UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Biochimica e Biologia Molecolare

Ciclo XXIV

The protective role of antioxidants and the aldo-keto reductase AKR7A2 against toxic aldehydes in cell lines

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Ⅰ ABSTRACT

Aldehydes and ketones are widely distributed in nature and can be very reactive, causing damage to DNA, proteins and lipids within the cell. A number of antioxidant molecules are able to react with reactive molecules to minimize their damaging effects. The human body also has a series of enzyme systems for protecting itself against reactive aldehydes and ketones including alcohol dehydrogenases (ADH), aldehyde reductases (AKR) and aldehyde dehydrogenases (ALDH). Some antioxidants are also able to increase the expression of these protective enzymes thereby leading to enhanced protection. In this thesis, intrinsic and extrinsic mechanisms of protection against the toxicity of aldehydes were investigated.

In the first part of this thesis, the toxicity of acetaldehyde to HepG2 human hepatoma and 1321N1 astrocytoma cells was investigated as a model for liver and brain cells. Acetaldehyde is a toxic metabolite of ethanol, and is thought to be a major determinant in alcohol toxicity. Acetaldehyde did not cause significant toxicity to 1321N1 or HepG2 at concentrations below 1 mM, but was toxic to 1321N1 cells at higher concentrations. However, acetaldehyde was able to cause an increase in activated caspase-ca3 and DNA fragmentation at concentrations between 5 and 25 μM in HepG2 cells, indicating an induction of apoptosis.

Three antioxidants (vanillin, gallic acid and resveratrol) were investigated for their capacity to protect cells against the toxicity of reactive aldehydes. Methylglyoxal and acrolein are products of the peroxidation of lipids and arise during exposure of cells to oxidants. The antioxidants vanillin and gallic acid are found in whisky in the non-volatile fraction and resveratrol is found in red wine. The antioxidants were not able to protect the cells against the aldehydes at the concentrations tested, but did increase cell viability. It appears that vanillin can inhibit ADH as well as ALDH activity by binding to the enzymes without altering their expression levels.

To investigate whether the AKR enzymes are involved in protection against aldehyde toxicity, human AKR7A2 and the mouse AKR7A5 were purified and characterized with different substrates including muconaldehyde, crotonaldehyde, acetaldehyde and methylglyoxal. Both AKR7A2 and AKR7A5 enzymes showed a high Km and low turnover number for all three of the aldehydes tested, indicating relatively low specificity.

To better understand the role of AKR7A2, the gene encoding the enzyme was transiently transfected into 1321N1 and HepG2 cell lines and exposed to crotonaldehyde, muconaldehyde and methylglyoxal. However, AKR7A2 was not able to protect cells against aldehyde toxicity, and, in some cases, its overexpression increased toxicity. Only when crotonaldehyde was tested at a relatively high concentration the transfected cells did show increased viability compared to the control, suggesting that the enzyme AKR7A2 could be involved in crotonaldehyde detoxification. Transfected HepG2 cells showed an increase in viability compared to the non-transfected control cells, at almost all the tested concentrations. Taken together these results show that AKR7A2 is able to protect HepG2 liver cells against the aldehydes, but is not able to protect 1321N1 brain cells.

II INTRODUCTION

ETHANOL OXIDATION

The consumption of alcohol (ethanol) is common in many countries around the world and in some places overconsumption has become a social problem. The potential dangerous effects caused by alcohol are influenced by the time and quantity present in the blood stream. Initially the alcohol, after ingestion, starts diffuse throughout the body. This is the first stage of alcohol metabolism. Only a small part of ethanol is able to leave the body by evaporation or during breathing. In the second stage, a low amount of ethanol estimated between 2 and 10% is metabolized by kidney and lung, but the highest amount is metabolized by the liver (1). It is difficult to establish a proper and exact rate for alcohol metabolism; different characteristics such as age, ethnicity and habits such as smoking and diet can interact with the metabolism rate. In a recent study it was established that an average rate for ethanol metabolism is between 50-80 mg/h/kg (2). However a major influence is made by the amount and efficiency of the enzymes involve in metabolism (3).

The metabolism of alcohol has been studied extensively and appears to be predominantly a series of oxidation reactions. The conversion of molecules through oxidation reactions is classified as Phase I metabolism (functionalization), and several different enzymes are involved in these reactions including alcohol dehydrogenases (ADH), cytochromes P450 and catalase. Hepatocytes have all three pathways for metabolizing ethanol. The three pathways are located in three different places inside the cell. The catalase enzymes are located inside the peroxisomes, the cytochrome P450 system in the endoplasmic reticulum (microsomes) and the ADH in the cell's cytosol.

Each metabolic pathway generates different products, but they share the same main metabolite, that is acetaldehyde. This metabolic product is reputed to be very dangerous and responsible for the main damage caused by alcohol abuse.. Figure 1 shows the main oxidative pathways involved in alcohol metabolism. Each of these enzyme families are described in detail below. Further metabolism is then carried out by aldehyde dehydrogenase (ALDH) enzymes, described in the next section.

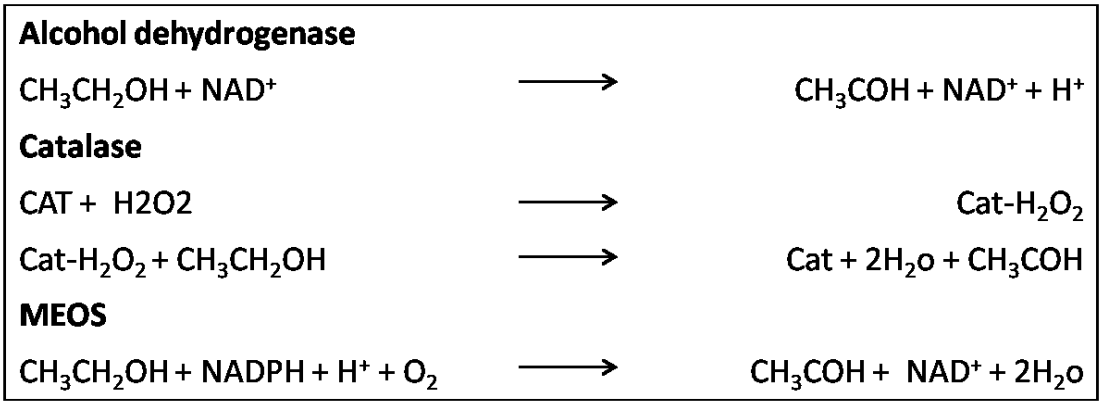


Figure 1: Main ethanol oxidation pathways in humans.

ALCOHOL DEHYDROGENASE

The enzymes of the alcohol dehydrogenase (ADH) family are responsible for alcohol oxidation and consequently they modify the time and the concentration of the alcohol in the human body. The alcohol dehydrogenases (ADH), are a group of enzymes and Class I is the most important class of ADH in ethanol oxidation. The ADHs are a group of enzymes composed of two subunits. Each subunit has a molecular weight around 40kDa, and each enzyme is made by two identical subunits. The ADH enzymes are classified in five different classes compared by sequence homology and the catalytic properties. All five classes are present in human (4). Each enzyme has two independent catalytic active sites, one for each subunit. In the core of the enzyme, there is a zinc atom. In Table 1, a summary of the different classes of ADH present in the human body and the different subunits made from different genes as well the distribution of the different enzymes in human body is given.

Table 1: Alcohol dehydrogenase classes and subunits differentiation and distribution in human body.

Class	Gene	Subunit	Distribution	K_m (mM)
I	ADH1A	α	Liver	4
	ADH1B*1	β1	Liver, lung, kidney	0.05-34
	ADH1B*2	β2		0.6-1
	ADH1B*3	β3		
	ADH1C*1	ɣ1	Liver, stomach	
	ADH1C*2	ɣ2		
II	ADH2	π	liver	34
III	ADH3	χ	Everywhere	1000
IV	ADH4	μ	Stomach, esophagus, liver, skin	20
V	ADH5	?	stomach	30

The first class of ADH (Class I) shows a very low K_m value for ethanol, indicating high affinity. These enzymes are very easily saturated but the high levels present in the liver indicate that they play a central role in ethanol detoxification. The first class of enzymes can be easily inhibited by pyrozilic derivatives (4).

The Class II ADH enzymes show a relatively high K_m and hence low affinity for ethanol, and in contrast to the Class I enzymes these can't be blocked by pyrozilic derivative as reported from (5).

The Class III ADH enzymes have a very high K_m and very low affinity for ethanol, and for this reason it is believed that they do not play an important role in the ethanol oxidation. Also experimental data has shown that the Class III prefers alcohols with a long chain or fatty acid, especially the ω (4). More recent studies have shown that this class of enzymes may be involved during the acute intoxication, because the expression of this class looks to be dependent on the amount of alcohol ingested (6).

The Class IV ADH are enzymes widely distributed in the body: stomach, esophagus, cornea are just some example of distribution. They have a high K_m for ethanol of around 1M and it is difficult to saturate them with the physiological alcohol concentrations found normally in the human body and also in hard drinkers.

The Class V ADH is identified with the name ADH6. This enzyme has not been purified yet, but its mRNA has been found in the stomach and liver. In *vivo* studies show it to have an apparent K_m around 30 mM. (7).

The Class VI ADH has been discovered only in rat and mouse.

CYTOCHROME P450 OR MEOS

Other than the ADH enzymes, other metabolic pathways are believed to play an important role in ethanol oxidation (6). This pathway, independent from ADH has been identified and it became relevant in acute intoxications and in subjects that are chronically addicted to alcohol. This pathway is also known as the Microsomal Ethanol Oxidizing System (MEOS), (8) because the oxidation process happens inside the endoplasmic reticulum (ER) (microsomal) lumen.

The enzymes responsible are members of the cytochrome 450 (CYP) super family, and the isoenzymes CYP2E1, 1A2 and 3A4 are mainly responsible for ethanol oxidation and are localized in the liver in the internal lumen of the ER. The enzymes utilize NADPH and O_2 for their metabolizing system (1). The isoform CYP2E1 is inducible by chronic alcohol consumption (9) for this reason this metabolic pathway is believed to play a major role in alcoholism, more so than in the occasional drinker. This pathway in the brain is very important because the ADH enzymes present there, show a very low affinity for alcohol metabolism, leaving brain cells more exposed to damaging effects of ethanol. The cytochromes P450 have a K_m for ethanol between 2-8 mM (2).

The MEOS is not only an exclusive ethanol metabolism system but can also interact with other hepatotoxins. This metabolic pathway however can produce some dangerous molecules known as Reactive Oxygen Species (ROS).

These molecules are derived from oxygen and can react with different molecules present in cells such as DNA, RNA, protein and lipids and cause significant damage. In particular, the end products of the reaction between lipids and ROS are very dangerous, and normally are aldehydes and ketones with a high reactivity against the macromolecules cited above (10). Hence, although the MEOS can potentially reduce the levels of ethanol in a system, the unwanted side effects can cause more extensive molecular damage than the ethanol itself.

