraction pH after SP sepharose separation to pH 7.5 with 240 µl 8 % $NaHCO_3$ solution. The experiment revealed surprising data, the results with pH adjusted to 7.5 were much worse than with the original buffer pH.

Table 22. Comparison of HPLC analysis results of SP sepharose fractions reductive activity in the original buffer A pH 5.0 and buffer adjusted with 8 % $NaHCO_3$ pH 7.5

fraction	total DHO [ng] original buffer	total DHO [ng] adjusted pH
A1	5.45	0.23
A2	9.30	6.10
A3	6.70	3.08
A4	6.95	4.93
A5	4.60	2.02
A6	6.75	1.74
A7	4.00	5.88
A8	5.60	0.00
A9	8.35	0.73
A10	4.30	1.51
A11	4.20	2.58
A12	13.05	2.63
B12	8.65	1.18
B11	7.05	8.62
B10	13.75	2.74
B9	234.70	23.07
B8	233.65	48.61
B7	62.40	26.04
B6	20.60	7.22
B5	14.65	3.81
B4	4.35	2.07
B3	9.20	3.98

- Separation conditions 4

Because of the results of previous experiment with pH adjustment being unsatisfactory, we tried to change the pH of the buffers A and B used during the desalting and SP sepharose separation.

Gradient pattern and flow rate were kept the same.

Buffer A – 50 mM sodium acetate buffer pH 6.0, 10 % glycerol and 0.2 mM mercaptoethanol

Buffer B – buffer A with 1 M NaCl

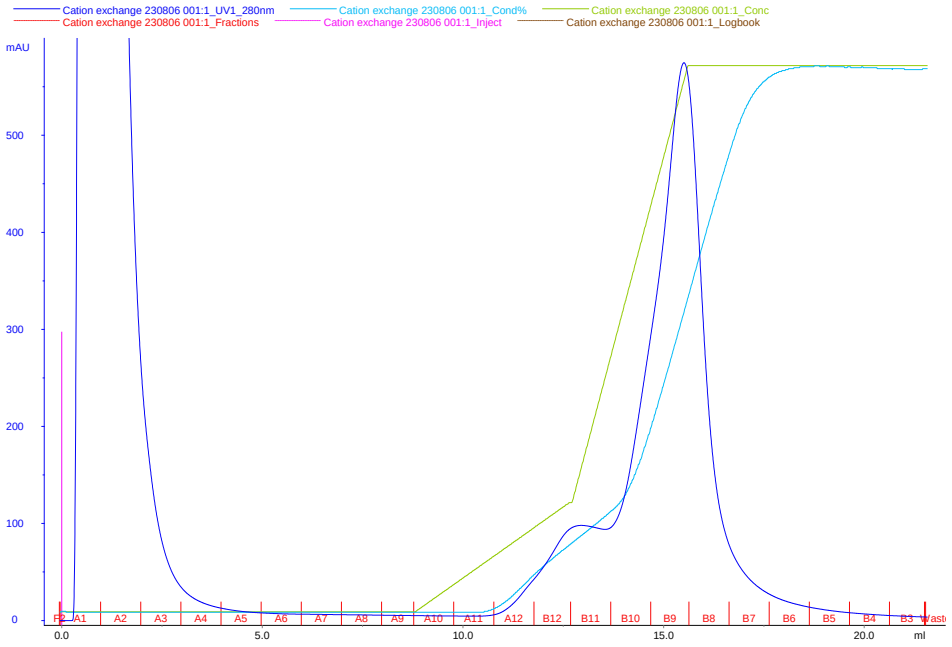


Fig 55. SP sepharose separation chromatogram (using separation conditions 4)

1 ml of concentrated (45.4 ml to 6.9 ml) desalted microsomes was injected in this experiment.

Table 23. Reductive activity assay of SP sepharose fractions

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
conc microsomes	20.94	5	52.36	1,0	10472.50
A1	0.47	50	1.17	1,0	23.45
A2	45.40	50	113.49	1,0	2269.85
A3	15.28	50	38.20	1,0	764.10
A4	2.77	50	6.93	1,0	138.60
A5	0.61	50	1.54	1,0	30.75
A6	0.32	50	0.80	1,0	16.05
A7	0.16	50	0.41	1,0	8.20
A8	0.10	50	0.25	1,0	5.05
A9	0.14	50	0.34	1,0	6.90
A10	0.14	50	0.35	1,0	7.00
A11	0.08	50	0.19	1,0	3.75
A12	0.18	50	0.44	1,0	8.80
B12	8.56	50	21.41	1,0	**428.15**
B11	35.43	50	88.59	1,0	**1771.75**
B10	30.89	50	77.23	1,0	**1544.60**
B9	23.93	50	59.83	1,0	**1196.55**
B8	16.05	50	40.12	1,0	**802.40**
B7	2.94	50	7.36	1,0	**147.25**
B6	0.78	50	1.94	1,0	38.85
B5	0.27	50	0.67	1,0	13.40
B4	0.07	50	0.18	1,0	3.65
B3	0.09	50	0.23	1,0	4.65

Total	9233.75
	88.17%

We performed seven runs of SP sepharose separations to obtain a larger volume of active fractions. All these runs showed the same pattern of activity in individual fractions and the same chromatogram.

The total volume of 21 ml (three most active fractions from each run) was concentrated to 4.1 ml.

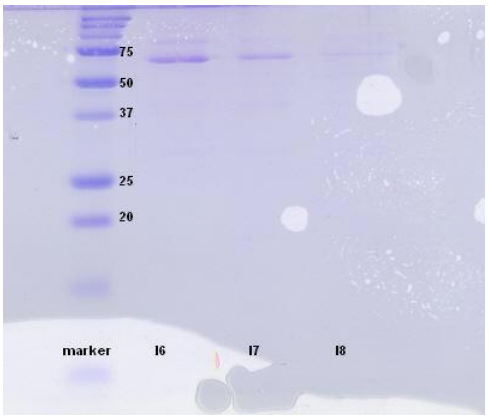


Fig 56. Coomassie blue stained SDS-PAGEof active fractions of SP sepharose separation (conditions 4), I6 corresponds to B11, I7 to B10 and I8 to B9

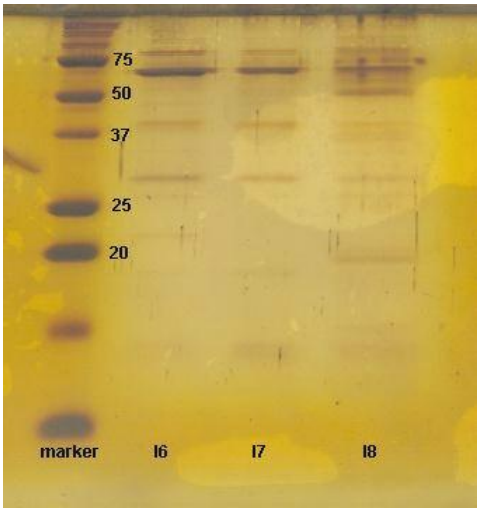


Fig 57. Silver stained SDS-PAGE of SP sepharose active fractions

The SDS-PAGE revealed several interesting bands. We concentrated our attention to bands of approx size of 27 kDa and 38 kDa.

Gel filtration 1

0.5 ml of concentrated SP sepharose active fractions was injected onto the gel filtration column using the same conditions as during previous GF experiments.

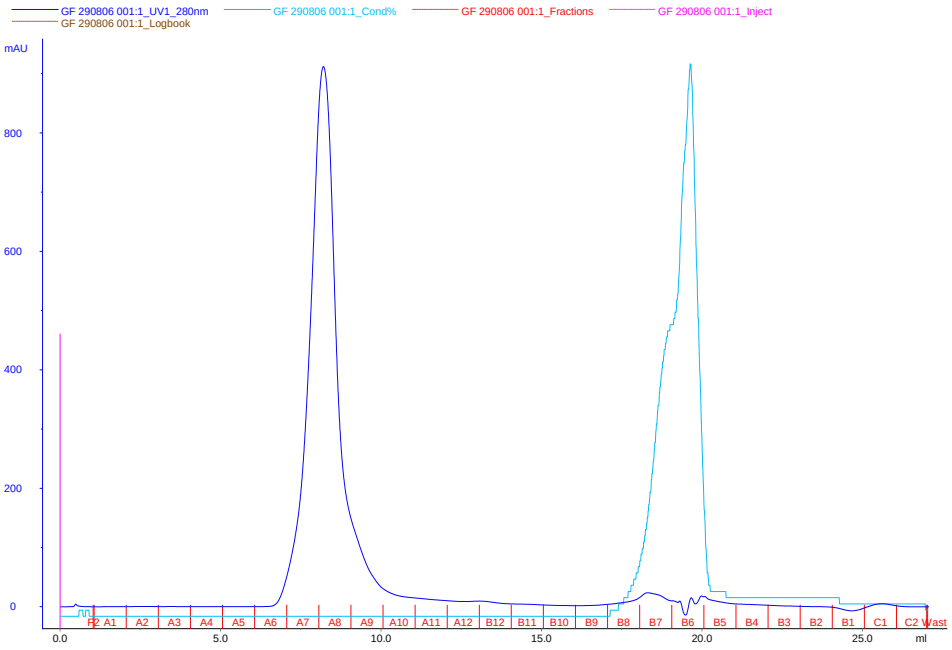


Fig 58. Gel filtration experiment chromatogram, the second step of purification after SP sepharose (using separation conditions 4)

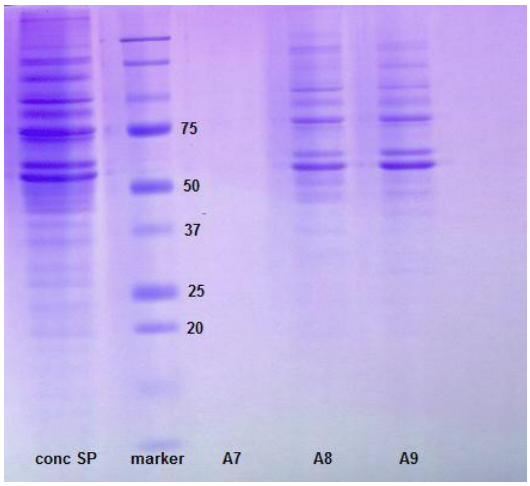


Fig 59. Coomassie blue stained gel of fractions A7 – A9 of GF separation, from the left: concentrated pooled active SP sepharose fractions, molecular marker, GF active fractions

Table 24. Gel filtration as a second purification step HPLC activity assay

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
SP conc	28.57	5	71.43	0.5	7143.25
A1	0.01	50	0.02	1.0	0.40
A2	0.00	50	0.00	1.0	0.00
A3	0.00	50	0.00	1.0	0.00
A4	0.00	50	0.00	1.0	0.00
A5	0.00	50	0.00	1.0	0.00
A6	0.00	50	0.00	1.0	0.00
A7	0.16	50	0.40	1.0	8.10
A8	10.51	50	26.27	1.0	525.35
A9	12.89	50	32.21	1.0	644.30
A10	24.53	50	61.32	1.0	**1226.45**
A11	29.22	50	73.06	1.0	**1461.15**
A12	10.51	50	26.29	1.0	525.75
B12	2.76	50	6.89	1.0	137.80
B11	1.65	50	4.13	1.0	82.70
B10	1.12	50	2.80	1.0	56.05
B9	0.95	50	2.38	1.0	47.65
B8	4.28	50	10.71	1.0	214.15
B7	5.16	50	12.91	1.0	258.15
B6	0.85	50	2.13	1.0	42.55
B5	0.07	50	0.18	1.0	3.70
B4	0.00	50	0.00	1.0	0.00
B3	0.00	50	0.00	1.0	0.00
B2	0.00	50	0.00	1.0	0.00
B1	0.00	50	0.00	1.0	0.00
C1	0.00	50	0.00	1.0	0.00
C2	0.00	50	0.00	1.0	0.00
				Total	5234.25
					73.28%

The most active fractions showed to be A10 and A11. They were loaded onto SDS-PAGE together with other surrounding fractions. This revealed a band of approximate molecular weight 27 kDa which was obvious even after the gel was stained only with Coomassie blue which is less sensitive than silver staining.