CATALASE

Catalase enzymes are also involved in alcohol oxidation although their role is less clear (6). Catalase is an enzyme that is located inside the peroxisomes. This enzyme has the capacity to oxidize ethanol and has been proposed to act in two different ways. In one route, during catalysis, catalase forms a complex with oxygen and peroxide, and this complex can react with a molecule of ethanol to give two molecules of water and acetaldehyde and the free enzyme. The second route involves the cofactor NADPH and a molecule of oxygen, which by the action of the NADPH oxidase gives oxygen, peroxide and $NADP^+$. In a second step the ethanol is oxidized and the molecule of peroxide is reduced by the action of catalase (9).

This metabolic pathway seems have an important role especially during the first phase of alcohol metabolism. Other studies carried out using rats shows an increase in hydrogen peroxide when the rats are driving to an chronic alcohol abuse. Other than catalase, the MEOS systems seem to increase its activity (11). Other mechanisms involving catalase are present in the human body, but they play a minor role.

ALDEHYDE METABOLISM

ALDEHYDE DEHYDROGENASE

The aldehyde dehydrogenase enzymes (ALDH) are a super family of enzymes that were discovered in the early 1940s. These enzymes serve to oxidize aldehydes and are involved in the second step of ethanol metabolism because they are able to oxidize the acetaldehyde derived from the first step of ethanol oxidation. The acetaldehyde is oxidized to acetate, which can be used by other metabolic pathways.

ALDH enzymes use the cofactor NAD^+ and have a huge range of substrate in addition to acetaldehyde. They can accommodate in their active site different molecules with different chemical structures: aliphatic and aromatic aldehydes that can be derived from different sources, both exogenous and endogenous. Many aldehydes and ketones are directly involved in the aroma of some fruits such as apples (12). The aldehydes made inside the body are normally derived from the metabolism of amino acid, lipids, steroids and during the metabolism of some drugs, including alcohol. Exogenous sources of aldehydes are cigarette smoke, pollution, and food.

Reactive aldehydes can be detoxified by both Phase I reactions (oxidation by ALDH or reduction by ADH and aldo-keto reductases) and Phase II reactions (conjugation to eg glutathione by glutathione S-transferases) (13).

The substrate needs to be very specific for some ALDH enzymes, but other enzymes can accept a wide range of different substrates. Some ALDH have an important role tissue development or to keep them perfectly operational. An example of this physiological role is the conversion of the aldehyde retinal (retinaldehyde) into retinoic acid which is essential for the brain development.

The ALDH enzymes are subdivided in different subclasses, as in the case for the ADH on the basis of the amino acid sequence similarity, which can be related to chemical, physical characterize, catalytic propriety and distribution in the different tissue (14). In the human genome, nineteen different aldehyde dehydrogenases have been identified (15). The most studied aldehyde dehydrogenases are the class I ALDH1 and the class II ALDH2. The ALDH1 and ALDH2 share 68% of sequence identity and they have a chain of 499-500 amino acid. These two enzymes are composed of four identical subunits (16). By multiple sequence alignment system and crystallography it was possible identify the conserved region of the catalytic site and the cofactor binding site. Four amino acids are essential for the catalysis Cys-302, Glu-268, Gly-299 and Asn-169.

Other amino acids are involved in the binding of the cofactor: Gly-245 and Gly-250. These amino acids are important for the Rossmann fold. (The amino acid numeration is based on the ALDH2 sequence present in the human species). Briefly the Cys-302 changes its position when the cofactor binds the subunit and this result in activation of the enzyme. The role of the Cys-302 is to be the nucleophile. The reaction between the nucleophile and the aldehyde results in an oxyanion thiohemiacetal intermediate. The intermediate is stabilized by Asn-169. The negative charge on the oxygen drives the next step, where the hydride is lost from the tetramer atom of carbon to bind the cofactor to change its redox state to NAD(P)H. A molecule of water hydrolyzes the thioacylenzyme intermediate helped by the Glu-268 that works as a base. This second nucleophilic attack gives the free enzyme and the production of the reaction the carboxylic acid. In this mechanism the cofactor is very important because it is the first to bind the enzyme, and the last to leave the enzyme. The interaction of the cofactor with the enzyme is important for the Vmax of the enzyme. In Figure 2 is the mechanism of action proposed by (15).

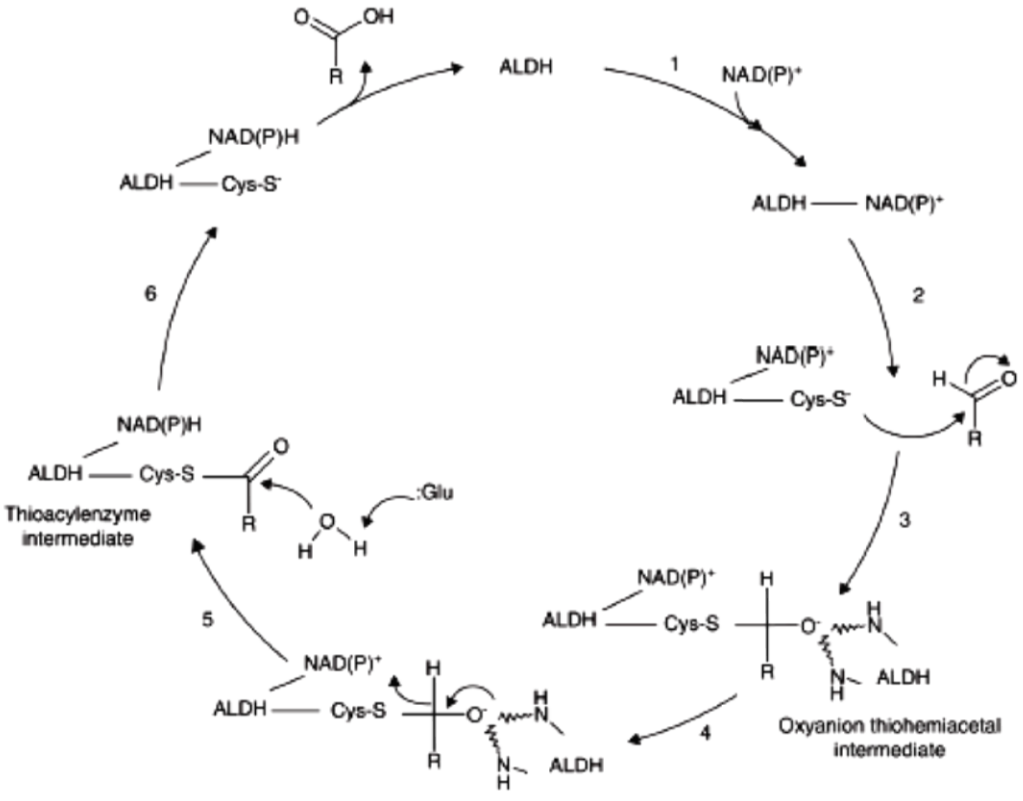


Figure 2: ALDH catalytic mechanism. 1, Cofactor binding results in a conformation change of the enzyme and activation of the catalytic thiol (Cys-S−); 2, Nucleophilic attack of the aldehyde substrate; 3, Oxyanion intermediate is stabilized by two NH groups of the ALDH peptide chain; 4, Hydride transfer to cofactor. 5; Glutamate residue acts as a base catalyst in the hydrolysis of the thioacylenzyme intermediate; 6, Release of carboxylic acid product followed by cofactor.

ALDH I FAMILY

ALDH1A1

The gene production *ALDH1A1* is a tetramer protein ALDH1A1 made by identically subunit. The subunits have a molecular weight around 55kDa. This enzyme is widely distributed in different tissue such as brain, kidney, liver and gut. It is present at a very high concentration (3%) in the cytosol of the retina (17). More specifically, this protein provides a protective role against ultra violet radiation (UVR). This protein is also called RALDH1 because show a very low value of K_m 0.1μM for retinaldehyde and catalyzes the irreversible oxidation of retinaldehyde to retinoic acid and acetate (18). The active site of this enzyme is also able to accommodate acetaldehyde but the enzymatic rate of this reaction is slow compared to retinaldehyde, and the affinity of the enzyme for acetaldehyde is relatively high, between 50-100 μM(19).

ALDH1A1 also plays a role in the detoxification of various reactive aldehydes generated during lipid peroxidation. These aldehydes are very reactive with almost all the macromolecules present in cells. ALDH1A1 is able to metabolize 4-hydroxy-2-nonenal (4-HNE) with a K_m of 17.9 μM, hexanal with a K_m of 14.3 μM, and malondialdehyde (MDA) with a K_m of 114.4 μM (15). Other than reactive aldehydes, this enzyme is also able to oxidize the aldehydes present in some anti cancer drugs. This results in reduced efficiency of the drugs. A rat variant of this enzyme known as ALDH1A2 can metabolize retinoids *in vitro* with a lower K_m (0.2μM) compared to the human ALDH1A1. However this variant has lower efficiency for acetaldehyde with a K_m of 0.65 mM (20).

ALDH1B1

In contrast to other enzymes of the ALDH1 family, ALDH1B1 has acetaldehyde as a favorite substrate, with a K_m of 30 μM. This enzyme is localized in the cytosol of many types of cells, including liver, kidney and brain (15). UV light has shown the capacity to up regulate this enzyme in vivo (21).

ALDH II FAMILY

ALDH2 is localized in the mitochondria, and has four subunits each with a mass of around 56 kDa. Based on sequence similarity this enzyme could be called ALDH1B and should be in the class I, but it is commonly reported in the scientific papers as ALDH2. Sequence similarity with the Class I enzymes is around 70%.

ALDH2 enzymes have a key role in the metabolism of the ethanol, and their best substrates are acetaldehyde. ALDH2 is present in the liver where the first step of the ethanol metabolism occurs, and can metabolize the acetaldehyde in acetate. Its K_m for acetaldehyde is 1 μM (15). ALDH2 enzyme is also expressed in kidney, heart, lung and brain. Of relevance, is the allele variant that is high diverse in the Asiatic population, the variant ALDH2*2. This variant is briefly discussed in section 0 THE ALDH2*2 IN ASIAN POPULATION. Other than acetaldehyde other molecules can be metabolize by ALDH2 including chloroacetaldehyde (13). Of importance is the capacity of this enzyme to metabolize 4-hydroxy-2-nonenal (4-HNE), and it is also able to metabolize some inorganic acids such as nitrate reductase in inorganic molecular as nitroglycerin (22).

ALDH III FAMILY

ALDH3B1

This enzyme plays a central role in the detoxification of long chain aldehydes such as octanal, but is not so efficient with short chain aldehydes such as acetaldehyde or malondialdehyde (MDA). The efficiency of this enzyme with different toxic substrates has been evaluated by transfecting human cells with the gene encoding the enzyme (23). This protein is able to use both the cofactor NAD^+ and $NADP^+$. This protein is also highly expressed in kidney and liver.

ALDH7A1

The aldehyde dehydrogenase found in black seabream fish (sbALDH7A1) was investigated to try and establish the putative role of the human enzyme because it shares 83% of identity. The fish enzyme is able to metabolize different aldehyde including acetaldehyde (K_m 3.6 mM), propionaldehyde (K_m 1.2 mM), and benzaldehyde (K_m 0.45 mM) (15). Later the gene encoding the human enzyme was cloned (24) and is role was investigated.

It was supposed to play a defensive role against aldehydes made during cell metabolism based on its sequence similarity with analogues in plants. This protein was tested with different products normally made during lipid peroxidation inside the cell. This protein is able to metabolize aliphatic molecules such as octanal and trans-2-nonenal, which appear to be the best substrate for this enzyme; but also with a K_m one thousand times higher with molecules such as glyceraldehyde. The human and the fish enzyme show a close K_m to each other for the substrate benzaldehyde, respectively 0.53mM and 0.45mM.

THE ALDH2*2 IN ASIAN POPULATIONS

A single amino acid substitution from lysine to glutamate in position 487 makes the variant ALDH2*2. The mutation does not involve the catalytic sites directly, but is able to change the conformation of the protein. The change in conformation reduces the nucleophilic power of the Cys and reduces the cofactor affinity. Compared to ALDH2, the ALDH2*2 has a very slow turnover (Kcat) for acetaldehyde. This low turnover value means this variant enzyme is not so efficient in removing acetaldehyde present in the blood stream (25). This natural variant is not generally widely distributed in the human population but is very common in the Asian population. It is estimated that around the 50% of this population express this slow variant of ALDH2 (16). The slow variant results in abnormal accumulation of acetaldehyde in blood after the ingestion of ethanol compared to the people with the normal ALDH2. The high and long permanence of the acetaldehyde in the blood stream induces some adverse effect to these people such as: red flushing in the face, warm face, nausea, dizziness and tachycardia. These adverse affect in half of the Asian people are proposed to be the reason for the low incidence of alcoholism in this country (26),(27).

ALDO-KETO REDUCTASE SUPERFAMILY

The aldo keto reductases (AKR) are a broad group of enzymes that form a superfamily. These enzymes are also widely distributed in nature, being present in human, invertebrate, protozoa, plants, yeast and in the bacteria (28). These enzymes are involved in different reactions from the biosynthesis of new molecules to reaction intermediate and detoxification of various aldehydes and ketones from endogenous and exogenous source. The AKR enzymes catalyze the reduction of aldehydes and ketones to their corresponding primary and secondary alcohol. NADH and NADPH are both cofactors for this family of enzymes.

More than one hundred proteins are believed belong to the AKR superfamily. This protein superfamily includes aldose reductases, aldehyde reductases, hydroxysteroid dehydrogenases and dihydrodiol dehydrogenases. All these proteins seem to have arisen from a single multi-functional gene; this gene has disappeared during the evolutionary process. These enzymes went on to develop different affinities for different substrates.

Based on sequence identity an official nomenclature has been accepted for these enzymes. The sign AKR is for aldo keto reductase enzyme; the first Arabic number is used for identify the subfamily and the following letter for the subclass, the last Arabic number is for identify a unique protein sequence. Each AKR subfamily differs from other AKR subfamilies by its members having less than 40% amino acid identity to other subfamilies. Inside the same subfamily, the identity is higher, and enzymes are in the same subclass when they have over 60% identity. A web page (29) is periodically updated with the new AKR discovered, and now it is possible count fifteen families (AKR1 to AKR15).

Different substrates can interact with the active site of these enzymes including steroids, glucose, many glycosilate molecules and many molecules derived from the lipid peroxidation. Other than protecting cells from aldehydes and ketones, this class of enzymes is responsible for the Phase I metabolism of a broad range of drugs and xenobiotics. In some cases this can be a problem, because interaction with some therapy drugs can reduce their efficacy before they react with their molecular target.

STRUCTURE AND KINETICS

The average size of the AKR enzymes is 350 amino acids. This chain forms a conserved $(\beta/\alpha)_8$ barrel structure in all the aldo keto reductase family, but some loops differ from enzyme to enzyme in order to determinate substrate specificity. Four amino acids are involved in the catalytic action mechanism: tyrosine, lysine, aspartate and histidine (30). The structure is usually monomeric and that is true for all most the family; however the families AKR2, AKR6 and AKR7 can all form multi subunit enzymes as dimers or tetramers (31).

The three-dimensional structure in Figure 3 shows the typical barrel structure for the AKRs. Eight β-sheets are responsible of the structure of the barrel. Every sheet is linked to the other with an α-helix anti parallel to the barrel. In total eight α-helices are used to link all eight β-sheets. Another two loops are present in this structure but they are not involved in the junction with the β-sheet. They seem to be involved in keeping the right three dimensional structure of the molecule (32). A multiple sequence alignment of many AKRs has shown that the least conserved regions are in the last three carboxy terminal loops. As proposed (28) the loops near the C-terminal are involved in determining substrate specificity, but it is not totally clear the mechanism by which these enzymes are able to distinguish between different substrates. The numeration of the following amino acid residues is based on the rat 3α-hydroxysteroid dehydrogenase (3α-HSD). The Asp-50, Tyr-55, Lys-84 and His-117 are the amino acids involved in the catalytic system in both the oxidation and reduction direction. Another five amino acids are conserved in the same family and are involved in cofactor binding (either NADH or NADPH). The AKR proteins do not show the typical Rossmann fold present in other kinds of proteins. (32). However, different studies have shown that the amino acids involved in the cofactor binding are much conserved. The position of the cofactor inside the AKR proteins is conserved and the nicotinamide ring is positioned in the core of the barrel. Many hydrogen interactions stabilize this interaction. These amino acids are much conserved also between different proteins with sequence identities of less than 30% (28).

Within the same family the AKR enzymes hold the same structure in space. Even a single amino acid changed in the catalytic site is able to switch the enzyme catalytic system from carbon oxidation to a double carbon bond oxidation (28).

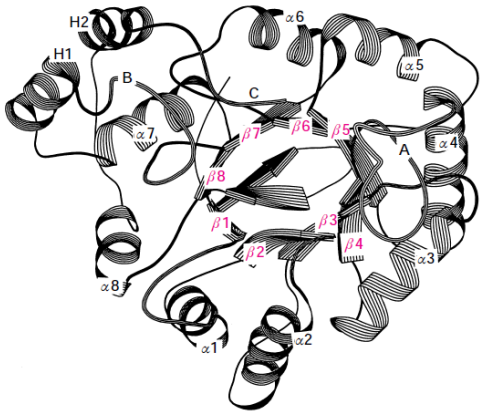


Figure 3: structure of the main AKRs. In pink the β- sheet structure that involve in the barrel. In black α-helix involve in the barrel structure. With H1 and H2 the two α-helix not involved in the barrel structure. From (28).

The catalytic reaction itself is divided into many steps. What has been shown for many AKRs is that the cofactor is the first molecule to bind to the enzyme (before the substrate) and is the last one to leave the enzyme (33). After this first step, the protein will be able to bind the right substrate. The oxygen of the aldehyde or ketone group involved in the reaction acquires a proton group from one of the amino acids involve in the catalytic process. Now the atom of carbon can be reduced by the hydride group donated by the cofactor. It is not clear which is the first hydrogen to be added to start the reduction process (28). The process that drives the carbon reduction that will accept the negative hydrogen is an amino acid in the catalytic system. This amino acid has an acid residue that is able to polarize the oxygen so that the carbon is active for the nucleophilic attack by negative hydrogen. For reduction the pKa and to make easier the polarization of the oxygen by the amino acid with an acidic residue from another amino acid, normally a tyrosine is very close to the acidic residue. This tyrosine has the role to make the surroundings around the acidic residue more favorable for donating a proton to carbon oxygen. This mechanism has been shown for many AKR.

After the reduction or oxidation, the substrate is let free and its dissociated to the enzyme. In the last step the oxidized cofactor is separated from the enzyme (34). The interaction between the enzyme and the cofactor seem to be the limited factor in the turnover of the substrate (35). This reaction mechanism has been identified in AKR1C2.

THE AKR7A FAMILY

THE RAT ENZYME AKR7A1

Aflatoxin B1 (AFB_1) is one of the most diffuse oxidative agents in the world. This dangerous toxin is made by *Aspergillus flavus toxin* and was found in many foods. The main target of this toxin is the liver (36). In the 1993 a research group from Scotland discovered that rat fed with a diet rich in ethoxiquin were more resistant to the AFB_1 (37). Ethoxyquin is able to increase one protein, an aldehyde reductase. This protein is able to interact with the aldehyde group present in the AFB_1 and change them into alcohols generating the AFB_1-dihydrodiol. This AFB_1-dihydrodiol is not able to react with proteins and DNA and for this reason is less dangerous that the dialdehyde form. Sequence analysis shows that this protein is a member of the aldo-keto-reductase family (38). In the same paper the authors suggest that this enzyme could have others physiological roles and they suggest the possibility to an analogous enzyme in human.

In a study carried out to characterize the enzyme, it was cloned and expressed in bacteria with a His-tag, and it was shown that it is able to work at a broad range of pH from 5 to 9 with the optimum around pH 6.6 estimated using as substrate the 4-nitro benzaldehyde (4-NBA) (39). 4-NBA is recognized is a model substrate for AKR, and AKR7A1 was also able to reduce a broad range of aldehydes and diketones.

The crystal structure of AKR7A1 has been determined and this enzyme was shown to be a dimer. It is one of the few AKR enzymes that are dimeric.

HUMAN AKR7A2

With a blast analysis based on the nucleotide sequence of the rat aldo-keto reductase AKR7A1, a human library was screened and a related protein identified. This protein shares with the rat a 78% identity and 87% similarity (40). Based on this data it was assumed that AKR7A2 is the corresponding protein to the rat AFB_1 aldehyde reductase (AKR7A1). A year later Schaller et al. cloned a succinic semialdehyde reductase (SSA) from human brain (41). The enzyme showed the same SDS-PAGE mobility as the SSA reductase.

They showed that their enzyme gave a very poor match with the AKR superfamily, showing less than the 20% identity with the AKR1 family and a bit higher with the AKR6 family known also as the K+ channel. Despite this, the enzyme was identified as part of the aldo-keto reductase family, being identical to the AKR7A2 cloned by Ireland et al., 1998 (40).

The AKR family are characterized by their ability to transfer the 4 H^- R donate by the cofactor, whereas the SSA reductases were originally thought to transfer the 4 H^- S (42). The enzyme characterized by (41) showed a very low K_m for SSA indicating that this enzyme plays a key role in the metabolism of the SSA in vivo. This enzyme shows a low K_m for aflatoxin B1, one of the original substrates identified for the rat enzyme AKR7A1.