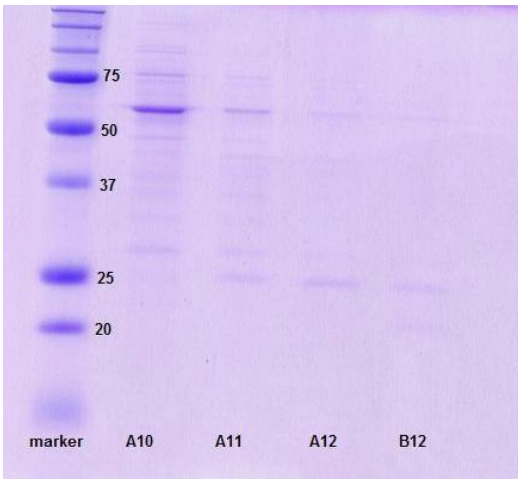


Fig 60. Coomassie blue stained gel with most active fractions A10 and A11 of Gel filtration experiment

The active fractions from two separate Gel filtration experiments were pooled and concentrated (nine fold).

Gel filtration 2

The second Gel filtration was added to achieve furher purification of the sample. We used the same conditions and column as in the first gel filtration step.

Five most active fractions from two runs GF 1 were pooled and concentrated (9.25 ml to 1.0 ml). 0.5 ml was injected.

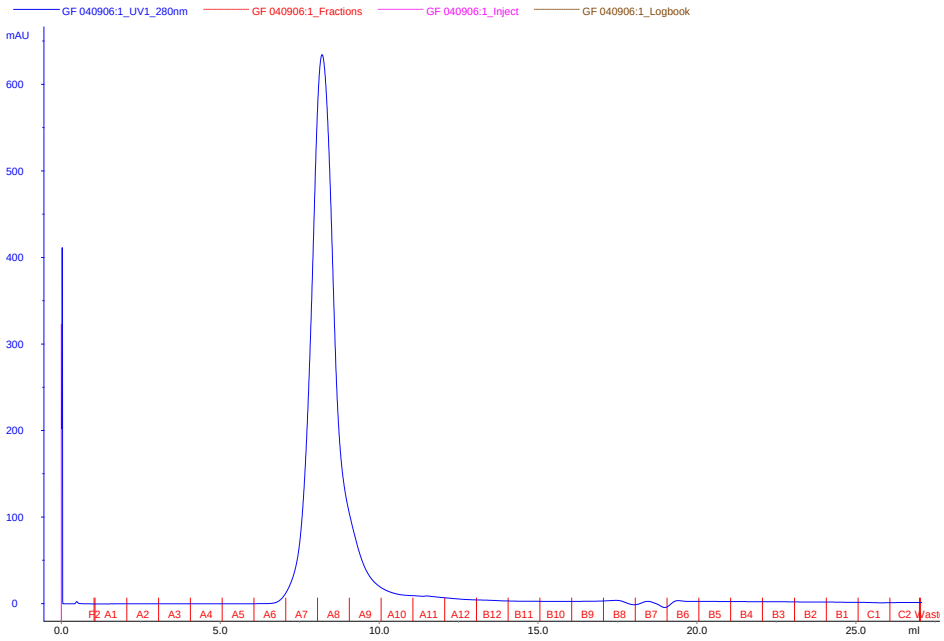


Fig 61. Chromatogram of Gel filtration as a third purification step after anion-exchange and size exclusion chromatography

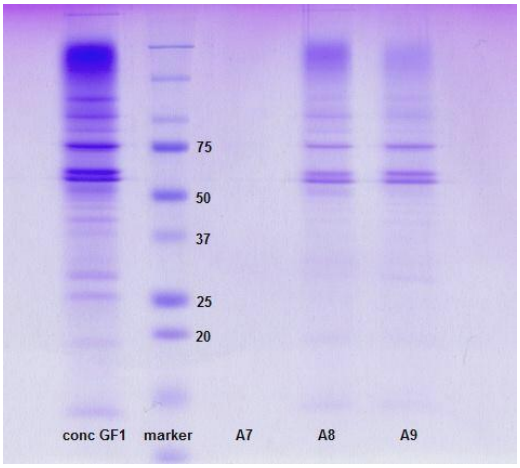


Fig 62. Coomassie blue stained SDS-PAGE of fractions A7-A9 from the second GF experiment, from the left: concentrated active GF1 fractions, marker, GF2 active fractions

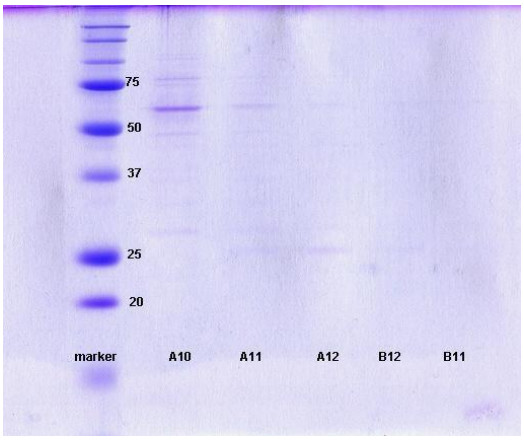


Fig 63. Coomassie blue stained SDS-PAGE of the most active fractions A10 and A11, Gel filtration 2 separation experiment

Adding the second gel filtration experiment did not show to be the best decision because the sample was diluted more and separation on the same column gave us the same results as the first gel filtration step without further purification of the active protein.

The results in Table 25 reveal that the distribution of activity throughout the fractions of GF2 is the same as in GF1 with slight decrease in activity. The reductive activity of fraction A11 is lower than A10 in this experiment which doesn't correspond with the first GF experiment. That might suggest that there are two active proteins present in these fractions and they differ in stability.

To separate these enzymes more, trying a Gel filtration column with different properties could be benefitial. The enzymes are apparently of similar molecular weight and the used column, Superdex 75 10/300, would not be able to separate them any further.

Table 25. Activity assay results of incubation with the second GF separation fractions

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
conc GF1	14.18	5	35.48	0.5	**3545.75**
A1	0.07	50	0.17	1.0	3.35
A2	0.12	50	0.31	1.0	6.15
A3	0.13	50	0.33	1.0	6.70
A4	0.07	50	0.17	1.0	3.35
A5	0.10	50	0.25	1.0	5.00
A6	0.09	50	0.22	1.0	4.45
A7	0.15	50	0.39	1.0	7.75
A8	3.04	50	7.60	1.0	152.05
A9	4.98	50	12.44	1.0	248.80
A10	20.51	50	51.28	1.0	**1025.60**
A11	17.64	50	44.09	1.0	**881.85**
A12	3.17	50	7.93	1.0	158.60
B12	1.19	50	2.98	1.0	59.60
B11	0.61	50	1.54	1.0	30.75
B10	0.39	50	0.98	1.0	19.65
B9	0.45	50	1.12	1.0	22.50
B8	0.49	50	1.22	1.0	24.40
B7	0.40	50	1.01	1.0	20.25
B6	0.49	50	1.23	1.0	24.65
B5	0.40	50	1.01	1.0	20.20
B4	0.42	50	1.04	1.0	20.85
B3	0.38	50	0.96	1.0	19.20
B2	0.40	50	0.10	1.0	19.95
B1	0.41	50	1.03	1.0	20.55
C1	0.38	50	0.94	1.0	18.90
C2	0.32	50	0.81	1.0	16.10

Total	2841.20
	80.13%

Table 26. Protein assay and specific activity results of purification pattern 3

fraction	protein [ug/ml]	DHO ng/ml	DHO nmol/ml	specific activity nmol/mg of protein
solubilized microsomes	1605.71	10472.50	31.17	19.41
SP sepharose	103.21	1504.30	4.48	43.38
Gel filtration 1	55.50	1343.80	4.00	72.06
Gel filtration 2	37.50	953.73	2.84	75.70

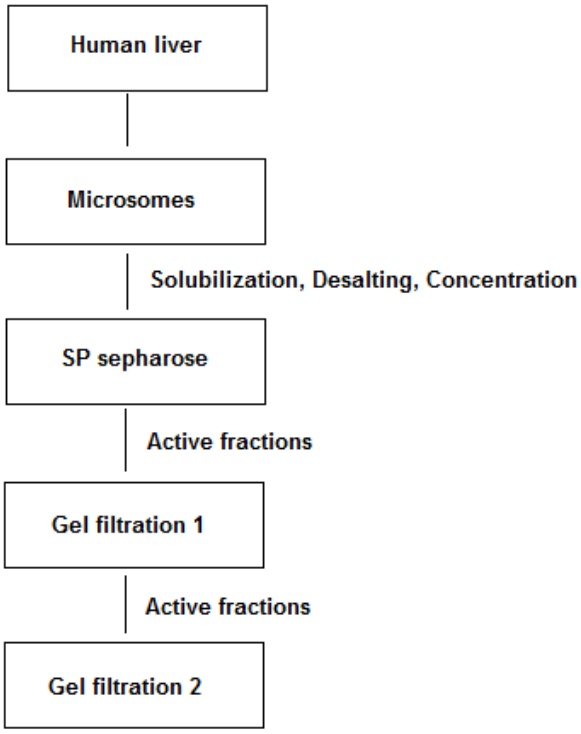


Fig 64. A new reductase purification scheme with anion-exchange as the first purification step

All methods used in purification steps mentioned above were successfully introduced and extablished at the Department of Biochemical Sciences. We achieved almost 100% ability to repeat the experiments with the same expected results.

5.2.3.4. Separation according to proteins pI

To separate proteins of similar moleacular weight, we needed to introduce a method which uses different technique.

Isoelectric point pI is the pH at which a particular molecule carries no net electrical charge or the positive and negative charges are equal.

Since proteins are amphoteric molecules and their charge depends on the functional groups present in their molecule and the pH of the surrounding

environment, purification techniques based on proteins' pI are very useful for separation of proteins similar in other ways.

Microrotofor isoelectric focusing cell

Microrotofor is an isoelectric focusing device which separates proteins in 2.5 ml samples. This enables avoiding dilution of sample.

The microrotofor cell consists of 10 compartments which are separated by nine parallel membranes made of monofilament polyester. The cell is placed in an integrated cooling block during the whole experiment to avoid protein denaturation.

Oscillation of the focusing chamber withing the cooling block around the focusing axes stabilizes the sample against disturbances and enables equal distribution of proteins in the analyzed sample.

After focusing experiment the fractions are collected by vacuum connected harvesting station to avoid mixing the compartments' content.

The manufacturer supplies an ampholyte solution which creates a pH gradient in the focusing cell. The amount of ampholyte depends on the protein content in the sample analyzed.

We performed the experiment according to manufacturers' instructions using 0.1 M H_3PO_4 and 0.1 M NaOH as electrode solutions for anode and cathode and 200 µl of ampholyte solution.

In Microrotofor experiments the manipulation with the focusing cell is very crucial. The holes in the cell compartments are sealed with sealing tape. Since the whole cell is oscillating during the duration of the experiment, it is very necessary to avoid its pealing and that way losing part of the sample. Also, the compartments must not contain any air bubbles because they would disrupt the

pH gradient formation. Since majority of purification buffers contain a detergent, it is quite difficult to achive.

In our first experiment we lost complete fraction 4 due to a tear in the sealing tape.

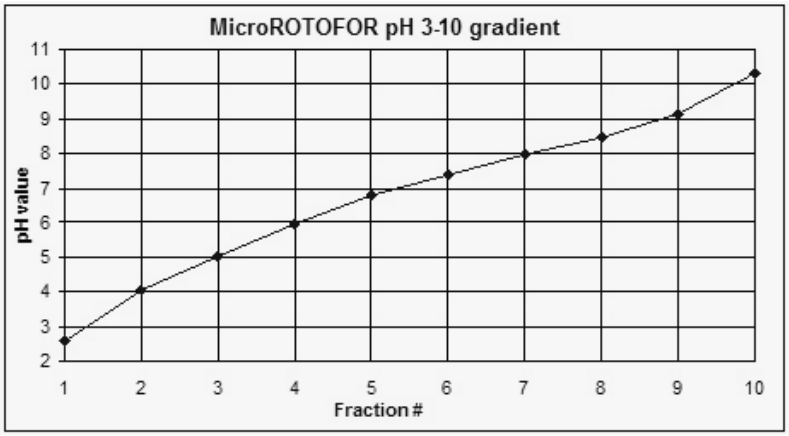


Fig 65. The microrotofor run pH profile

We repeated the experiment with 20 µl of ampholyte solution; the analysis took about 3 hours to complete at 1 W constant power

Table 27. Activity assay of the second isoelectric focusing experiment

fraction	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
pooled GF	17.94	3.0	1076.40
1	0.64	0.3	3.86
2	0.46	0.3	2.77
3	1.32	0.3	7.90
4	49.14	0.3	**294.84**
5	26.30	0.3	**157.80**
6	24.74	0.3	**148.44**
7	0.81	0.3	4.87
8	0.26	0.3	1.58
9	0.25	0.3	1.50
10	0.32	0.3	1.93

total	625.49
	58.11%

This analysis showed the highest activity in fraction 4 which corresponds to pI 5.2-5.8.