In the paper by (40), they discovered that AKR7A2 is the principal enzyme responsible for metabolizing 2-carboxybenzaldehyde (2-CBA) to its alcohol in the liver. 2-CBA is an important drug and is metabolized to its corresponding alcohol by AKR7A2 (40). Studying different substrates to understand the physiological role of this enzyme showed that AFB_1 is not the best substrate and that the then enzyme has a K_m for SSA that is lower than for the toxin, and more importantly SSA is a normal chemical present in the body. GABA-transaminase, the precursor of SSA for the SSA-reductase is widely spread throughout the body. The AKR7A2 enzyme is also widely distributed in many tissue and organs like liver kidney, pancreas, skeletal muscle and in more moderate amount in the liver, heart, testis and ovary. Lower amounts of mRNA were found in the brain and colon. These data, the low K_m for SSA and the wide distribution led to the proposal (40) that AKR7A2 has a specific role in SSA metabolism not just in the brain. Often the gene encoding the AKR7A2 in the chromosome I is missing in many kinds of cancer (43). This evidence suggests that this enzyme is able to protect cells against the cancer. Other evidence shows a high level of expression of this enzyme in the brain and its role is in GHB biosynthesis in response to a degenerative stress such as Alzheimer's disease. More works need to be done to discover the real role of this enzyme that is widely expressed in the human body.

Another point that needs to be clarified is the relationship between the antioxidant and the expression of this human enzyme, especially after it was discovered is that the rat AKR7A1 is inducible by the antioxidant ethoxyquin (37).

MOUSE AKR7A5

The enzyme known as AKR7A5 was cloned from mouse, and shares 78% identity with the rat AKR7A1, but is more closely related to the human AKR7A2 with 89% of identity. A multiple sequence alignment of this new class of enzymes revealed they show a very high identity to each other placing them in a proper category the AKR7 family, that are quite distant to most other mammalian AKR enzymes (44). To study the role of this enzyme, it was cloned and a his tag purification, dynamic light scattering showed this enzyme is also dimer (45).

This enzyme also is versatile for the cofactor and is able to use NADPH and NADH, but with a strong preference for NADPH (45). AKR7A5 showed a very high affinity for the aromatic aldehydes, but a very low activity for the benzaldehyde. The aliphatic aldehyde SSA gave a very high rate that is almost double the rate of the 4 nitrobenzaldehyde. In contrast, the specific activity with aldose sugars is very low.

More analysis is needed to discover the physiological role of this enzyme in the mouse. This enzyme is also able to metabolize some powerful and reactive aldehydes such as trans-trans muconaldehyde that is derived from benzene detoxification in the liver. Trans-trans muconaldehyde is considered an exogenous aldehyde, whereas acrolein is a powerful toxic aldehyde made endogenously during the oxidative stress. For both these aldehydes this enzyme shows a very low activity (44).

The structure of the AKR7A enzymes is the typical for the family of aldo keto reductases. It has a barrel made with eight β-sheets that are linked by many α-helices. Two α-helices H1 and H2 are involve in the three dimensional structure of the enzyme. A reported for other aldo keto reductases each subunit has a cofactor molecule bound that makes various interaction with amino acid chain. The nicotinamide ring is deeply inserted in the core of the barrel. The reaction site is conserved and is typical for the AKR. Tyr 45 and His 109 (base on the human AKR7A1 numeration) are involve in the interaction with the substrate, Asp40 and Lys73 have the role to made a more favorable environment for the proton transfer give way from Tyr45 during the reduction reaction. As in other AKR the more important loops A-C play a crucial role in the substrate recognition (46).

The structure of the AKR7A5 enzyme is show in Figure 4 with the cofactor $NADP^+$ and the citrate used in the crystallization process.

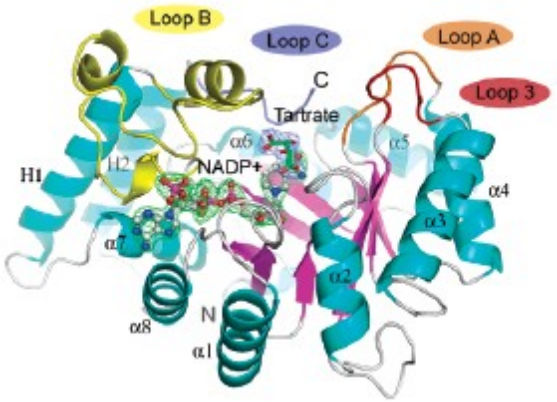


Figure 4: structure of mouse aldo keto reductase AKR7A5. Loops A, B and C are shown for their important in the substrate identification and interaction. The nicotinamide ring cofactor $NADP^+$ is localize in the core of the barrel in violet. In light blue the α-helices. H1 and H2 α-helices involve in the right structure of the protein from (46).

This crystal structure is very close to human AKR7A1, just some amino acid difference in the loops involved in the substrate recognition, these amino acids difference seem be the answer to the different activity of these enzymes for the same substrate (46).

ACETALDEHYDE TOXICITY

Acetaldehyde CH_3CHO, is the main metabolite of ethanol, generated during the first step of the ethanol oxidation. Acetaldehyde is also present in a wide range of food and beverages and pollution. Also cigarette smoke is able to produce this dangerous aldehyde that is breathed during the smoking. The bacteria present in the gut and in the mucosa is also a source of acetaldehyde. The bacteria in the gut make acetaldehyde during the metabolism of different molecules and expel it into the gut. The bacteria present in the teeth and mouth are also a source of acetaldehyde; an experiment showed that the acetaldehyde concentration in the mouth after the ingestion of ethanol is around 50 to 200 μM. This value is influenced by the oral hygiene of the mouth (47).

The Environmental Protection Agency (EPA) had classified acetaldehyde as a class II component. This means that is probably able to generate cancer in the human. This is supported by some experiments carried out using rats - these animals develop cancer in the nose and throat after being exposed to air and acetaldehyde (48). The acetaldehyde in the human body is metabolized to acetate. Different enzymes are able to carry out this reduction but particularly the acetaldehyde reductase. A study made by (49) using human cells and animals proved that this toxic aldehyde is mutagenic and is also carcinogenic.

O6-methylguanosine transferase is an enzyme involve in the DNA repair when damage by alkylating agents. Acetaldehyde in very low amounts (0.01 μM)is able to stop the activity of this enzyme (50). A study using plasma serum showed that acetaldehyde is also able to interact with membrane proteins. When the concentration of acetaldehyde is 100 μM a small amount of it (38 nM) had reacted with membrane proteins (51). Damage to DNA was much greater. Other than protein damage the acetaldehyde is able to interact directly with DNA and damage it. The *TP53 gene* is a tumor suppressor gene. After acetaldehyde exposure this gene showed a modification in the guanidine that is swapped with an adenine, leading to a common mutation found, which can have drastic effects on the proteins function and folding. Sometimes the mutation in the gene makes it not able to encode the protein. (52). The minimal amount of acetaldehyde able to induce this modification is 100 μM (53); however 100 μM is a value higher than normal acetaldehyde found in the blood stream in heavy drinkers (10 μM).

LIPID PEROXIDATION PRODUCTS

Living cells are enclosed by a double layer of phospholipids. These lipids are esterified with a molecule of glycerol. The lipids can be saturated or not saturated. Indirectly different pathways such as protein turnover, sugar metabolism and respiratory chain in the mitochondria are able to affect the lipid membrane. These pathways produce some harmful oxidant. The oxidant are able to remove an hydrogen to the lipid for made itself more stable but they leaving the fats chains more reactive to the oxygen. This is the starter of a long chain of lipid oxidations, were every radical lipid try to stabilize itself stolen a hydrogen from a neighbor lipid more unsaturated than it. This oxidative process is able to made some very dangerous molecule in the body, some of them still remaining in the lipid membrane linked with the glycerol but others are able to move around the cells. The oxidizing process is able to break the lipid chain giving rise to two different molecules. The molecules generated during this long series of reactions are aldehydes and ketones. It has been reported that these molecules are able to form adducts with other macromolecules present in the human body such as DNA, proteins or lipids. The interaction with DNA leads to the generation of mutations during DNA replication. In Figure 5 it is possible to see the main products derived from the oxidation of the linoleic acid. 4-hydroxy 2(E) nonenal (HNE), malondialdehyde (MDA) and 4-oxo 2(E) nonenal (ONE) are all α-β-unsaturated aldehydes derived from the homolytic breakdown of the polyunsaturated fatty acids. Not only are the unsaturated fats able to give rise to reactive aldehydes and ketones, the homolytic breakdown of the polyunsaturated fatty acid (PUFA) are able to generate a large amount of other reactive molecules (54). These electrophiles are able to react readily with proteins and DNA. This process is continuous in human body and its metabolite generate during the DNA repair after the adduction with the reactive aldehydes can be monitored in the urine. Some factors such as smoking are able to increase the amount of metabolites that can be detected in the urine. It is also possible using this metabolite as cancer biomarker (55).

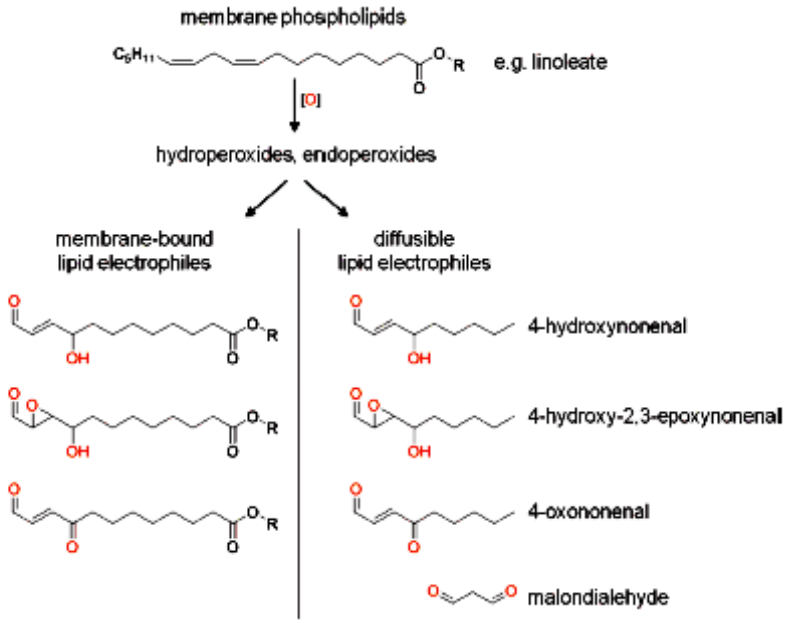


Figure 5: diffusible lipid electrophiles and membrane-bound lipid electrophiles made by membrane phpspholipids from (56).

Cells have developed different ways for protect itself from these dangerous molecules. Glutathione (GSH) is an important defense inside the cells, and is able to supply reducing equivalents to these aldehydes. Helped by the glutathione S-transferase (GST) it is able to forms adducts between the active lipids and GSH. These adducts are easy transport out to the cells. Some molecules directly derived from the lipid peroxidation such as HNE are so reactive with GSH, they have the effect of lowering the amount of GSH inside the cells. Other enzymes such as the aldo-keto reductases and aldehyde dehydrogenases can reduce aldehydes to alcohols. The alcohols are less reactive than the aldehydes. The importance of these aldehydes and ketones generated during the peroxidation process is apparent as when their amount is to high inside the cells and the cell is not able to eliminate them, this leads to cellular damage and can contribute to the aging process (57).

ACROLEIN

Drinking a normal wine can expose the human body to a various toxic compounds. Other than acetaldehyde in the wine it is possible to find another more toxic aldehyde, acrolein (2-propenal). The precursor is the 3-hydroxypropinaldehyde formed during the glycerol fermentation by bacteria (58). Wine is not the only external source of acrolein, and many foods are a source of it as well as cigarette smoke. Fried food is a source of acrolein because of the reaction between the fatty acid (oil) at high temperature used during the process (59).