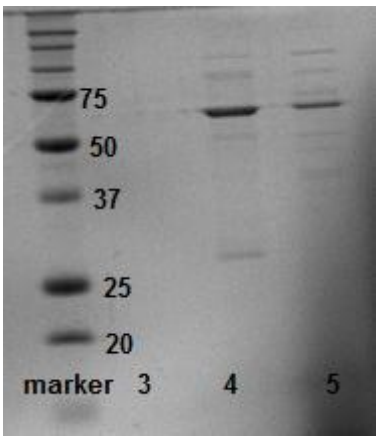


Fig 66. Coomassie blue stained gel of two out of three active fractions, molecular marker on the left side

There is a surprisingly well visible band in fractions 4 of approximate size 27 kDa which is not present in fraction 5 though.

To identify the active band and avoid sending a wrong band for the MALDI-TOF analysis, we introduced a new method, native electrophoresis.

This method doesn't use SDS and that's why proteins travel through the gel different way. The native state of proteins enabled us to test the activity directly in the gel. The native electrophoresis apparatus had to be extensively cooled to preserve as much enzymatic activity as possible.

At first we faced several problems, expecially with the solutions used for incubation.

- Solution 1 – 1.5 mM NADPH with 2.3 mM oracin - oracin precipitated
- Solution 2 – 1.5 mM NADPH with 1.5 mM oracin – precipitation
- Solution 3 – 1.5 mM NADPH with 1.0 mM oracin – precipitation

- Solution 4 – 1.5 mM NADPH with 0.5 mM oracin – did not precipitate

The final experiment conditions were set to 25 ml of solution 4 (in phosphate buffer pH 7.5) and incubation time 1 hour.

The active band showed as a transparent stain on a purple backround (gels are stained overnight in a solution of meldola blue with p-iodonitrotetrazolium which color NADPH).

All gels were prepared twice; one was stained with Coomassie blue, the other with Meldola blue.

After quite succesful native electrophoresis experiment, we decided to perform microrotofor separation with SP sepharose actve fractions. The active band was sent for MALDI-TOF analysis.

The protein present in the band with the highest probability was identified as carboxylesterase 1. This was very surprising and needed further confirmation. That’s why we tested a recombinant carboxylesterase 1 for a reductive activity by incubation it with oracin.

We prepared 3 lots of samples

- Blank samples without enzyme
- Samples with 1 µl of enzyme
- Samples with 3 µl of enzyme

Unfortunatelly, the MALDI-TOF results were not confirmed. There was no activity found in samples with enzyme.

The enzyme, carnboxylesterase 1, has never ben reported to have any reductive properties. MALDI-TOF analysis results are therefore not expected and hard to explain.

The possible explanations of the inability to confirm the results could be either the fact that we used a recombinant enzyme for our activity assay (could have slightly different properties than the native enzyme), enzyme was ordered from Hong Kong and could have lost its activity on the way or simply the active enzyme in the tested band was not found and correctly identified.

Chromatofocusing

This separation technique is performed with use of Äkta purifier. The column we used in our experiment was Mono P 5/50. Chromatofocusing is a special type of ion-exchange chromatography which separates proteins accrording to their pI.

The pH gradient is formed inside the column matrix (special media substituted with charged, buffering amines) by use of specifically designed buffers supplied by the manufacturer.

We only performed this experiment several times; the separation conditions buffers were used according to manufacturers' recommendations.

Buffer A – 0.025 M BisTris pH 7.1 (adjusted with iminodiacetic acid)

Buffer B – Polybuffer 74 (10ml in 100ml) pH 4.0 (adjusted with iminodiactetic acid)

The electrodes needed to be calibrated before the experiment to pH 4.0 and 9.0 and column washed by 20 % ethanol, purified water and stabilized with buffer A after injecting 1 ml 5 M NaOH.

The electrodes needed to be calibrated three times and even so they still showed lower pH than pH of the buffers. The test run with albumin pI 5.82 and ovalbumin pI 4.6 did not give us results satisfactory enough for us to continue working with this chromatographic technique.

5.2.3.5. Blue sepharose

Blue sepharose is a type of affinity media. The dye ligand, Cibacron Blue F3G-A, is covalently attached to the Sepharose High Performance matrix via the triazine part of the dye molekule and shows certain structural similarities to NAD^+ and $NADP^+$. It is mainly used for albumin removal from various biological samples but in our case it can be used because our enzymes of interest are NADPH dependent.

- Separation conditions 1

The initial separation conditions we used are described in the column manual, flow rate 0.5 ml/min.

Buffer A – 50 mM KH_2PO_4 pH 7.0

Buffer B – buffer A with 1.5 M KCl

Gradient pattern – 11ml 0 % B

8 ml 100 % B

2 ml gradient delay 100 % B

Here we injected 0.75 ml of pooled most active fractions from GF2 experiment (A10 and A11).

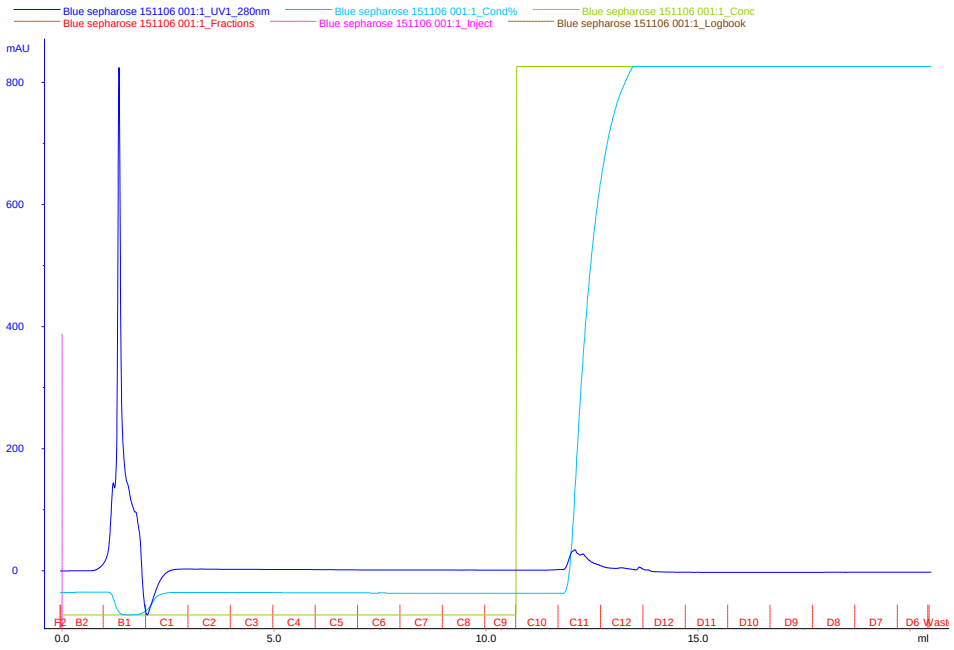


Fig 67. Chromatogram of Blue sepharose experiment using separation conditions 1

The decrease of activity to 22.10 % in total and no significant activity in separation fractions was found. The buffer B caused precipitation of buffers used for SDS-PAGE.

In the second experiment with the same separation conditions, we injected 1 ml pooled flow through A1+A2 fractions from Q sepharose step.

The yield of 27.81 % and amount of DHO the fractions were able to produce seemed to be quite discouraging.

As mentioned above, the buffer B precipitated the electrophoresis buffers. That's why we do not have any SDS-PAGE results. Considering the activity in individual fractions, performing SDS-PAGE would probably be wasting of time since the activities were really low and the active bands would not be visible on Coomassie blue stained gels.

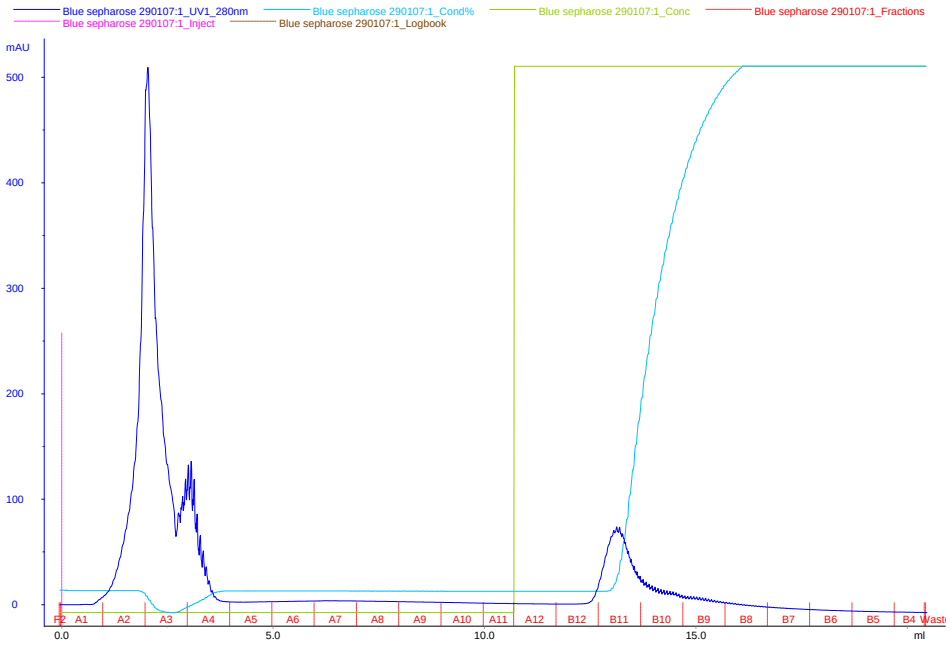


Fig 68. Blue sepharose experiment chromatogram, Q sepharose flow through fraction was injected

- Separation conditions 2

Buffer A – sodium phosphate buffer pH 6.0

Buffer B – buffer A with 2 M NaCl

The other conditions were kept the same. We injected 0.5 ml of pool active fractions of GF2.

There was a peak washed out during washing the column after the experiment was finished.

Table 28. Activity assay of Blue sepharose separation fractions using separation conditions 2

fraction	DHO/inj [ng]	incubation [µl]	DHO/ incubation [ng]	total volume [ml]	total DHO [ng]
GF A10+A11	8.32	50	20.80	0.5	**208.05**
A1	0.06	50	0.16	1.0	3.15
A2	0.16	50	0.40	1.0	8.10
A3	0.58	50	1.44	1.0	**28.90**
A4	0.20	50	0.50	1.0	10.00
A5	0.18	50	0.45	1.0	8.95
A6	0.17	50	0.43	1.0	8.55
A7	0.22	50	0.54	1.0	10.90
A8	0.18	50	0.45	1.0	9.05
A9	0.16	50	0.40	1.0	8.00
A10	0.16	50	0.40	1.0	7.95
A11	0.17	50	0.41	1.0	8.30
A12	0.16	50	0.40	1.0	8.05
B12	0.16	50	0.40	1.0	8.10
B11	0.14	50	0.36	1.0	7.25
B10	0.31	50	0.78	1.0	**15.55**
B9	0.15	50	0.39	1.0	7.75
B8	0.11	50	0.27	1.0	5.35
B7	0.10	50	0.25	1.0	5.05
B6	0.06	50	0.16	1.0	3.15
B5	0.06	50	0.15	1.0	3.00
B4	0.12	50	0.30	1.0	6.05

total	181.15
	87.07%

Further Blue sepharose experiments were performed by Adam Skarka, at the time fifth year student, at this moment a PhD student at the Deparment of Biochemical Sciences.

5.2.3.6. Protein assays

We faced various problems trying to determine the protein content in the samples.

Protein purification buffers often contain 2-mercaptoethanol and/or detergents. That can cause problems with protein assays. We had to use two different types of protein assay methods for our experiments.

BCA protein assay

The reagent used for this assay contains copper which interacts with mercaptoethanol in purification buffers and that compromises the spectrophotometric results. The method is though compatible with detergents.

Using method of Smith et al. (1995) as a starting point, we have solved the problem. Adding iodoacetamide into the reaction mixture resulted into elimination of the interaction.

The reaction conditions needed to be optimized.

- 10 µl of 20 mM iodoacetamide in purified water incubated
    1. with 25 µl of sample
    2. with 10 µl of sample
    3. with 5 µl of sample
- 10 µl of 20 mM iodoacetamide in Tris HCl buffer pH 8.0 incubated
    1. with 10 µl of sample
    2. with 25 µl of sample
- 10 µl of 2 mM iodoacetamide in Tris HCl buffer pH 8.0 incubated
    1. with 10 µl of sample
    2. with 25 µl of sample

All samples were preincubated with iodoacetamide for 15 minutes and reaction was started by adding the BCA reagent. Results were read after 30 minutes incubation at 562 nm.