Acrolein is present in the air as a product of the fuel combustion. This means that acrolein can enter the human body by ingestion or inhalation.

Acrolein is also present in the body naturally. Acrolein is derived from the oxidation of lipids, especially during stress condition; but this source is very low compared to that in tobacco smokers (60). The organ most targeted by volatile acrolein is the stomach mucosa. The body protects itself by conjugating acrolein with GSH and cysteine groups, and this seems to the decrease the toxicity of acrolein (60). Conjugation with the glutathione could result in a low availability in free glutathione reductase and consequently a major expose to the ROS for the cell. Liver cytosol or the mitochondria are able to oxidize this toxic aldehyde to acrylic acid or reduce to allyl alcohol. Acrylic acid can be incorporate into different metabolic pathways. Lung and liver are also able to metabolize the acrolein by oxidation, the molecule that result is the glycidaldehyde, which is also toxic (61). In the review paper (59) presents a good scheme about the source of acrolein made by human body and also proposes the metabolic pathways for its detoxification.

METHYLGLYOXAL

An imbalance between radicals (especially ROS) and antioxidants drives the cell to a state called oxidative stress. Oxidative stress is looks to play a major role in the aging process (62). The mitochondrial electron transport chain and oxidative metabolism are sources of ROS. Methylglyoxal (MG) looks to play a central role in the aging process. This aldehyde is very reactive with protein and DNA causing damage, and when a cell is not able to neutralize the aldehydes and the ketones made, the aging process starts. This reactive aldehyde has origins in lipid oxidation that can occur in two different ways: the random lipid oxidation made by ROS or during the lipid cycle oxidation that occurs at the cell membrane. Another source of MG is from the oxidation of sugars. In subjects with diabetes, where the metabolic pathway is not properly regulated, MG is present in higher concentration than people not affected by this illness. Lactic acid is the final product of MG, and two enzymes glyoxalase-I and glyoxalase-II (glyoxalase system), and the cofactor glutathione are responsible for its conversion (57). In a recent study (63) used a human histiocytic cell line and proposed a different pathway for the MG. They gave attention to formic acid (FA), a sub product of MG degradation by oxidative stress. They showed that FA could be involved in the detoxification of the MG interacting with H_2O_2.

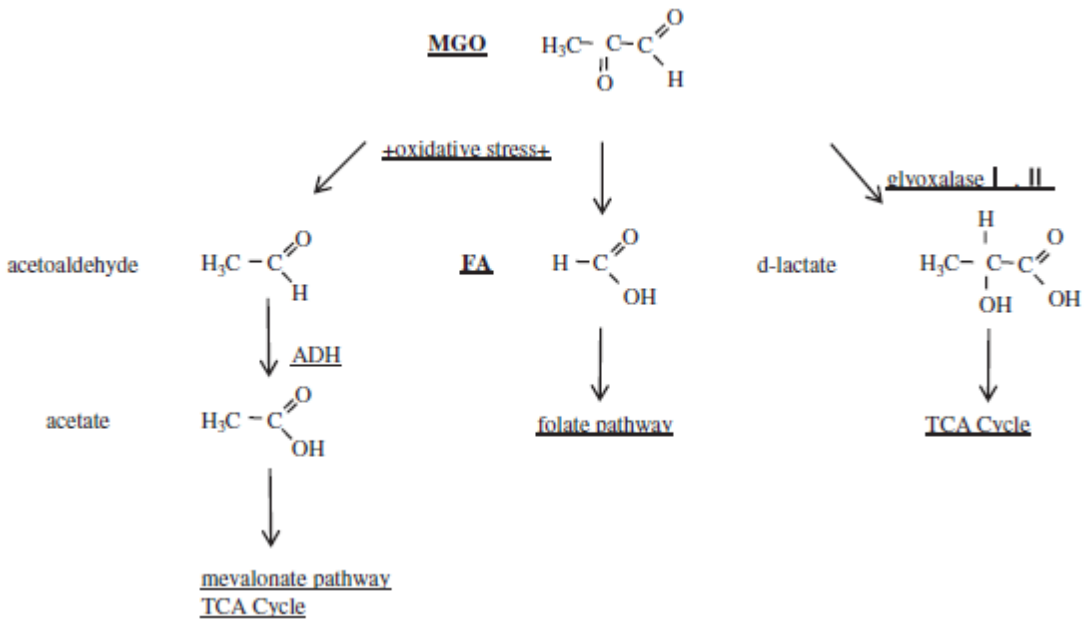


Figure 6: the propose MG system of detoxification proposed (63)

In Figure 6 the possible pathways for MG degradation are shown; in this scheme it is possible see that one of the products of the MG degradation is the acetaldehyde another aldehyde known as damaging to cells.

MALONALDEHYDE (MALONDIALDEHYDE; MDA)

In 1974 it was reported that malonaldehyde was formed in animal tissue from lipid oxidation and a low presence of antioxidant. In the same study, (61) showed that this aldehyde when administered to the rat is able to cause cancer. This dangerous di-aldehyde is the final product of the phospholipid oxidation. It is able to diffuse into the cell far from the lipids membrane (64). This electrophile like others is able to interact with DNA, RNA and as well with proteins where a classic example is the group SH, acting as a good nucleophile. MDA adducts were found in humans showing that this di-aldehyde is potentially dangerous for humans and not only for rats (65), (66). Malonaldehyde present in the human serum is used as an oxidation marker (67). The level of malonaldehyde correlates with some disease processes such as atherosclerosis and cardiovascular (68).

MUCONALDEHYDE

Muchonaldehyde (MUC) is a reactive aldehyde that is a metabolite of benzene whose degradation is initiated by cytochrome P450. Muconic acid is around 10-20% of the metabolite made by benzene. The benzene ring is oxidized and oxygen forms an epoxide. This instable ring after opening will give muconaldehyde (69). It is known that the human liver is able to open the aromatic ring of the benzene. Muconoaldehyde like other reactive aldehydes is able to react with DNA and proteins. Benzene is recognized as a tumor promoter in the development of cancer, but the way in which it promotes this mechanism remains unclear. MUC is associated with hematopoietic damage leading to leukaemia. In 2008 (70) showed that z-z MUC is more potent than benzene and is responsible for protein damage. MUC can be metabolized by aldehyde dehydrogenase isolated from yeast. This enzyme looks have a biphasic manner with a apparent K_m of 0.48 and 3.2 μM and Vmax of 604 and 1227 respectively nmol/minute/mg of enzyme (71). Also a reduction reaction can occur carried out by a purified ADH from yeast (72). In animal studies two enzymes in mouse liver are mainly responsible for the MUC detoxification: an NADH dependent ADH1 and an NADPH-dependent AKR1A4. These enzymes show a very similar K_m for MUC but the ADH1 shows a much greater rate. The AKR could have a major role under stress condition when the NADH is in low concentration and the rate between NADPH/NADH is increased (73).

AFLATOXIN B1

Aflatoxin B1 (AFB_1) is a metabolite produce by *Aspergillus* in particular conditions such as high humidity and temperature. This fungus is able to colonize corn and grain stored and consequently food as well. This toxin is responsible for the high incidence of liver cancer in the Asian and African population, where the storage conditions are not so good. The toxin is not dangerous in itself but its metabolites are. The first of them is the AFB_1 exo 8,9 oxide. This metabolite made by cytochrome P450 is able to forms adducts with DNA and causing mutations. This component is made less dangerous by the action of glutathione S-transferase (GSTs) that are able to conjugate the epoxide. The epoxide could also hydrolyze to give the AFB_1 dihydrodiol. This can then rearrange to give a double aldehyde that is very reactive especially with amino groups of the protein to give the Schiff base (74). The double aldehyde can be reduced to a dialcohol by members of the AKR7 family, AKR7A1, AKR7A2, AKR7A3 and AKR7A5. In the Figure 7 is schematic of AFB1 metabolism in the liver.

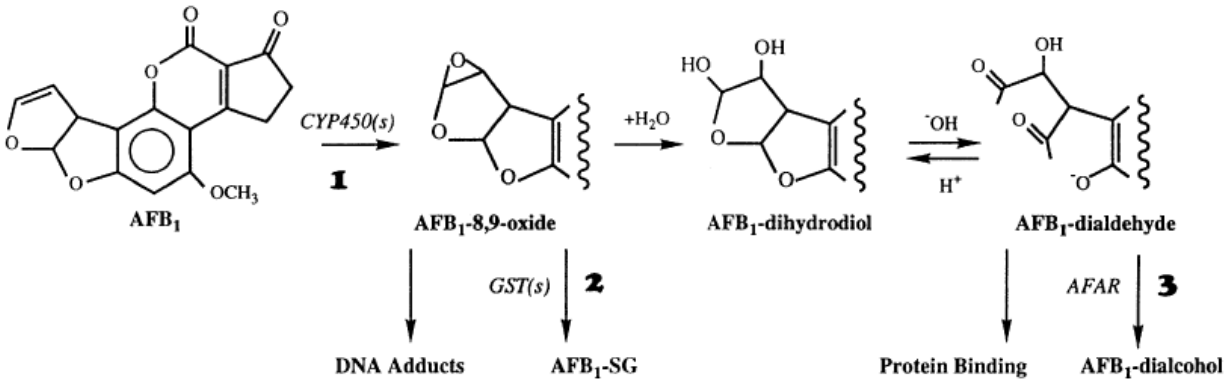


Figure 7: the metabolism by the liver of the toxic AFB_1 . With the number they show the succession of the passage catalyzed by enzyme. 1 the formation of the AFB_1 catalyze by cytochrome P450. 2 the adduction between the glucation and AFB_1 catalyze by Glucation S-Transferase. 3 the reduction of two aldehyde present in the AFB_1 dialdehyde to two alcohol for give the AFB_1 dialcohol, the reaction is catalyze by an Aldo-Keto reductase. In this figure is also possible see which molecule is able to reactive with the macromolecules present in the human body. There is also show the non enzymatic molecular transformation like the hydrolysis.

ɣ HYDROXYBUTYRATE AND SUCCINIC SEMIALDEHYDE METABOLISM

Gamma hydroxybutyrate (Gamma hydroxybutanoic acid; GHB) is an illegal drug that has increased in prevalence in rave parties and so-called "date-rape" abuse. Analogues of this substance include 1-4 butanediol (1,4BD) and the ɣ-butyrolactone (GBL). These substances are also classified as illegal because they are metabolized to ɣ-hydroxybutyrate, but they are easy to obtain in the black market (75). GHB is a metabolite physiologically present in the human body. GHB is supposed to be a moderator and regulator of itself in the central nervous system. When is taken in high dosage it can interact with the gamma-aminobutyric receptor (76). This could result in low response of the GABA receptor against the effect of neurotransmitters. The effect of these substance to the human brain are typical of the benzodiazepines and alcohol, and are able to cause dysregulation to the central nervous system (77). The classic effects of GHB are sedation, euphoria, decreased inhibitions, enhanced sex drive, and amnesia (78). GHB is commonly reported as a date-rape drug, as the victims are not able to remember what happened to them after the ingestion of high amounts of GHB (79). In Figure 8 the metabolic pathway for GHB from different precursors such as 1,4 BD and ɣ-butyrolactone is shown.

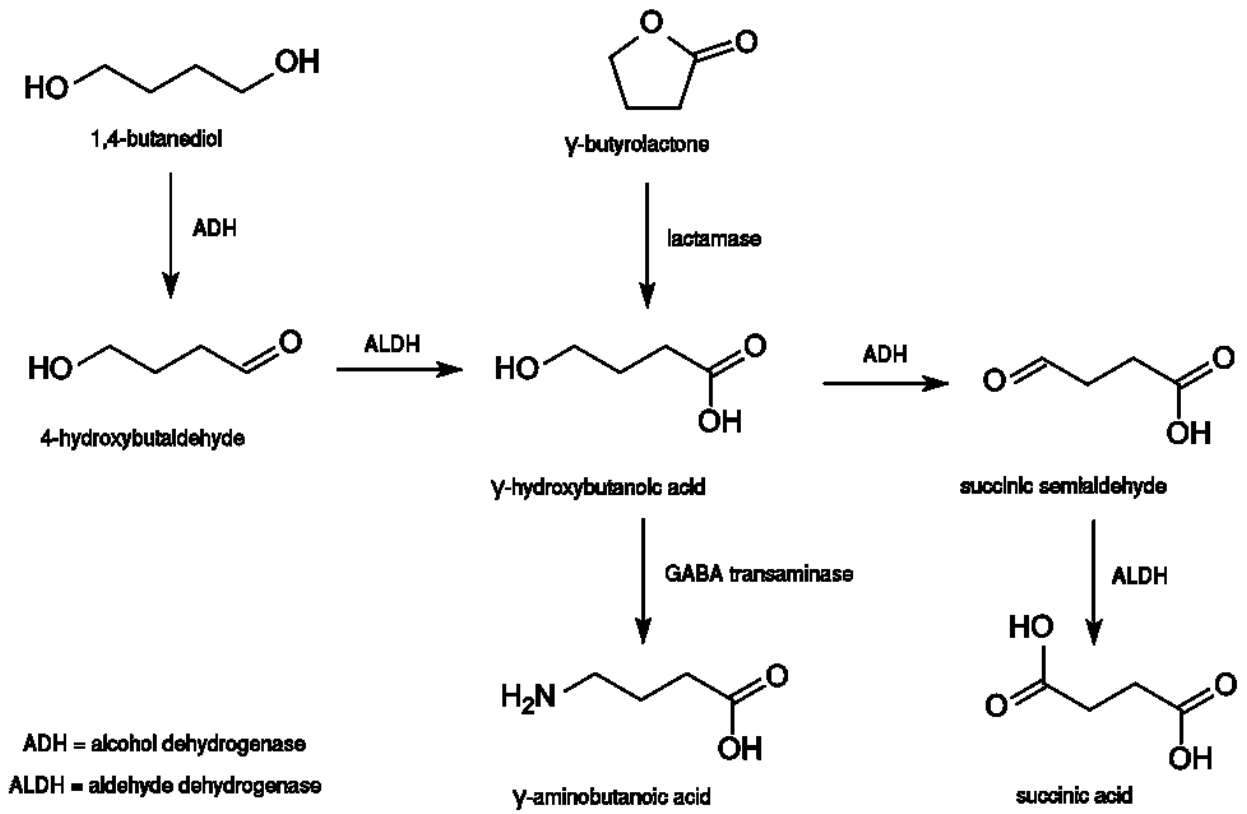


Figure 8: metabolic pathway of GHB.

The reaction can occur in both directions to and from GHB for both the substrates. The human body is able to make GHB from the GABA or SSA in a physiological equilibrium that involves the concentration of the precursor. Following the ingestion of GHB or derivatives as "fun drugs or different purposes" the concentration is much higher, and the human body is not able to convert all the GHB to its metabolites. In a physiological system this fatty acid GHB is mainly synthesized from GABA. GABA-transaminase a mitochondrial enzyme, converts the GABA into succinic semialdehyde (SSA). Two enzymes are able to convert the SSA to GHB. One is the aldehyde reductase AKR1A1 and the other is AKR7A2, both from the aldo keto reductase superfamily, both being NADPH-dependent. These enzymes have a different K_m for SSA, AKR1A1 has a K_m ten times higher than AKR7A2; the low K_m for AKR7A2 and localization to the brain indicates that the key role of this enzyme is the reduction of the SSA to GHB (80). In the brain there is more than one AKR present, and it is clear that AKR7A2 is not the only enzyme that reduces SSA. In neuroblastoma cells SH-SY5Y silenced for the AKR7A2 gene, it was shown that this enzyme is responsible for the majority of SSA reduction at low concentrations of SSA (10 μM) . The same study shows that at high concentrations of SSA such as 1mM AKR1A1 plays a more important role in metabolizing SSA (80). The same paper tried to establish the role of this enzyme inside the cell, and it was shown that this enzyme is not responsible for the oxidation GHB to SSA, because the silenced cells show the same level of GHB dehydrogenase as the control.

After this evidence the focus for GHB oxidation is look for an alcohol dehydrogenase that is NADH dependent and localized in the mitochondria. After this controversial evidence, the physiological role of AKR7A2 in the human brain is still open.

In the Table 2 are summarized some of the enzymes known to be able to detoxify the dangerous aldehydes described above. The enzymes are taken from different sources, but a particular focus is gave to the enzymes in the AKR family.

Table 2: enzymes able to metabolize aldehydes. The enzymes present are from different source.

Substrate	Enzyme	Source	K_m	Vmax (nmol/min/mg)	Vmax/K_m	Referemce
Muconaldehyde	ALDH	yeast	0.48 / 0.32 µM	604 / 1227		(72)
	ADH1 (NADH)	Mouse liver	11 µM	2141+/- 500		(73)
	AKR1A4 (NADPH)	Mouse liver	15µM	115+/-16		
Acrolein	AKR7A1	Rat liver		17.5 [1mM]		(39)
SSA	AKR7A1	Rat liver	0.14mM	1.46 µM/min/mg	10.43	(39)
4-nitrobenzaldehyde	AKR7A1	Rat liver	0.72mM	2.58 µM/min/mg	3.58	(39)
Methglyoxal	AKR7A1	Rat liver	7.22mM	1.13 µM/min/mg	0.16	(39)
Menadione	AKR7A1	Rat liver		< 1.3 [1mM]		(39)
Crotonaldehyde	AKR7A1	Rat liver		48.4 [1mM]		(39)

MENADIONE AND NAPHTHOQUINONE

Muconaldehyde is not the only chemical derived from modification accord to benzene ring. Other modifications can occur created without breaking the aromatic ring, but breaking the double bond between the two carbon atoms, adding a double bound with oxygen to one of the carbon atoms involved in the double bond. This modification changes the aromatic structure of the ring. The same modification can happen to other aromatic rings such as naphthalene. The names of these changed molecules are quinones. Several of these molecules are present in human body, where they play different physiological roles such as electron transport in the aerobic respiration for example ubiquinone. A well known quinone is vitamin K1. This large class of components have show a wide range of functions from anti microbial proprieties to protein synthesis, and some of them are also studied for their anti-tumor activity.

Menadione is a quinone and its IUPAC name is 2-Methylnaphthalene-1,4-dione. It is commonly called vitamin K3. This molecule is a precursor of vitamin K and is considered toxic (81). Using human cell lines it was establish that vitamin K3 has anticancer properties in carcinoma cell lines by generating free radicals (82). When K3 is associated with vitamin C in the ratio 1:100 it can generate a redox cycle to kill tumor cells and leave unchanged the other cells (83). So this vitamin could be part of anticancer cocktail.

5-hydroxy-1,4-naphthalenedione its generally called Juglone, this chemical is present in different trees and leaves in the Juglandacea family. Juglone is used for several different purposes as a herbicide and cosmetic, and also in the food industry for is brown color. When the 5-hydroxy in Juglone is exchanged for 2-hydroxy-1,4-naphthalenedione, the new chemical takes the name of Lawsone. This molecule is particularly abundant in the henna herb. The color of the molecule is black and is used to dye hair, nails and skin (84). Both these molecules have the capacity to reduce significantly the glutathione inside the cells, as well as start a redox cycle able to generate a large amount of ROS. Their toxicity was establish in isolated hepatocytes cells (85). 5-hydroxy-1,4-naphthalenedione is more toxic for hepatocytes. For cosmetic use especially for skin tattoos the 2-hydroxy-1,4-naphthalenedione is considered safe (86). The mechanism that the human body use for reduce the toxicity of these two toxic compounds is not clear, especially in the liver, the organ most involved in their toxicity.

WHISKY AND ITS POLYPHENOLS

Alcohol is consumed everywhere in the world and is metabolized as a food. The high ingestion of alcoholic beverages is associate with different kinds of illness; contrarily, a moderate consumption of alcoholic beverage is associated with the prevention of some illnesses. A low intake of alcohol every day has shown improve the cardiovascular system (4), (87). A recent study has shown that moderate drinkers are less affected to same pathology for degenerative diseases such as Alzheimer (88). A study conducted in the same year was able to connect the amount of ethanol ingested with the protection to cardiovascular diseases in the Asiatic population (89). Many of the drinks normally consumed during the day such as beer and wine are also rich in secondary metabolite derived from the original fruit or during the manufacturing process. These molecules derived from the plants are associated with different pharmacology properties, and it is also believed that some of these molecules are able to promote an improved state of health. This is one of the reason that led to the belief that a moderate alcohol consummation is healthy: it is not the ethanol that is able to promote a moderate state of wellness but all the secondary metabolites that are in many alcoholic beverages. Of these beneficial molecules great interest is given to the polyphenols. Polyphenols are a class of organic molecules that possess several phenol groups. They components are divided into different classes: catechins, pro-anthocyanidins and phenolics. Many of these molecules can react with different species of reactive oxygen. The oxidation of polyphenols could be chemical or enzymatic. The oxidation of these molecules can happen inside the plant during the ripening process of some fruits where the oxidized polyphenols contributes to the smell of these fruit. Sometimes the oxidization process starts in humans following ingestion. During the maceration process, the plant material is broken and some of the enzymes stored in different parts in the cell came in contact with the polyphenols and begin their oxidization process. The change in oxidation of the phenols is responsible for black tea (90). This high capacity to be oxidized leads to the concept that in the human body these molecules can prevent the oxidation of macromolecules by being oxidized first themselves.

Scottish whisky is particularly rich in aromatic components. Many of these components don't derive directly from the malt but from the wood barrels (usually oak) used for the "spirit maturation". The maturation process could be as long as twenty years. During this time some of the components of the wood are dissolved by the spirit and move into it. These components are then subject to other chemical modification including oxidation. The barrel is also able to promote exchange of oxygen between the environmental and the inside of the barrel. The diversity and quantity of these modified components can be influenced by many factors such as the contact time between the wood and fresh spirit, the barrel treatment before the contact with the spirit, the age of the barrel and others.