The reaction conditions were tested with samples in Q sepharose buffer and gel filtration buffer.

According to the results, the reaction mixture containing 10 µl of 2 mM iodoacetamide and 25 µl of sample showed to be the only possibility working for samples in GF buffer.

Unfortunately, none of the tested conditions worked for Q sepharose buffer.

Bradford protein assay

Bradford assay showed to be the only option to determine protein content in other than GF buffer. Since the buffers do not contain detergent, this assay can be used. This was confirmed by a test.

5.2.3.7. Protein purification summary

Purification of enzymes has always been complicated by their fragile native state and easy loss of activity. A slight change of purification conditions can make a big difference, not only in the purification results but also in the activity of protein of interest.

We tested a large number of purification methods, most of them based on low pressure liquid chromatography, and also a lot of separation conditions. We not only introduced these methods at the Department of Biochemical Sciences but also achieved reproducibility of the results.

Several years of hard work of the whole team led to publication of our results in Toxicology in 2009.

6. Conclusions

Human body gets in contact with many types of xenobiotics and these are metabolized by various pathways. Enzymes which take part in such reactions can influence the effect of xenobiotics which can also act as enzyme inhibitors or inductors.

Anticancer drugs are such xenobiotics. We turned our attention to the reduction of carbonyl group present in molecules of doxorubicin, anticancer drug used in practice, and oracin, potential anticancer agent for peroral administration.

Some of the carbonyl reductases are known and partially characterized, some still avait their discovery. We focused on one knows cytosolic enzyme, AKR1C3, and one unknown microsomal reductase.

- We determined the extent of reduction of oracin to its main metabolite dihydrooracin by AKR1C3. This aldo-keto reductase is able to metabolize oracin with K_m 110 μM and V_{max} nmol/min/mg.

- AKR1C3 is also able to deactivate doxorubicin to form doxorubicinol which is probably one of the main reasons for chronic anthracycline-induced cardiotoxicity, as mentioned before. This reaction runs with K_m 355 μM and V_{max} 53 nmol/min/mg.

- We managed to introduce several purification techniques and achieved almost 100% ability to repeat our experiments with the same results.

- We tested a large number of separation conditions for various techniques and selected the most suitable ones.

- After a long time of searching and trying various purification conditions, the team has succeded in purifying a novel membrane-bound reductase

7. Abbreviations

ACT	anthracycline-induced cardiotoxicity
AKR	aldo keto reductase
ATP	adenosine triphosphate
BCA	bicinchonic acid
BPH	benign prostate hyperplasy
CBR	carbonyl reductase
cDNA	complementary deoxyribonucleic acid
CV	column volume
DD2	dihydrodiol dehydrogenase 2
DHEA	dihydroepiandrosterone
DHO	dihydrooracin
DHT	dihydrotestosterone
FT	flow through
GF	gel filtration
GSH	glutathione
GST	glutathione S transferase
HIC	hydrophobic interaction chromatography
HMG-CoA	hydroxymethylglutaryl Coenzyme A
HNF	hepatocyte nuclear factor
HPLC	high pressure liquid chromatography
HSD	hydroxysteroid dehydrogenase
IEX	ion exchange
KSI	ketosteroid isomerase
LDH	lactate dehydrogenace
MDR	multidrug resistance
mRNA	messenger ribonucleic acid
NAD(H)	nicotine amide dinucleotide
NADP(H)	nicotine amide adenine dinucleotide phosphate
NNK	4-methyl-nitrosamino-1-(3-pyridyl)-1-butanone
NSAID	non-steroidal anti-inflamatory drug
PBS	phosphate buffered saline
PGD	prostaglandine D
PGF	prostaglandine F
PGH	prostaglandine H
ROS	reactive oxygen species
SDR	short chain dehydrogenase/reductase
SDS	sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine

8. Abstract

Reduction is the reverse of oxidation and therefore it can involve loss of oxygen atom or the addition of two hydrogen atoms. The reduction of carbonyl groups in xenobiotics was the main topic of this thesis.

We tried to identify and characterize human carbonyl reductases responsible for anticancer drugs deactivation. When cancer is among the most common death causes in the developed world, it is necessary to look for new and efficient ways of its treatment. Inhibition of enzymes, which may contribute to disease development or relapses and/or treatment efficacy decrease by drug inactivation, could be a possible way of treatment improvement and might also lead to decrease of drug doses and side effects of cytostatics.

In the first part of our project, we focused on a soluble cytosolic reductase AKR1C3. This enzyme is involved in sex hormone metabolism and might play an important role in breast and prostate cancer development. We tested its ability to metabolize anticancer drugs by its incubation with oracin and doxorubicin with subsequent metabolite determination with use of HPLC. Our experiment proved that it can deactivate these two drugs with K_m 355 μM for doxorubicin and 110 μM for oracin, respectively. AKR1C3 can therefore influence the anticancer therapy, expecially when overexpressed.

The second part of our project was targeted on purification of a novel human liver microsomal reductase with help of ÄKTA design chromatography system. We used several chromatographic techniques (ion-exchange, affinity, and size exclusion chromatography, electrofocusing) and separation modes. Oracin was used as a model substrate to evaluate reductive activity. SDS-PAGE and Native PAGE were necessary to determine the purity of fractions obtained after protein separation, active bands were analyzed by means of MALDI-TOF. To determine the protein content in the active fractions, Bradford protein assay and BCA assay were used.

Various separation methods and conditions were tested and introduced, some of them were ruled out, and some were used in further experiments. In this long process we followed several leads and thanks to the hard work of the whole team, we managed to partially purify a new human membrane bound reductase which is able to metabolize anticancer agent oracin.

9. Abstrakt

Redukce je opakem oxidace a může jí být buď ztráta atomu kyslíku, nebo získání dvou atomů vodíku. Redukce karbonylové skupiny xenobiotik byla hlavním tématem této práce.

V naší práci jsme se pokusili identifikovat a charakterizovat lidské karbonylreduktázy zodpovědné za deaktivaci protinádorových léčiv. Rakovina je jednou z nejčastějších příčin úmrtí v rozvinutých zemích, proto je hledání nových a efektivních způsobů léčby velmi důležité. Inhibice enzymů, které se mohou podílet na rozvoji choroby nebo jejích relapsů a/nebo snížení účinnosti terapie deaktivací léčiv, může být způsobem, jak zlepšit léčbu a snad i snížit dávky léčiv a tím i incidenci nežádoucích účinků cytostatik.

V první části našeho projektu jsme se soustředili na rozpustnou cytosolickou reduktázu, AKR1C3. Tento enzym se účastní metabolismu pohlavních hormonů a může hrát důležitou roli v rozvoji rakoviny prsu a prostaty. Testovali jsme její schopnost metabolizovat protinádorová léčiva inkubací s oracinem a doxorubicinem a následným stanovením metabolitů pomocí HPLC. Podařilo se nám prokázat, že AKR1C3 je schopná deaktivovat doxorubicin s K_m 355 μM a oracin s K_m 110 μM. AKR1C3 tedy může ovlivnit protinádorovou terapii, zejména pokud je její exprese v zasažené tkáni zvýšená.

Druhá část projektu byla zeměřena na purifikaci nové lidské jaterní mikrosomální reduktázy použitím ÄKTA chromatografického systému. Použili jsma řadu chromatografických technik (iontově výměnná, afinitní chromatografie, gelová filtrace, elektrofocusing) a separačních metod. Oracin byl použit jako modelový substrát pro stanovení redukční aktivity. SDS-PAGE a Nativní PAGE byly důležité pro určení čistoty frakcí získaných purifikací, aktivní proužky byly analyzovány použitím Maldi-Tof. Pro stanovení obsahu proteinu ve vzorcích byly byužity dvě metody, BCA a stanovení bílkoviny dle Bradforda.

Testovali jsme řadu separačních metoda podmínek, některé byly na pracovišti zavedeny a použity v dalších experimentech, jiné byly vyloučeny. Během tohoto dlouhého procesu jsme sledovali několik vodítek a díky práci celého týmu se nám podařilo částečně purifikovat novou lidskou membránově vázanou reduktázu, která je schopná metabolizovat protinádorové léčivo oracin.

10. References

Agapova OA, Yang P, Wang WH, Lane DA, Clark AF, Weinstein BI, Hernandez MR: Altered expression of 3α-hydroxysteroid dehydrogenases in human glaucomatous optic nerve head astrocytes. *Neurobiol. Dis.,* 14, 63-73, 2003.

Agarwal AK, Mune T, Monder C, White PC: NAD^+-dependent isoform of 11β-hydroxysteroid dehydrogenase: cloning and characterization of cDNA from sheep kidney. *J. Biol. Chem.,* 269, 25959-25962, 1994.

Ahmed NK, Felsted RL, Bachur NR: Comparison and characterization of mammalian xenobiotic ketone reductases. *J. Pharmacol. Exp. Ther.,* 209, 12-19, 1979.

Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS: Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell. Endocrinol.,* 105, R11-R17, 1994.

Atalla A, Maser E: Characterization of enzymes participating in carbonyl reduction of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human placenta. *Chem. Biol. Interact.,* 130-132, 737-748, 2001.

Bauman DR, Rudnick SI, Szewczuk LM, Jin Y, Gopishetty S, Penning TM: Development of nonsteroidal anti-inflammatory drug analogs and steroid carboxylates selective for human aldo-keto reductase isoforms: potential antineoplastic agents that work independently of cyclooxygenase isozymes. *Mol. Pharmacol.,* 67, 60-68, 2005.

Bauman DR, Steckelbroeck S, Penning TM: The roles of aldo-keto reductases in steroid hormone action. *Drug News Perspect.,* 17, 563-578, 2004.

Ben-Zimra M, Koler M, Melamed-Book N, Arensburg J, Payne AH, Orly J: Uterine and placental expression of steroidogenic genes during rodent pregnancy. *Mol. Cell. Endocrynol.,* 187, 223-231, 2002.

Blouin K, Blanchtte S, Richerd C, Dupont P, Luu-The V, Tchernof A: Expression and activity of steroid aldoketoreductases 1C in omental adipose tissue are possitive correlates of adiposity in women. *Am. J. Physiol.,* 288, E398-E404, 2005.

Bonnesen C, Eggleston IM, Hayes JD: Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res.,* 61, 6120-6130, 2001.

Boudet AM, Lapierre C, Grima-Petternati J: Biochemistry and molecular biology of lignifications. *New phytol.* 129, 203-236, 1995.

Branden CI, Jornvall H, Eklund H, Furugren B: Alcohol dehydrogenases. *The Enzymes*, 3rd edn. (boyer PD, ed), pp. 104-190. Academic Pres, New York, 1975.

Bray JE, Marsden BD, Oppermann U: The human short-chain dehydrogenase/reductase (SDR) superfamily: A biotransformatics summary, *Chem.Biol. Interact.* 178, 99-109, 2009.

Breyer-Pfaff U, Nill K: High affinity stereoselective reduction of the enantiomers of ketotifen and of ketonic nortriptyline metabolites by aldo-keto reductases from human liver. *Biochem. Pharm.,* 59, 249-260, 2000.

Breyer-Pffaf U, Nill K: Carbonyl reduction of naltrexone and dolasetron by oxidoreductases isolatedfrom human liver cytosol. *J. Pharm. Pharmacol.,* 56, 1601-1606, 2004.

Brown RW, Chapman KE, Edwards CR, Seckl JR: Human placental 11β-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* 132, 2614-2621, 1993.

Brown RW, Chapman KE, Murad P, Edwards CRW, Seckl JR: Purification of 11β-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity label technique. *Biochem. J.,* 313, 997-1005, 1996.

Burczynski ME, Lin HK, Penning TM: Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophyles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res.,* 59, 607-614, 1999.

Burczynski ME, Sridhar GR, Palackal NT, Penning TM: The reactive oxygen species and Michael acceptor-inducible human aldo-keto reductase AKR1C1 reduces the α, β-unsaturated aldehyde 4-hydroxy-2-nonenal to 1,4-dihydroxy-2-nonene. *J. Biol. Chem.,* 276, 2890-2897, 2001.

Campbell MM, Sederoff RR: Variation in lignin content and composition. Mechanisms of control and implications for genetic improvements of plants. *Plant Physiol.* 110, 3-13, 1995.

Chakrabarti S, Cukiernik M, Hileeto D, Evans T, Chen S: Role of vasoactive factors in the pathogenesis of early changes in diabetic retinopathy. *Diabetes Metab. Res. Rev.* 16, 393-407, 2000.

Chetrite GS, Cortes-Prieto J, Phillipe JC, Wright F, Pasqualini JR: Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and cancerous, human breast tissues. *J. Steroid. Bioch. Mol. Biol.,* 66, 295-302, 2000.

Ciaccio PJ, Jaiswal AK, Tew KD: Regulation of human dihydrodiol dehydrogenase by Michael acceptor xenobiotics. *J. Biol. Chem.,* 269, 15558-15562, 1994.

Cirillo R, Sacco G, Venturella S, Brightwell J, Giachetti A, Manzini S: Comparison of doxorubicin and MEN 10755-induced long-term progressive cardiotoxicity in the rat. *J. Cardiovasc. Pharmacol.,* 35, 100-108, 2000.

Couture JF, Legrand P, Cantin L, Luu-The V, Labrie F, Breton R: Human 20α-hydroxysteroid dehydrogenase: Crystallographic and site-directed mutagenesis studies lead to the identification of an alternative binding site for C21-steroids. *J. Mol. Biol.,* 331, 593-604, 2003.

Czajka MC, Lee RE Jr.: A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster. J. Exp. Biol.* 148, 245-254, 1990.

Dell´Acqua G, Polishchuck K, Fallon JT, Gordon JW: Cardiac resistance to adriamycin in transgenic mice expressing a rat α-cardiac myosin heavy chain/human multiple drug resistance 1 fusion gene. *Hum. Gene Ther.,* 10, 1269-1279, 1999.

Desmond JC, Mountford JC, Drayson MT, Walker EA, Hewison M, Ride JP, Luong QT, Hayden RE, Vanin EF, Bunce CM: The aldo-keto reductase AKR1C3 is a novel suppressor of cell differentiation that provides a plausible target for the non-cyclooxygenase-dependent antineoplastic actions of nonsteroidal anti-inflammatory drugs. *Cancer Res.,* 63, 505-512, 2003.

Deyashiki Y, Taniguchi H, Amano T, Nakayama T, Hara A, Sawada H: Structural and functional comparison of two human liver dihydrodiol dehydrogenases associated with 3α-hydroxysteroid dehydrogenase activity. *Biochem. J.,* 282, 741-746, 1992.

Dianzani I, de Sanctis L, Smooker PM, Gough TJ, Alliaudi C, Brusco A, Spada M, Blau N, Dobos M, Zhang HP, Yang N, Ponzone A, Armarego WL, Cotton RG: Dihydropteridine reductase deficiency:

psychical structure of the QDPR gene, identification of two new mutations and genotype-phenotype correlations. *Hum. Mutat.,* 12, 267-273, 1998.

Doorn JA, Maser E, Blum A, Claffey DJ, Petersen DR: Human carbonyl reductase catalyzes reduction of 4-oxonon-2-enal. *Biochemistry,* 43, 13106-13114, 2004.

Doroshow JH: Anthracyline antibiotic-stimulated superoxide hydrogen peroxide and hydroxyl radical production by NADH dehydrogenase. *Cancer Res.,* 43, 4543-4551, 1983.

Dufort I, Labrie F, Luu-The V: Human types 1 and 3 3α-hydroxysteroid dehydrogenases: Differential lability and tissue distribution. *J. Clin. Endocrinol. Metab.,* 86, 841-846, 2001.

Dufort I, Rheault P, Huang XF, Soucy P, Luu-The V: Characteristics of a highly labile human type 5 17β-hydroxysteroid dehydrogenase. *Endocrinology,* 140, 568-574, 1999.

Dufort I, Soucy P, Labrie F, Luu-The V: Molecular cloning of human type 3 3α-hydroxysteroid dehydrogenase that differs from 20α-hydroxysteroid dehydrogenase by seven amino acids. *Biochem. Biophys. Res. Commun.,* 228, 474-479,1996.

Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C: localization of 11β-hydroxysteroid dehydrogenase – tissue specific protector of mineralocorticoid receptor. *Lancet II,* 986-989, 1988.

El-Kabbani O, Wilson DK, Petrash M, Quiocho FA: Structural features of the aldose reductase and aldehyde reductase inhibitor-binding sites. *Mol. Vis.,* 4, 19, 1998.

Flowers-Geary KD, Harvey RG, Penning TM: Examination of polycyclic aromatic hydrocarbon o-quinones produced by dihydrodiol dehydrogenase as substrates for redox cycling in rat liver. *Biochem. (Life Sci. Adv.),* 11, 49-58, 1992.

Flowers-Geary L, Bleczinski W, Harvey RG, Penning TM: Cytotoxicity and mutagenicity of polycyclic aromatic hydrocarbon ortho-quinones produced by dihydrodiol dehydrogenase. *Chem. Biol. Interact.,* 99, 55-72, 1996.

Fogli S, Danesi R, Innocenti F, Di Paolo A, Bocci G, Barbara C, Del Tacca M: An improved HPLC method for therapeutic drug monitoring of daunorubicin, idarubicin, doxorubicin, epirubicin and their 13-dihydro metabolites in human plasma. *Ther. Drug. Monit.,* 1, 367-375, 1999.

Forrest GL, Gonzales B, Tseng W, Li X, Mann J: Human carbonyl reductase overexpression in the heart advances the development of doxorubicin-induced cardiotoxicity in transgenic mice. *Cancer Res.,* 15, 5158-5164, 2000.

Forrest GL, Gonzales B: Carbonyl reductase. *Chem. Biol. Interact.,* 129, 21-40, 2000.

Funder JW, Pearce PT, Smith R, Smith AI: Mineralocorticoid action: target specifity is enzyme, not receptor mediated. *Science,* 242, 583-585, 1988.

Fung KM, Samara ENS, Wong C, Metwalli A, Krlin R, Bane B, Liu CZ, Yang JT, Pitha JV, Culkin DJ, Kropp BP, Penning TM, Lin HK: Increased expression of type 2 3α-hydroxysteroid dehydrogenase/ type 5 17β-hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma. *Endocr. Relat. Cancer,* 13, 169-180, 2006.

Gabrielli F, Donadel G, Bensi G, Heguy A, Melli M: A nuclear protein, synthetised in growth-arrested human hepatoblastoma cells, is a novel member of the short-chain alcohol dehydrogenase family, *Eur. J. Biochem.,* 232, 473-477, 1995.

Gersl V, Mazurová Y, Bajgar J, Melka M, Hrdina R, Palicka V: Lack of cardiotoxicity of a new antineoplastic agent, a synthetic derivative of indenoisochinoline: comparison with daunorubicin in rabbits. *Arch. Toxicol.,* 70, 645-651, 1996.

Gonzales P, Rao PV, Nunez SB, Zigler JS Jr.: Evidence for independent recruitment of ζ-crystallin/quinone reductase (CRYZ) as a crystalline in camelids and hydricomorph rodents. *Mol. Biol. Evol.* 12, 773-781, 1995.

Gurates B, Bulun SE: Endometriosis: The ultimate hormonal disease. *Semin. Reprod. Med.,* 21, 125-134, 2003.

Habig WH, Jakoby WB: Assay for differentiation of glutathione S-transferases. *Methods in Enzymology* 77, 398-405, 1981.

Hara A, Taniguchi H, Nakayama T, Sawada H: Purification and properties of multiple forms of dihydrodiol dehydrogenase from human liver. *J. Biochem. (Tokyo),* 108, 250-254, 1990.

Harkonen P, Torn S, Kurkela R, Porvari K, Pulkka A, Lindfors A, Isomaa V, Vihko P: Sex hormone metabolism in prostate cancer cells during transition to androgen-independent state. *J. Clin. Endocrinol. Metab.,* 88, 705-712, 2003.

Higaki Y, Usami N, Shintani S, Ishikura S, El-Kabbani O, Hara A: Selective and potent inhibitors of human 20α-hydroxysteroid dehydrogenase (AKR1C1) that metabolizes neurosteroids derived from progesterone. *Chem. Biol. Interact.,* 143-144, 503-513, 2003.

Holloway RW: Treatment options for endometrial cancer: experience with topotecan. *Gynecol. Oncol.,* 90, 28-33, 2003.

Hsu NY, Ho HC, Chow KC, Lin TY, Shih CS, Wang LS, Tsai CM: Overexpression of dihydrodiol dehydrogenase as a prognostic marker of non-small cell lung cancer. *Cancer. Res.,* 61, 2727-2731, 2001.

Hukkanen J, Lassila A, Paivarita K, Valanne S, Sarpo S, Hakkola J, Pelkonnen O, Raunio H: Induction and regulation of xenobiotic-metabolizing cytochrome P450s in the human A549 lung adenocarcinoma cell line. *Am. J. Respir. Cell Mol. Biol.,* 22, 360-366, 2000.

Hult M, Nobel CS, Abrahmsen L, Niccoll-Griffith DA, Jornvall H, Oppermann U: Novel enzymological profiles of human 11β-hydroxysteroid dehydrogenase type 1. *Chem. Biol. Interact.,* 130-132, 805-814, 2001.

Hyndman D, Bauman DR, Heredia VV, Penning TM: The aldo-keto reductase superfamily homepage. *Chem. Biol. Interact.,* 143-144, 621-631, 2003.

Ishikura S, Nakajima S, Kaneko T, Shinitani S, Usami N, Yamamoto I, Carbone V, El-Kabbani O, Hara A: Comparison of stereoselective reduction of 3- and 20-oxosteroids among mouse and primate 20α-hydroxysteroid dehydrogenase. In Weiner H, Plapp B, Lindahl R, Maser E (ed): Enzymology and Molecular Biology of Carbonyl Metabolism 12. West Lafayette, Purdue University Press, 2005, pp. 341-351.

Iwata N, Inazu N, Satoh T: The purification and properties of NADPH-dependent carbonyl reductases from rat ovary. *J. Biochem.,* 105, 556-564, 1989.

Jamieson PM, Chapman KE, Edwards CR, Seckl JR: 11β-hydroxysteroid dehydrogenase in an exclusive 11-β reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology,* 136, 4754-4761, 1995.

Jarabak J, Harvey RG: Studies on three reductases which have polycyclic aromatic hydrocarbon quinones as substrates. *Arch. Biochem. Biophys.,* 303, 394-401, 1993.

Jez JM, Bennett MJ, Schlegel BP,Lewis M, Penning TM: Comparative anatomy of the aldo-keto reductase superfamily. *Biochem. J.,* 326, 625-636, 1997a.

Jez JM, Flynn TG, Penning TM: A new nomenclature for the aldo-keto reductase superfamily. *Biochem. Pharmacol.,* 54, 639-647, 1997b.

Jez JM, Penning TM: The Aldo-keto reductase (AKR) superfamily: an update. *Chem. Biol. Interact.,* 130-132, 499-525, 2001.

Ji Q, Aoyama C, Nien YD, Liu PI, Chen PK, Chang L, Stanczyk FZ, Stoltz A: Selective loss of AKR1C1 and AKR1C2 in breast cancer and their potential effect on progesterone signaling. *Cancer Res.,* 64, 7610-7616, 2004.

Jin Y, Penning TM: Steroid 5α-reductase and 3α-hydroxysteroid dehydrogenases: key enzymes in androgen metabolism. *Best Pract. Res. Clin. Endocrinol. Metab.,* 15, 79-94, 2001.

Jornvall H, Hoog J-O, Persson B, Pares X: Pharmacogenetics of the alcohol dehydrogenase system. *Am. J. Pharmacol.* 61, 184-191, 2000.

Jornvall H, Hoog J-O, Persson B: SDR and MDR: completed genome sequences show protein families to be large, of old origin, and of complex nature. *FEBS Lett.* 445, 261-264, 1999.

Jornvall H, Persson B, Krook M, Atrian S, Gonzales-Duarte R, Jeffery J, Ghosh D: Short-chain dehydrogenases/reductases (SDR). *Biochemistry*, 34, 6003-6013, 1995.

Kallberg Y, Persson B: Prediction of coenzyme specificity in dehydrogenases/

reductases: a hidden Markov model-based method and its application

on complete genomes. *FEBS J.,* 273, 1177–1184, 2006.

Kallberg Y, Oppermann U, Jornvall H, Persson H: Short-chain dehydrogenases/

reductases (SDRs). *Eur. J. Biochem,.* 269, 4409–4417, 2002.

Kavanagh KL, Klimacek M, Nidetzky B, Wilson DK: The structure of apo and holo forms of xylose reductase, a dimeric aldo-keto reductase form *Candida tenuis. Biochemistry,* 41, 8785-8795, 2002.

Kelly VP, Ireland LS, Ellis EM, Hayes JD: Purification from rat liver of a novel constitutively expressed member of aldo-keto reductase 7 family that is widely distributed in extra hepatic tissues. *Biochem. J.,* 348-389-400, 2000.