However of great importance is the time of contact between the spirit and the barrel, as this seems to influence directly the amount of polyphenol in the final product (91). This evidence is support by many authors (92),(93). The effect is to change the polyphenol profile of the whisky (94). It's difficult to establish the total amount of polyphenol in whisky because of the different methods used and the type of maturation. However some groups have quantified the amount in a range between 25 and 75 ppm (95).

The main polyphenols present in whisky are ellagic acid (29-50)%, gallic acid (13-23)%, 5-hydroxy methyl 2 formaldehyde (HMF) (2-38)%, and syringaldehyde (6-15)%. In Table 3 are shown the amount of the principal polyphenols found in whisky (91).

Table 3: polyphenol constituent the non volatile fraction of the whisky.

Component	µg/ml	µM/ml
Eallic acid	263.1	1.54
Syringaldehyde	1.01	5.54
B-Pentil alcohol	145	1.11
Gallic acid	0.66	3.87
Vanillin	103.7	0.68

Figure 9 shows some of the many polyphenols found in whisky. These component are found in the non volatile part of the whisky.

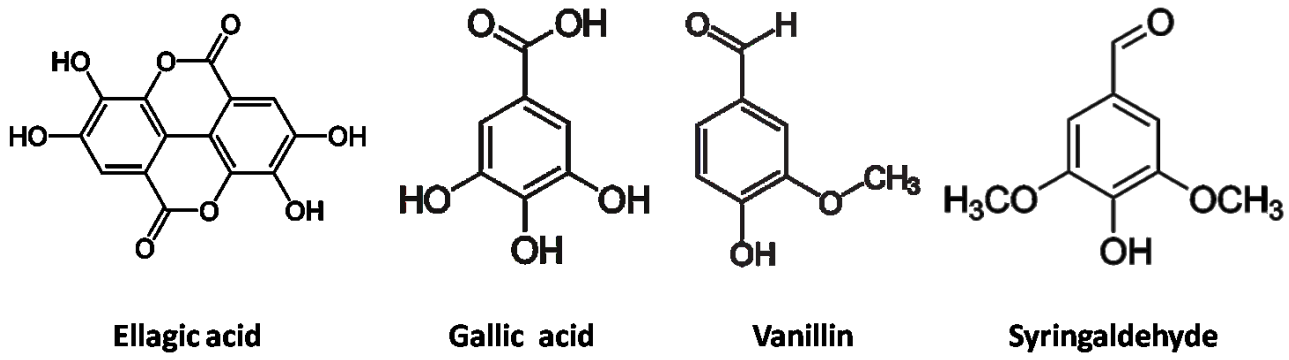


Figure 9: many polyphenols found in the whisky. These polyphenol have shown antioxidant propriety.

Inside cells these components seem to play an important role in the defense against reactive oxygen species (ROS). ROS can be generated during alcohol metabolism, especially in the pathway involving the MEOS. The polyphenols are readily able to cross the gut barrier and enter the circulation. They have been found in the blood stream of people after the ingestion of wine or whisky (92).

An evaluation of the antioxidant properties of many polyphenols found in whisky has been made. Gallic acid and ellagic acid have shown the best capacity to neutralize some radical molecules.

Significant interest is given to ellagic acid because of its high availability in whisky. However, not much is known about the bioavailability of the components, and which is the predominant form detectable in the human body after ingestion. Some components can be modified by the bacteria present in the human gut, during the absorption process. Also some enzymes are able to change the chemical structure of the polyphenols, to make them easier to be eliminated from the human body. Hence its difficult to establish which are the predominant forms present in the human body and which are the main polyphenols responsible for their antioxidant property.

Not only are these polyphenols able to act as radical scavengers, some are also able to alter the expression of some of the protective enzymes. It is well know that some xenobiotics are able to affect the levels of some proteins in the cell (38). The mechanism of action is not always known, but one antioxidant ethoxyquin is able to increase in rat the mRNA that encodes the aldo keto reductase AKR7A1 (37). This antioxidant can increase the expression of AKR7A1 20 fold, although the increase is lower with other antioxidants.

Experiment conduced using rat had shown that these polyphenols are able to change the rate in which the alcohol is metabolized in the rat body. In particular, these polyphenols and especially the vanillin seem to be able to influence the first step that is ethanol oxidation. In the same study has been shown that this effect is increases with the age of the whisky used in the experiment and correlates with increased levels of polyphenols. The researchers noticed that in rat blood after the administration of ethanol and polyphenol together, the ethanol has a longer half-life compared to the control group (no polyphenol). The ethanol levels remain high in the blood stream, but acetaldehyde remains low in rat treated with polyphenol. This evidence indicates that the polyphenol can inhibit the oxidation of alcohol by ADH. Experiments conducted with single polyphenol show that strong inhibition of ethanol oxidation was also made by vanillin (91), (96). However they did not evaluate the effect on the second metabolite made during the ethanol metabolism that is the acetaldehyde. Acetaldehyde is considerate more toxic and powerful than ethanol, so knowledge of its levels are important to understand ethanol toxicity.

RESVERATROL

3,5,4' –hydroxystilbene is the chemical component known as resveratrol. This is a polyphenol present in a wide variety of vegetables and fruits, but a particularly rich source is the grape skin, in particular the red grape cultivar, and peanuts and cranberries. In vegetables, the main role of this phenol is to protect them from UV radiation as well as bacteria and yeast (97). Many good qualities are attributed to resveratrol, especially its ability to prevent cardiovascular disease. Resveratrol is able to protect low density lipoprotein (LDL) from oxidation, and also able to increase the quantity of high density lipoprotein (HDL) in the blood. Vasodilatation is another attribute of this molecule (98). This antioxidant is normally introduced in the human body through the diet but is also available as supplement. A recent study (99) reported a very low bioavailability for this component, and less then 1% of resveratrol was detected in the blood stream in subjects treated with a single oral dose of 25mg (around the average intake with red wine in a day). The maximum amount of resveratrol found in the blood stream is less then 10ng/ml after no more than two hours after the oral ingestion. Other studies were made to evaluate the tolerability of resveratrol when is integrated in the diet. The intake of 5g/day of resveratrol was studied and some subjects were not able to tolerate such a high amount. They were affected by adverse reaction such as vomiting, dizziness, and headache. There is not however any advice on intake but it is reputed that 1 mg/day is the right amount (100). In terms of metabolism, resveratrol is simply glycosylated by the liver to make it more easily eliminated from the human body; this is probably an explanation for the low amount of free resveratrol found in the blood and its low bioavailability (101).

In *vitro* studies using HepG2 have shown that resveratrol is able to stop cell proliferation in a concentration between the range of 12.5 and 25 μM. Increasing the amount of resveratrol in the cell's media in a range between 50 and 100μM and exposing for more than 24 hours leads to a typical apoptotic phenotype such as DNA fragmentation (102). However it should be noted that the cells used in this experiment are carcinoma cells and they are not able to express all the enzymes normally found in liver cells. It also been reported that the capacity of some secondary metabolites to interact in a specific way is effective only against cancer cells and an example is gallic acid (103).

VANILLIN

Vanillin is produced by the vanilla orchid pods from Mexico. Other plants are able to produce this interesting metabolite such as the tobacco plants. Vanillin is present in the whisky and it passes into the distilled product through contact with the wooden barrel during maturation. Vanillin is recognized as one of the most important aromas worldwide. It is now possible to obtain vanillin by chemical synthesis. Vanillin displays some interesting properties and is able to interact with bacteria and yeast. Vanillin can be used as an antimicrobial, however is use for this purpose is limited by the high amount of vanillin required, so high as to make its aroma very strong, but it is used in low amounts to prevent the bacteria growing in food (104).

Many studies have been carried out using cell lines in order to better understand the general properties of this flavour molecule; part of these studies were made using cancer cells. A study made with hamster lung fibroblast V79 cells line (105) showed that vanillin can protect against DNA damage as well against uv-induced mutation (106). The ability of vanillin to prevent mutagenesis was studied in vivo using mouse in order to show its ability to block cancer breast metastasis (107). An important consideration needed to be made, that is, vanillic acid the main metabolite of the vanillin does not show the same properties as vanillin. For this reason great importance is given to the aldehyde group present in the vanillin (108).

Vanillin has the ability to interact with ADH, one of the enzymes responsible for alcohol metabolism, by steric inhibition. This inhibition is proportional to the amount of vanillin present in the distilled whisky used in the experiment (96). Not only is vanillin able to inhibit enzymes but it is also able to change the gene expression profile, particularly for those genes that are involved in the cell cycle and apoptosis (109).

Only the property to interact with ADH has been investigated but there has been no other study made to evaluate the possible interaction with other enzymes involved in alcohol metabolism, such as the acetaldehyde reductase or other enzymes involve in the Kreb's cycle where the acetate made from the acetaldehyde is directed.

GALLIC ACID

Gallic acid is a polyhydroxyphenolic. This natural component is present in different fruits as pineapple and apple skin, strawberry, bananas and lemon. Not only are fruits rich in this secondary metabolite but also cereals such as oat bark, tea leaves and consequently also the products derived from them such as red and white wine and tea. This molecule has a high bioavailability. The presence of free gallic acid can be detected systemically after the ingestion of foods rich in it (110). Many virtues are given to this molecule. It has been shown to have cytotoxic properties against cancer cells (103). This is mediated by increasing the amount of ROS inside the cell, an increase of Ca^{2+} inside the cell, and abnormal mitochondrial function. It is not totally clear how the gallic acid is able to kill the cells. Gallic acid seems to have a strong influence on the cancer cells to induce apoptosis. In some cell lines (human lung cancer NCIH460) it has been reported that caspase-3 is directly involved in mediating gallic acid induced apoptosis (111). However as caspase-3 is not involved in cell death of lung cancer cells Calu-6 and A549, and also as caspase-9 is not involved in the death of these cells this suggests that another pathway is involved in cell death (112). Over the years a wide range of papers have accumulated in which is shown the anti-inflammatory effect of this molecule other than cancer prevention (113).

In non cancer cells, gallic acid is able to protect cells against glucolipotoxicity (112). In a recent study it has been established that gallic acid is able to offer a good protection against the formation of protein adducts with aldehydes and ketones. This experiment shows the ability of gallic acid to reduce the amount of adduction between the BSA (Bovine Serum Albumin) used as reference protein and the methylglyoxal used as an aldehyde (114). In animal studies, when the rats were fed with a gallic acid supplements they showed an increase in the levels of antioxidant enzymes and more resistance to the lipid oxidation (115). Supplements with gallic acid were also able to prevent some cytotoxic effects on the liver. Based on single cell migration (Comet assay) it was possible to establish that gallic acid could prevent DNA fragmentation by gallic acid. This protective effect was shown when gallic acid was added to human lymphocytes in the range 0.015 and 0.15 μg/ml (115). So this molecule is able to increase ROS when present at high concentrations but in low concentration it prevents the damage made by the ROS.