Khanna M, Qin KE, Wang RW, Cheng KC: Substrate specifity, gene structure and tissue distribution of multiple human 3α-hydroxysteroid dehydrogenase. *J. Biol. Chem.,* 270, 20162.20168, 1995.

Kitawaki J, Kado N, Ishihara H, Koshiba H, Kitaoka Y, Honjo H: Endometriosis: The patophysiology as an estrogen-dependent disease. *J. Steroid. Biochem. Mol. Bio.,* 83, 149-155, 2003.

Klucar J, Al-Rubeai M: G2 cell cycle arrest and apoptosis are induced in Burkitt's lymphoma cells by the anticancer agent oracin. *FEBS Lett.,* 400, 127-130, 1997.

Kozma E, Brown E, Ellis EM, Lapthorn AJ: The crystal structure of rat liver AKR7A1: a dimeric member of the aldo-keto reductase superfamily. *Biochem. Pharmacol.,* 54, 639-647. 2002.

Krazeisen A, Breitling R, Moller G, Adamski J: Phytoestrogens inhibit human 17β-hydroxysteroid dehydrogenase type 5. *Mol. Cell. Endocrinol.,* 171, 151.162, 2001.

Labrie F, Luu-The V, Lin SX, Simard J, Labrie C, El-Alfy M, Pelletier G, Belager A: Intracrinology: role of the family of 17β-hydroxysteroid dehydrogenases in human physiology and disease. *J. Mol. Endocrinol.,* 25, 1-16, 2000.

Lai CH, Chou CY, Ch´ang LY, Liu CS, Lin WC: Identification of novel human genes evolutionarily conserved in caenorhabditis elegans by comparative proteomics. *Genome Res.,* 10, 703-713, 2000.

Lang DS, Becker S, Devlin RB, Koren HS: Cell-specific differences in the susceptibility of potential cellular targets of human origin derived from blood and lung following treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Cell Biol. Toxicol.,* 14, 23-38, 1998.

Lanisnik Rizner T, Lin KM, Peehl DM, Steckelbroeck S, Bauman DR, Penning TM: Human type 3 3α-hydroxysteroid dehydrogenase (aldo-keto reductase 1C2) and androgen metabolism in prostate cells. *Endocrinology,* 144, 2922-2932, 2003.

Legha SS, Benjamin RS, Mackay B, Ewer M, Wallace S, Valdivieso M, Rasmussen SL, Blumenschein GR, Freireich EJ: Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. *Ann. Intern. Med.,* 96, 133-139, 1982a.

Legha SS, Wang YM, Mackay B, Ewer M, Hortobagy GN, Benjamin SR, Ali MK: Clinical and pharmacologic investigation of effects of α-tocopherol on adriamycin cardiotoxicity. *Ann. NY. Acad. Sci.,* 393, 411-418, 1982b.

Lewis MJ, Wiebe JP, Heathcote G: Expression of progesterone metabolizing enzyme genes (AKR1C1, AKR1C2, AKR1C3, SRD5A1, SRD5A2) is altered human breast carcinoma. *BMC Cancer*, 4, 27-38, 2004.

Lin HK, Jez JM, Schlegel BP, Peehl DM, Pachter JA, Penning TM: Expression and characterization of recombinant type 2 3α-hydroxysteroid dehydrogenase (HSD) from human prostate: Demonstration of bifunctional 3α/17β-HSD activity and cellular distribution. *Mol. Endocrinol.,* 11, 1971-1984, 1997.

Lin HK, Steckelbroeck S, Fung KM, Jones AN, Penning TM: Characterization of a monoclonal antibody for human aldo-keto reductase AKR1C3 (type 2 3α-hydroxysteroid dehydrogenase/type 5 17β-hydroxysteroid dehydrogenase); immunohistochemical detection in breast and prostate. *Steroids,* 69, 795-801, 2004.

Lovering AL, Ride JP, Bunce CM, Desmond JC, Cummings SM, White SA: Crystal structures of prostaglandin D(2) 11-ketoreductase (AKR1C3) in complex with the nonsteroidal anti-inflammatory drugs flufenamic acid and indomethacin. *Cancer Res.,* 64, 1802-1810, 2004.

Low SC, Chapman KE, Edwards CR, Seckl JR: “Liver-type” 11β-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. *J. Mol. Endocrinol.,* 13, 167-174, 1994.

Luu-The V, Dufort I, Pelletier G Labrie F: Type 5 17β-hydroxysteroid dehydrogenase: Its role in the formation of androgens in women. *Mol. Cell Endocrinol.,* 171, 77-82, 2001.

Maceratesi P, Daude N, Dallapiccola B, Novelli G, Allen R, Okano Y, Reichardt J: Human UDP-galactose 4´epimerase (GALE) gene and identification of five missense mutations in patients with epimerase-deficiency galactosemia. *Mol. Genet. Metab.,* 63, 26-30, 1998.

Marhan J: Mutagenicity in bacterial system. I. Ames test. *Folia Microbiol (Praha)*. 40(5):457-61, 1995.

Marschall H-U, Oppermann UC, Svensson S, Nordling E, person B, Hoog J-O, Jornvall H: Human liver class I alcohol dehydrogenase γγ isoenzyme: the sole cytosolic 3β hydroxysteroid dehydrogenase of iso bile acids. *Hepatology* 31, 990-996, 2000.

Maser E and Breyer-Pfaff U: Competing roles of reductase in the detoxification of the tobacco-specific nitrosamine ketone NNK In Penning TM and Petrash JM (ed): Aldo-keto reductases and toxicant metabolism, Washington DC, American Chemical Society, pp 67-82, 2004.

Maser E, Friebertshauser J, Volker B: Purification, characterization and NNK carbonyl reductase activities of 11β-hydroxysteroid dehydrogenase type 1 from human liver: enzyme cooperativity and significance in the detoxification of tobacco-derived carcinogen. *Chem. Biol. Interact.,* 143-144, 435-448, 2003.

Maser E, Oppermann U: Role of type 1 11β-hydroxysteroid dehydrogenase in detoxification processes. *Eur. J. Biochem.,* 249, 365-369, 1997.

Maser E, Volker B, Frieberthauser J: 11β-Hydroxysteroid dehydrogenase type 1 from human liver: dimerization and enzyme cooperativity support its postulated role as glucocorticoid reductase. *Biochemistry*, 41, 2459-2465, 2002.

Matsunaga T, Shintani S, Hara A: Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug. Metab. Pharmacokinet.* 21, 1-18, 2006

Matsuura K, Deyashiki Y, Sato K, Ishida N, Miwa G, Hara A: Identification of amino acid residues responsible for differences in substrate specifity and inhibitor sensitivity between two human liver dihydrodiol dehydrogenase isoenzymes by site-directed mutagenesis. *Biochem. J.,* 323, 61-64, 1997.

Matsuura K, Hara A, Kato M, Deyashiki Y, Miyabe Y, Ishikura S, Sugiyama T, Katagiri Y: Activation of human liver 3α-hydroxysteroid dehydrogenase by clofibrate derivatives. *J. Pharmacol. Exp. Ther.,* 285, 1096-1103, 1998.

Matsuura K, Hara A, Sawada H, Bunai Y, Ohya I: Localization of pulmonary carbonyl reductase in guinea-pig and mouse: Enzyme histochemical and immunochemical studies. *J. Histochem. Cytochem.,* 38, 217-223, 1990.

Matsuura K, Shiraishi H, Hara A, Sato K, Deyashiki Y, Ninomiya M, Sakai S: Identification of a principle mRNA species for human 3α-hydroxysteroid dehydrogenase isoform (AKR1C3) that exhibits high prostaglandin D2 11-ketoreductase activity. *J. Biochem.,* 124, 940-946, 1998.

Matsuura K, Tamada Y, Deyashiki Y, Miyabe Y, Nakanishi M, Ohya I, Hara A: Activation of human liver 3α-hydroxysteroid dehydrogenase by sulphobromophtalein. *Biochem. J.,* 313, 179-184, 1996.

McCoull KD, Rindgen D, Blair IA, Penning TM: Synthesis and characterization of polycyclic aromatic hydrocarbon o-quinone depurinating N7-guanine adducts. *Chem. Res. Toxicol.,* 12, 237-246, 1999.

Melka M: Oracin-preclinical summary report of the Research Institute for Pharmacy and Biochemistry, Prague, Czech Republic, 1993.

Miko M, Poturnajová M, Soucek R: Cytotoxicity and mode of action of the potential cytostatic drug oracin. *Neoplasma,* 49, 167-171, 2002.

Mills KI, Gilkes AF, Sweeney M, Choudhry MA, Woodgate LJ, Bunce CM, Brown G, Burnett AK: Identification of a retinoid acid responsive aldoketoreductase expressed in HL60 leukaemic cells. *FEBS Lett.,* 440, 158-162, 1998.

Miyoshi Y, Ando A, Shiba E Taguchi T, Tamaki Y, Noguchi S: Involvement of up-regulation of 17β-hydroxysteroid dehydrogenase type I in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer,* 94, 685-689, 2001.

Moeller LC, Dumitrescu AM, Walker RL, Meltzer PS, Refetoff S: Thyroid hormone responsive genes in human fibroblasts. *J. Clin. Endocrinol. Metab.,* 90, 936-943, 2005.

Molowa DT, Shayne AG, Guzelian PS: Purification and characterization of chlordecone reductase from human liver. *J. Biol. Chem.,* 261, 12624-12627, 1986.

Myers C, Bonow R, Palmieri S, Jenkins J, Corden B, Locker G, Doroshow J, Epstein SA: A randomized controlled trial assessing the prevention of doxorubicin cardiomyopathy by N-acetylcysteine. *Semin. Oncol.,* 10, 53-55,1983.

Nakayama T, Yashiro K, Inoue Y, Matsuura K, Ichikawa H, Hara A, Sawada H: Characterization of pulmonary carbonyl reductase of mouse and guinea pig. *Biochem. Biophys. Acta,* 882, 220-227, 1986.

Napolitano A, Voice MW, Edwards CR, Seckl JR, Chapman KE: 11β-hydroxysteroid dehydrogenase type 1 in adipocytes: expression is differentiation-dependent and hormonally regulated. *J. Steroid. Biochem. Mol. Biol.,* 64, 251-260, 1998.

Nishizawa M, Nakajima T, Yasuda K, Kanzaki H, Sasaguri Y, Watanabe K, Ito S: Close kinship of human 20α-hydroxysteroid dehydrogenase gene with three aldo-keto reductase genes. *Genes Cells,* 5, 111-125, 2000.

Nordling E, Jornvall H, Persson B: Medium-chain dehydrogenases/reductases (MDR). Family characterizations including genome comparisons and active site modeling. *Eur. J. Biochem.* 269, 4267-4276, 2002.

Ohara H, Miyabe Y, Deyashiki Y, Matsuura K, Hara A: reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. *Biochem. Pharmacol.,* 50, 221-227, 1995.

Ohlendieck K: Extraction of membrane protein, in Cutler P: Methods in molecular biology volume 244, Protein purification protocols, Second edition, Human Press, Inc., Totowa, New Yersey, USA, 2003.

Olson LE, Bedja D, Alvey SJ, Cardounel AJ, Gabrielson KL, Reeves RH: Protection from doxorubicin-induced cardiac toxicity in mice with null allele of carbonyl reductase 1. *Cancer Res.,* 63, 6602-6606, 2003.

Olson RD, Mushlin PS, Brenner DE, Fleischer S, Chang BK, Cusack BJ, Boucek RJ Jr.: Doxorubicin cardiotoxicity may be due to its metabolite doxorubicinol. *Proc. Natl. Acad. Sci. USA,* 85, 3585-3589, 1988.

Olson RD, Mushlin PS: Doxorubicin cardio-toxicity: analysis of prevailing hypotheses. *FASEB J.*, 4, 3076-3086, 1990.

Oppermann U, Filling C, Hult M, Shafgat NY, Persson B, Jornvall H: Short-chain dehydrogenases/reductases (SDR): the 2002 update. *Chem. Biol. Interact.* 143-144, 247-253, 2003.

Oppermann U, Filling C, Jornvall H: Forms and functions of SDR enzymes. *Chem. Biol. Interact.,* 130-132, 699-705, 2001.

Oppermann U, Maser E: Molecular and structural aspects of xenobiotic carbonyl metabolizing enzymes. Role of reductases and dehydrogenases in xenobiotic phase I reactions. *Toxicology,* 144, 71-81, 2000.

Oppermann U, Persson B, Filling C, Jornvall H: Structure-function relationships of SDR hydroxysteroid dehydrogenases. *Adv. Exp. Med. Biol.,*414, 403-415, 1997.

Oritani H, Deyashiki Y, Nakayama T, Hara A, Sawada H, Bunai Y, Ohya I: Purification and characterization of pig lung carbonyl reductase. *Arch. Biochem. Biophys.,* 292, 539-547, 1992.

Ozeki T, Takahashi Y, Kume T,Nakayama K, Yokoi T, Nunoya K, Hara A, Kamataki T: Co-operative regulation of the transcription of human dihydrodiol dehydrogenase (DD)4/aldo-keto reductase (AKR)1C4 gene by hepatocyte nuclear factor (HNF) 4 α/γ and HNF-1α. *Biochem. J.,* 355, 537-544, 2001.

Ozeki T, Takahashi Y, Nakayama K, Funayama M, Nagashima K, Kodama T, Kamataki T: Hepatocyte nuclear factor-4α/γ and hepatocyte nuclear factor-1α as causal factors of interindividual difference in the expression of human dihydrodiol dehydrogenase 4 mRNA in human livers. *Pharmacogenetics,* 13, 49-53, 2003.

Ozeki T, Takahashi Y, Nakayama K, Kamataki T: Hepatocyte nuclear factor (HNF) 4 α/γ, and HNF-1α regulate the cell-specific expression of the human dihydrodiol dehydrogenase (DD)4/AKR1C4 gene. *Arch. Biochem. Biophys.,* 405, 185-190, 2002.

Palackal NT, Lee SH, Harvey RG, Blair IA, Penning TM: Activation of polycyclic aromatic hydrocarbon *trans*-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. *J. Biol. Chem.,* 277, 24799-24808, 2002.

Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M, Maloche C: Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J. Clin. Endocrinol. Metab.,* 81, 1460-1464, 1996,

Pasqualini JR: The selective estrogen enzyme modulators in breast cancer: a review. *Biochem. Biophys. Acta,* 1654, 123-143, 2004.

Pelletier G, Luu-The V, Tetu B, Labrie F: Immunocytochemical localization of type 5 17β-hydroxysteroid dehydrogenase in human reproductive tissues. *J. Histochem. Cytochem.*, 47, 731-738, 1999.

Peltoketo H, Luu-The V, Simard J, Adamski J: 17β-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol.,* 23, 1-11, 1999.

Penning TM, Burczynski ME, Jez JM Lin HK, Ma H, Moore M, Ratnam K, Palackal N: Structure-function aspects and inhibitor design of type 5 17β-hydroxysteroid dehydrogenase (AKR1C3). *Mol. Cell Endocrinol.,* 171, 137-149, 2001.

Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N, Ratnam K: Human 3α-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: Functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem. J.,* 351, 67-77, 2000.

Penning TM, Jin Y, Steckelbroeck S, Lanisnik Rizner T, Lewis M: Structure-function of human 3α-hydroxysteroid dehydrogenases: genes and proteins. *Mol. Cell. Endocrinol.,* 215, 63-72, 2004.

Penning TM: Aldo-keto reductases and formation of polycyclic aromatic hydrocarbons *o*-quinones. *Method Enzymol.,* 378, 31-67, 2004.

Persson B, Krook M, Jornvall H: Characteristics of short-chain alcohol dehydrogenases and related enzymes. *Eur. J. Biochem.* 200, 537-543,1991.

Persson B, Zigler JS Jr., Jornvall H: A super-family of medium-chain dehydrogenases/reductases (MDR). Sub-lines including ζ-crystallin, alcohol and polyol dehydrogenases, quinine oxidoreductase, enoyl reductases, VAT-1 and other proteins. *Eur. J. Biochem.* 226, 15-22, 1994.

Persson B, Kallberg Y, Bray JE, Bruford E, Dellaporta SL, Favia AD, Gontalez Duarte R, Jornvall H, Kavanagh KL, Kedishvili N, Kisiela M, Maser E, Mindnich R, Orchard S, Pennig TM, Thornton JM, Adamski J, Opperman U: Ths SDR (short-chain dehydrogenase/reductase and realted enzymes) nomenclature initiative. *Chem. Biol. Interact.,* 178, 94-98, 2009.

Piccinni MP, Beloni L, Livi C, Maggi E, scarselli G, Romagnani S: Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat. Med.,* 4, 1020-1024, 1998.

Pilka ES, Niesen FH, Lee WH, El-Hawari Y, Dunford JE, Kochan G, Wsol V, Martin HJ, Maser E, Oppermann U.: Structural basis for substrate specificity in human monomeric carbonyl reductases. *PlosOne*, 4(10), e7113, 2009.

Poirier D: Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* 10 (6), 453-477, 2003.

Protein Purification Handbook, Amersham Biosciences, USA, 2001

Qin KN, New MI, Cheng KC: Molecular cloning of multiple cDNAs encoding human enzymes structurally related to 3α-hydroxysteroid dehydrogenase.*J. Steroid. Biochem. Mol. Biol*., 46,673-679, 1993.

Rao PV, Gonzales P, Persson B, Jorvall H, Garland D, Zigler JS Jr.: Guinea pig and bovine ζ-crystallins have distinct functional characteristics highlighting replacements in otherwise similar structures. *Biochemistry* 36, 5353-5362, 1997:

Riveros-Rosas H, Julian-Sanchez A, Villabolos-Molina R, Pardo JP, Pina E: Diversity, taxonomy and evolution of medium-chain dehydrogenase/reductase superfamily. *Eur. J. Biochem.* 270, 3309-3334, 2003.

Russell DW: The enzymes, regulation, and genetics of bile acid synthesis. *Ann. Rev. Biochem.,* 72, 137-174, 2003.

Russo IH, Russo J: Role of hormones in mammary cancer initiation and progression. *J. Mammary Gland Biol. Neoplasia,* 3, 49-61, 1998.

Sandeep TC, Walker BR: Patophysiology of modulating of local glucocorticoid levels by 11β-hydroxysteroid dehydrogenases. *Trends Endocrinol. Metab.,* 12, 446-453, 2001.

Sawada H, Hara A: The presence of two NADPH-linked aromatic aldehyde-ketone reductases different from aldehyde reductase in rabbit liver. *J. Biochem.,* 86, 883-892, 1979.

Seckl JR, Walker BR: Minireview: 11β-hydroxysteroid Dehydrogenase type 1 – a tissue-specific amplifier of glucocorticoid action. *Endocrinology,* 142, 1371-1376, 2001.

Seckl JR: 11β-Hydroxysteroid dehydrogenase in the brain: a novel regulator of glucocorticoid action. *Front. Neuroendocrinol.,* 18, 49-99, 1997.

Shan K, Lincoff AM, Young JB: Anthracycline-induced cardiotoxicity. *Ann. Intern. Med.,* 125, 47-58, 1996.

Shiraishi H, Ishikura S, Matsuura K, Deyashiki Y, Ninomyia M, Kakai S, Hara A: Sequence of the cDNA of a human dihydrodiol dehydrogenase isoform (AKR1C2) and tissue distribution of tits mRNA. *Biochem. J.,* 334, 399-405, 1998.

Schlegel, B. P., Jez, J. M., and Penning, T. M. Mutagenesis of 3_-hydroxysteroid

dehydrogenase reveals a “push-pull” mechanism for proton transfer in aldo-keto

reductases. Biochemistry, *37:* 3538–3548, 1998.

Shou M, Harvey RG, Penning TM: Reactivity of benzo[a]pyrene-7,8-dione with DNA. Evidence for the formation of deoxyguanosine adducts. *Carcinogenesis,* 14, 475-482, 1993.

Simpson ER, Clyne C, Speed C, Rubin G, Bulun S: Tissue-specific estrogen biosynthesis and metabolism. *Ann NY Acad. Sci.*, 949, 58-67, 2001.

Singal PK, Iliskovic N: Doxorubicin-induced cardiomyopathy. *New Engl. J. Med.,* 339, 900-905, 1998.

Skarydova L, Zivna L, Xiong G, Maser E, Wsól V: AKR1C3 as a potential target for the inhibitory effect of dietary flavonoids. *Chem. Biol. Interact.* 178 (1-3), 138-144, 2009.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC: Measurement of protein using bicinchoninic acid. *Anal. Biochem.,* 150, 76-85, 1985.

Smithgall TE, Harvey RG, Penning TM: Regio- and stereospecificity of homogeneous 3 alpha-hydroxysteroid-dihydrodiol dehydrogenase for trans-dihydrodiol metabolites of polycyclic aromatic hydrocarbons. *J. Biol. Chem.,* 261, 6184-6191, 1986.

Soyal S, Ismail PM, Li J, Mulac-Jericevic B, Conneely OM, Lydon JP: Progesterone’s role in mammary gland development and tumorigenesis as disclosed by experimental mouse genetics. *Breast Cancer Res.,* 4: 191-1996, 2002.

Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, Febbo PG, Balk SP: Increased expression of genes converting adrenal androgens to testosterone in androgen-independent cancer. *Cancer Res.,* 66, 2815-2824, 2006.

Steckelbroeck S, Jin Y, Gopishetty S, Oyeshanmi B, Penning TM: Human cytosolic 3α-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3β-hydroxysteroid dehydrogenase activity. *J.Biol.Chem.,* 279, 10784-10795, 2004.

Steward PM, Valentino R, Wallace AM, Burt D, Shackleton CHL, Edwards CRW: Mineralocorticoid activity of liquorice: 11β-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet II*, 821-824, 1987.

Stoltz A, Hammond L, Lou H, Takikawa H, Ronk M, Shively JE: cDNA cloning and expresson of the human hepatic bile acid-binding protein. *J. Biol. Chem.,* 268, 10448-10457, 1993.

Suto K, Kajihara-Kano H, Yokoyama Y, Hayashi M, Kimura J, Kumano T, Takahata T, Kudo H, Tsuchida S: Decreased expression of the peroxisomal bifunctional enzyme and carbonyl reductase in human hepatocellular carcinomas. *J. Cancer Res. Clin. Oncol.,* 125, 83-88, 1999.

Suzuki T, Darnel AD, Akahira JI, Ariga N, Ogawa S, Kaneko C, Takeyama J, Moriya T, Sasano H: 5α-reductases in human breast carcinoma: possible modulator of in situ androgenic actions. *J. Clin. Endocrinol. Metab.,* 86, 2250-2257, 2001.

Suzuki T, Miki Y, Nakamura Y, Moriya T, Ito K, Ohuchi N, Sasano H: Sex steroid-producing enzymes in human breast cancer. *Endocr. Relat. Cancer,* 12, 701-720, 2005.

Suzuki T, Moriya T, Ishida T, Kimura M, Ohuchi N: In situ production of estrogens in human breast carcinoma. *Breast Cancer,* 9, 296-302, 2002.

Suzuki-Yamamoto T, Nishizawa M, Fukui M, Okuda-Ashitaka E, Nakajima T: cDNA cloning, expression and characterization of human prostaglandin F synthase. *FEBS Lett.,* 462, 335-340, 1999.

Takenaka K, Ogawa E, Oyanagi H, Wada H, Tanaka F: Carbonyl reductase expression and its clinical significance in non-small-cell lung cancer. *Cancer Epidemiol. Biomarkers Prev.,* 14, 1972-1975, 2005.

Takikawa H, Stoltz S, Sugiyama Y, Yoshida H, Kaplowitz N: Relationship between the newly identified bile acid binder and bile acid oxidoreductases in human liver. *J. Biol. Chem.,* 265,2132-2136, 1990.

Tanaka M, Bateman R, Rauh D, Vaisberg E, Ramachandani S, Zhang C, Hansen KC, Burlingame AL, Trautman JK, Shokat KM, Adams CL: An unbiased cell morphology-based screen for new, biologically active small molecules *PLoS Biol,* 3, e128, 2005.

Terada T, Sugihara Y, Nakamura K, Mizobuchi H, Maeda M: Further characterization of Chinese hamster carbonyl reductases (CHCRs). *Chem. Biol. Interact.,* 143-144, 373-381, 2003.

Tomlinson W: 11β-hydroxysteroid dehydrogenase type 1 in human disease: a novel therapeutic target. *Minerva. Endocrinol.,* 30, 37-46, 2005.

Umemoto M, Yokoyama Y, Sato S, Tsuchida S, Al-Mulla F, Saito Y: Carbonyl reductase as a significant predictor of survival and lymph node metastasis in epithelial ovarian cancer. *Br. J. Cancer,* 85, 1032-1036, 2001.

Usami N, Kitahara K, Ishikura S, Nagano M, Sakai S, Hara A: Characterization of a major form of human isatin reductase and the reduced metabolite. *Eur. J. Biochem.,* 268, 5755-5763, 2001.

Usami N, Yamamoto T, Shintani S, Higaki Y, Ishikura S, Katagiri Y, Hara A: Substrate specifity of human 3(20)α-hydroxysteroid dehydrogenase for neurosteroids and its inhibition by benzodiazepines. *Biol. Pharm. Bull.,* 25, 441-445, 2002.

Utsunomiya H, Suzuki T, Ito K, Moryia T, Konno R, Sato S, Yaegashi N, Okamura K, Sasano H: The correlation between the response to progestogen treatment and the expression of progesterone receptor B and 17β-hydroxysteroid dehydrogenase type 2 in human endometrial carcinoma. *Clin. Endocrinol.,* 58, 696-703, 2003.

Vihko P, Herrala A, Harkonen P, Isomaa V, Kaija H, Kurkela R, Li Y, Patrikainen L, Pulkka A, Soronen P, Torn S: Enzymes as modulators in malignant transformation. *J. Steroid. Bioch. Mol. Biol.,* 93, 277-283, 2005.

Vihko R, Apter D: Endogenous steroids in the patophysiology of breast cancer. *CRC Crit. Rew. Oncol./Hematol.,* 9, 1-15, 1989.

Voice MW, Seckl JR, Edwards CR, Chapman KE: 11β-Hydroxysteroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is hormonally regulated: a model system for the study of hepatic glucocorticoid metabolism. *Biochem. J.,* 317, 621-625, 1996.

Von Hoff DD, Layard MW, Basa P, Davis HL Jr., Von Hoff AL, Rozencweig M, Muggia FM: Risk factors for doxorubicin-induced congestive heart failure. *Ann. Intern. Med.,* 91, 710-717, 1979.

Walsh PC, Wilson JD: The induction of prostatic hypertrophy in the dog with androstanediol. *J. Clin. Invest*. 57(4), 1093–1097, 1976.

Wang M, He Y, Eisenman LN, Fields C, Zeng CM, Mathews J, Benz A, Fu T: 3β-hydroxy-pregnane steroids are pregnenolone sulfate-like $GABA_A$ receptor antagonists. *J. Neurosci.,* 22, 3366-3375, 2002.

Watanabe K, Sugawara C, Ono A, Fukuzumi Y, Itakura S, Yamazaki M, Tashiro H, Osoegawa K, Soeda E, Nomura T: Mapping of a novel human carbonyl reductase, CBR3), and ribosomal pseudogenes to human chromosome 21q22. *Genomics,* 52, 95-100, 1998.

Weihua Z, Lathe R, Warner M, Gustafsson JA: An endocryne pathway in the prostate, Erβ, AR, 5α-androstane-3β, 17β-diol, and CYP7B1, regulates prostate growth. *Proc. Natl. Acad. Sci. USA,* 99, 13589-13594, 2001.

Weiss RB: The anthracyclines: will we ever find a better doxorubicin. *Semin. Oncol.,* 19, 670-686, 1992.

Wermuth B, Bohren KM, Heinermann G, Von Wartburg J-P, Gabbay KH: Human carbonyl reductase: nucleotide sequence analysis of a cDNA and amino acid sequence of the encoded protein. *J. Biol. Chem.,* 263, 16185-16188, 1988.

Wermuth B, Platt KL, Seidel A, Oesch F: Carbonyl reductase provides the enzymatic basis of quinine detoxication in man. *Biochem. Pharmacol.,* 33, 1277-1282, 1986.

Wermuth B: Purification and properties of an NADPH-dependent carbonyl reductase from human brain. Relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase. *J. Biol. Chem.,* 256, 1206-13, 1981.

Wiebe JP, Muzia D, Hu J, Szwajcer D, Hill SA, Seachrist JL: The 4-pregnene and 5α-pregnane progesterone metabolites formed in nontumorous and tumorous breast tissue have opposite effect on breast cell proliferation and adhesion. *Cancer Res.,* 60, 936, 943, 2000.

Wiebe JP, Muzia D: The endogenous progesterone metabolite, 5α-pregnane-3, 20-dione, decreases cell-substrate attachment, adhesion plaques, vinculin expression, and polymerized F-actin in MCF-7 breast cancer cells. *Endocrine*,16, 7-14, 2001.

Wilson JD, Leihy MW, Shaw G, Renfree MB: Unsolved problems in male physiology: studies in a marsupial. *Mol. Cell. Endocrinol.,* 211, 33-36, 2003.

Wilson RC, Dave-Sharma S, Wei JQ, Obeyesekere VR, Li K, Ferrari P, Krozowski ZS, Shackleton CH, Bradlow L, Wiens T, New MI: A genetic defect resulting in mild low-renin hypertension. *Proc. Natl. Acad. Sci. USA,* 95, 10200-10205, 1998.

Winters CJ, Molowa DT, Guzelian PS: Isolation and characterization of cloned cDNAs encoding human liver chlordecone reductase. *Biochemistry,* 29, 1080-1087, 1990.

Wirth H, Wermuth B: Immunohistochemical localization of carbonyl reductase in human tissues. *J. Histochem. Cytochem.* 40, 1857-1863, 1992.

Wojnowski L, Kulle B, Schirmer M, Schluter G, Schmidt A, Rosenberger A, Vonhof S, Bickeboller H, Toliat MR, Suk EK, Tzvetkov M, Kruger A, Seifert S, Kloess M, Hahn H, Loeffler M, Nurnberg P, Pfreundschuh M, Trumper L, Brockmoller J, Hasenfuss G: NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. *Circulation,* 112, 3754-3762, 2005.

Wolfe GR, Smith CA, Hendrix DL, Salvucci ME: Molecular basis for thermoprotection in *Bemisia*: structural differences between whitefly ketose reductase and other medium-chain dehydrogenases/reductases. *Insect. Biochem. Mol. Biol. 2*9, 113-120, 1999.

Wsól V, Kvasnicková E, Szotáková B, Hais IM: High-performance liquid chromatographic assay for the separation and characterization of metabolites of the potential cytostatic drug oracin. *J. Chromatogr. B. Biomed. Appl.,* 681, 169-75, 1996.

Wsól V, Skálová L, Szotáková B, Kvasnicková E: Sex-related differences in stereospecifity of oracin reductases in rat in vitro and in vivo. *Chirality,* 11, 505-509, 1999.

Wsól V, Szotáková B, Kvasnicková E, Fell AF: High performance liquid chromatography study of stereospecific microsomal enzymes catalyzing the reduction of a potential cytostatic drug, oracin. Interspecies comparison. *J. Chromatogr. A.,* 797, 197-201, 1998.

Wsól V, Szotáková B, Martin HJ, Maser E: Aldo-keto reductases (AKR) from the AKR1C subfamily catalyze the carbonyl reduction of the novel anticancer drug oracin in man. *Toxicology,* 238, 111-118, 2007.

Wsól V, Szotáková B, Skálová L, Cepková H, Kvasnicková E: The main metabolic pathway of oracin, a new potential cytostatic drug, in human liver microsomes and cytosol. Stereoselectivity of reoxidation of the principal metabolite 11-dihydrooracin to oracin. *Enantiomer,* 5, 263-270, 2000.

Wsól V, Szotáková B, Skálová L, Maser E: Contribution of aldo-keto reductases to the metabolism of the novel anticancer drug oracin in man. In Weiner H, Plapp B, Lindahl R, Maser E: Enzymology and Molecular Biology of Carbonyl Metabolism 12. West Lafayette, Purdue University Press, 249-255, 2005a.

Wsól V, Szotáková B, Skálová L, Maser E: Contribution of aldo-keto reductases to the metabolism of the novel anticancer drug oracin in man. *Enzymol. Mol. Biol. Carbonyl. Metab.*, 12, 249-255, 2005b.

Wsól V, Szotáková B, Skálová L, Maser E: Stereochemical aspects of carbonyl reduction of the original anticancer drug oracin by mouse liver microsomes and purified 11β-hydroxysteroid dehydrogenase type 1. *Chem. Biol. Interact.,* 143-144, 459-468, 2003.

Wsól V, Szotáková B, Skálová L, Maser E: The novel anticancer drug oracin: different stereospecificity and cooperativity for carbonyl reduction by purified human liver 11beta-hydroxysteroid dehydrogenase type 1. *Toxicology*, 197, 253-261, 2004.

Yamamoto T, Matsuura K, Shintani S, Hara A, Miyabe Y, Sugiyama T, Katagiri Y: Dual effects of anti-inflammatory 2-arylpropionic acid derivatives on a major isoform of human liver 3α-hydroxysteroid dehydrogense. *Biol. Pharm. Bull.,* 21, 1148-1153, 1998.

Yamazoe M, Shirahige K, Rashid MB, Kaneko Y, Nakayama T, Ogasawara N, Yoshikawa H: A protein which binds preferentially to single-stranded core sequence of autonomously replicating sequence is essential for respiratory function in mitochondrial of *Saccharomyces cerevisiae. J. Biol. Chem..* 269, 15244-15252, 1994.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN: Living with water stress: evolution of osmolyte systems. *Science* 217, 1214-1222, 1982.

Zimmerman RA, Dozmorov I, Nunlist EH, Tang Y, Li X, Cowan R, Centola M, Frank MB, Culkin DJ, Lin HK: 5α-Androstane-3α, 17β-diol activates a pathway that resembles the epidermal growth factor

responsive pathways in stimulating human prostate cancer LNCaP cell proliferation. *Prostate Cancer Prostatic Dis.*, 7, 364-374, 2004.

Zucchi R, Danesi R: Cardiac toxicity of antineoplasic anthracyclines. *Curr. Med. Chem. Anti-Canc. Agents,* 3: 151-171, 2003.

Uetrecht JP, Trager W: Drug Metabolism. Chemical and Enzymatic Aspects. Informa Healthcare USA, Inc., 2007.

Scopes RK: Protein purification, principles and practice. Third edition, Springer-Verlag New York, Inc., USA, 1994.

www6.gelifesciences.com

www.bio-rad.com

www.enzymes.me.uk

www.med.upenn.edu/akr

11. Publications and posters

Publications

- *Included in this thesis*

Novotná R, Wsól V, Xiong G, Maser E: Inactivation of the anticancer drugs doxorubicin and oracin by aldo-keto reductase (AKR) 1C3. *Toxicol Lett.*, 181(1), 1-6, 2008

Skarydová L, Skarka A, Novotná R, Zivná L, Martin HJ, Wsól V, Maser E.: Partial purification and characterization of a new human membrane-bound carbonyl reductase playing a role in the deactivation of the anticancer drug oracin. *Toxicology*, 264(1-2), 52-60 2009.

- *Not included in this thesis*

Gavelová M, Hladíková J, Vildová L, Novotná R, Vondrácek J, Krcmár P, Machala M, Skálová L.: Reduction of doxorubicin and oracin and induction of carbonyl reductase in human breast carcinoma MCF-7 cells. *Chem Biol Interact.* 176(1), 9-18, 2008.

Cvilink V, Kubícek V, Nobilis M, Krízová V, Szotáková B, Lamka J, Várady M, Kubenová M, Novotná R, Gavelová M, Skálová L.: Biotransformation of flubendazole and selected model xenobiotics in Haemonchus contortus. *Vet Parasitol*., 151(2-4), 242-248, 2008.

Link M, Novotná R, Suchanová B, Skálová L, Wsól V, Szotáková B.: The stereoselective biotransformation of the anti-obesity drug sibutramine in rat liver microsomes and in primary cultures of rat hepatocytes. *J Pharm Pharmacol.,* 57(3), 405-410, 2005.

Posters

R Novotná, L Škarydová, V Wsól: Reduction of anticancer drugs doxorubicin and oracin by recombinant AKR1C3, RDPA 2007, Island of Elba, Italy, 2007.

V Wsól, R Novotná, L Škarydová: Inhibition of AKR1C3 human reductase activity by selected flavonoids and NSAID, RDPA 2007, Island of Elba, Italy, 2007.

R Novotná, L Škarydová, V Wsól: Reduction of anticancer drug doxorubicin by recombinant AKR1C3, XXVI. Xenobiochemický sjezd, Liptovský Ján, 2007.

V Wsól, R Novotná, L Škarydová: Role of AKR1C3 in metabolism of anticancer drug oracin, XXVI. Xenobiochemický sjezd, Liptovský Ján, 2007.

L Škarydová, R Novotná, M Hubálek, V Wsól: Carboxylesterase 1 – human liver microsomal carbonyl reductase?, XXVI. Xenobiochemický sjezd, Liptovská Ján, 2007.

R Novotná, V Wsól: Purification of novel human liver microsomal carbonyl reductase, XX. Biochemický sjezd, Piešťany, 2006

V Wsól, R Novotná, HJ Martin, E Maser: Phase II biotransformation enzymes involved in phase I biotransformation of the anti-cancer drug oracin in man, Hawai, 2005.