HEPG2 CELL LINE

During this thesis works the cell line HepG2 was used. This cell line is derived from human liver. This cell line expresses almost of the enzyme that are responsible for the detoxification of xenobiotic by activation or detoxification Phase I and Phase II (116), However the profile expression of cytochrome P450 enzymes is much lower in the cell lines than in primary culture (117). For this characteristic this cell line is ideal for study different toxic aldehydes especially the acetaldehyde that is made in the liver during the ethanol oxidation and aldehydes generated during the lipid peroxidation. This cell line is particularly useful in the studying the oxidant defense mechanism, because it expresses many of the enzyme involve in the protection against it, including different superoxide-dismutases (118). The HepG2 cells line has kept the capacity to metabolize acetaldehyde by acetaldehyde dehydrogenase (ALDH) (119). In one of the few papers published (120), it has been shown that acetaldehyde is able to decrease the viability of these cells by around 14% when exposed to 1mM of acetaldehyde for four hours. In the same study it was also demonstrated that acetaldehyde showed greater toxicity compared to ethanol. Increasing concentrations of acetaldehyde to 200 mM for the same exposure time is able to kill these cells (121).

1321N1 CELL LINE

Astrocytes are one of the main types of cells present in the human brain. They have a primary role during the development of the neuron, and they support them by providing nutrients. Astrocytes are also the primary place for the detoxification of many neurotoxins (122). What is not totally understood is the metabolism of ethanol in the brain and the localization of it. However astrocytes are able to metabolize ethanol through three pathways: catalase; cytochrome P450 2E1 and ADH. ADH does not seem to play a great role in this metabolize so the other two are more important, especially the catalase (123),(124). The metabolism of ethanol is the main source of acetaldehyde in the brain. The acetaldehyde produced in other part of the body (mainly in the liver) it does not cross the encephalic barrier because is metabolized before it reaches it. High amounts of acetaldehyde 10-40 mg/kg administered to rats are able to saturate the enzymes involved in the conversion of acetaldehyde to acetate. In this condition the acetaldehyde is able to reach the blood brain barrier and cross it (125).

Several studies have used the astrocytes as a model for targeting different molecular toxicities and effects.

A study with the purpose of evaluating the toxicity of ethanol exposed rat astrocytes to concentrations ranging from 20 to 50mM. The viability of the cells was unchanged even at the high concentrations; the DNA did not show any damage indicating that ethanol is not very toxic. The same study evaluated the toxicity of acetaldehyde in astrocytes, using an exposure of three hours with different concentrations 0.25, 0.5 and 1 mM. There was no difference in the cells' viability but DNA damage was observed (126). Using rat primary cultures the lowest concentration of acetaldehyde able to lower the viability of cells is just less than 50 mM. This value is very high compared to the amount of acetaldehyde found in the blood stream of drinkers (127).

III AIM OF THE WORK

The aim of the work described in this thesis is to elucidate the role of the AKR7A enzymes in acetaldehyde metabolism. This will be achieved through the following objectives:

- Characterization of the human AKR7A2 and AKR7A5 enzymes.
- Evaluation of the role of the AKR7A2 enzyme in detoxication in human cell lines.
- Evaluation of the effect of the main antioxidants found in whisky and wine in the protection against aldehydes generate by lipid peroxidation.
- Evaluation of acetaldehyde toxicity in the cell line 1321N1 and HepG2.

IV RESULTS AND DISCUSSION

EVALUATION OF ACETALDEHYDE TOXICITY

The main metabolite made during the ethanol oxidation is acetaldehyde. This metabolite is believed to be responsible for diseases related to alcoholism. Acetaldehyde may interact with other component present in alcoholic beverages. Normally the amount of acetaldehyde in drinkers is around 4-10 μM in the blood stream. A second study showed a considerably higher amount of acetaldehyde in the blood stream in a drinker of 27 μM. This was the average value after the ingestion of 1g of ethanol for kg of body weight (128).

Several descriptions of the toxicity of acetaldehyde can be found, particularly the interaction with DNA and proteins. Acetaldehyde can affect cells in different ways. It could exercise its toxic effects over a long period of time or initiate processes able to damage the cell or kill it. This aldehyde is able to react with different macromolecules such as DNA, proteins and lipids. Also the metabolism of this compound at high concentrations may be able to unbalance the NAD^+/NADH radio in the cell.

However there are only a few papers that discuss acetaldehyde toxicity in cell lines. One of the purposes of this project is to evaluate acetaldehyde toxicity in the human cells line 1321N1 and liver HepG2, especially with respect to their viability. Other than the viability of the cells, the capacity to induce apoptosis inside the cells was also investigated, and in particular the role of the caspase-3, a protein commonly involved in apoptosis was investigated. Agarose gel electrophoresis was used to investigate another marker of apoptosis, DNA fragmentation.

ACETALDEHYDE TOXICITY

The first step was to evaluate the total toxicity in the cells line 1321N1 and HepG2. These parameters could be simply determined using an MTT assay, to understand the viability of the cells after being exposed to different concentrations of aldehyde. In the Figure 10 and Figure 11 it is possible follow the loss of viability caused by acetaldehyde over increasing concentrations and during different times of exposure.

During these viability experiments, to prevent the high volatility of acetaldehyde a layer of plastic film was used between the 96 well plate and its lid. The results without using the plastic film were quite different to the results shown in this thesis. In particularly, the acetaldehyde looks less toxic. This reduction in toxicity was attributed to the reduction of toxic concentration over the time, especially at the normal incubator temperature 37ºC where evaporation would take place.

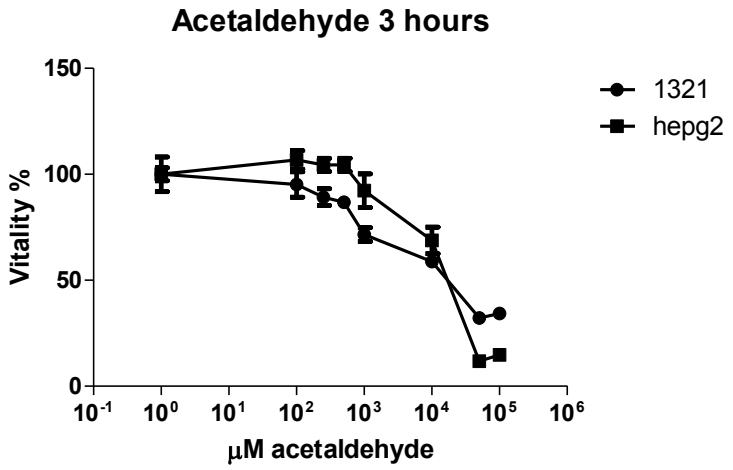


Figure 10: viability of the two different cells line studied, in different range of toxic concentration, after a short exposition, three hours. Three samples for every measurement. N=4, p<0,05.

For the brain cells 1321N1, there is no significant difference in viability between the control cells and the acetaldehyde-treated cells concentration up to a concentration of 10 mM for 3 hours. Increasing the concentration up to 100 mM gives a significant decrease in cell viability. The HepG2 cells start showing a decrease in viability at 10mM of acetaldehyde, and this loss of viability becomes more pronounced at higher concentrations. It is possible to see from Figure 10 that the two curves are very close to each other. In this short time frame, HepG2 looks more resistant to acetaldehyde showing a reduction of viability of 8% at 10mM and 89% at 50mM.

1321N1 cells are more sensitive to acetaldehyde exposure, showing a reduction of viability of 29% at 1mM and 30% at 50 mM. However at higher concentrations these cells are more resistant than the HepG2.

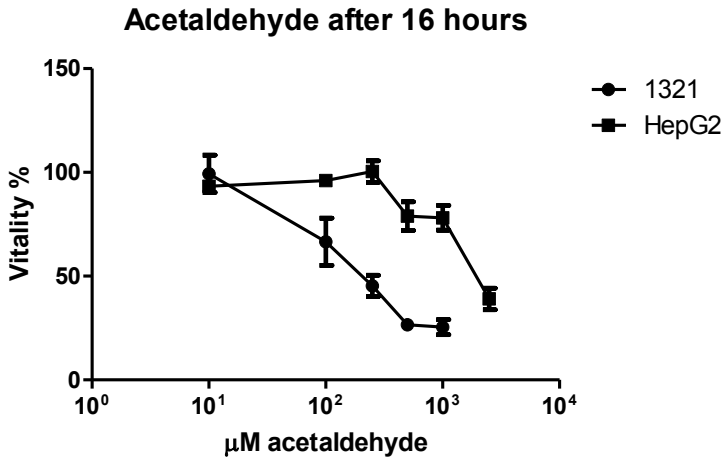


Figure 11: viability of the two different cells line studied, in different range of toxic concentration, after a long exposition, 16 hours. Three samples for every measurement. N=4, p<0,05.

In Figure 11 a longer acetaldehyde exposure time was used and the two cells lines show clearly different behavior. The HepG2 cells seem very resistant to acetaldehyde, the only concentration being able to reduce the cells viability is the highest used 25mM. This data are particularly hard to explain especially when the concentration of 10mM is able to cause a significant drop in viability in three hours exposure. It should be born in mind that the MTT assay is strictly related to mitochondrial activity as the mitochondria are the catalytic site for the colorimetric reaction. It seem that the effects of acetaldehyde on mitochondrial activity (viability) is particularly apparent at an early stage but that over time, the cells begin to recover as the cells metabolism is able to eliminate / metabolize the aldehyde and the mitochondria regains function to give a strong viability signal.

A different situation is observed for 1321N1 cells. The lowest concentration of acetaldehyde that is able to cause a loss of viability is 250 µM. Increasing the exposure time to acetaldehyde reduces cell viability by 4 times compared to the shorter exposure, indicating that these cells are not able to metabolize the acetaldehyde over time and hence are not able to recover mitochondrial function.

Overall, the data presented for acetaldehyde toxicity at both exposure times indicates that acetaldehyde does not play a great role in the death of HepG2 cells, but does have an effect on 1321N1 cells. However the high concentrations required for significant cell death (250 µM) is greater than would be expected to occur following ethanol consumption. The brain cells may be more sensitive because they are not normally exposed to aldehydes, unlike the liver which plays a major role in drug metabolism. Liver possesses a wide range of enzymes that can metabolize acetaldehyde whereas brain may not have these enzymes.

The metabolism of ethanol in the brain is a controversial topic. This metabolism could be a source of acetaldehyde (129), and for this reason the human brain cells need to have some resistance to this chemical. It is relevant to note that the concentration of acetaldehyde produced in the brain is likely to be much lower than the concentration found in the liver, and all the concentration tested are therefore much higher than would be encountered physiologically.

A few other studies have looked at acetaldehyde toxicity in human cells as a model. In one paper the viability of cells HepG2 treated with 180 μM of acetaldehyde plus liposomes showed a reduction of only of 10% compared to the cells used as control (130). These data are different to the data presented here. A concentration of 100 μM was able to reduce the cells viability by only 4% and this value was not statistically different to the control. The next concentration tested was 250 μM and at this concentration there wasn't any further decrease in viability. One of the reasons could be the different time of contact between the aldehyde and the cells. In the experiment reported previously, (130) cells were kept in contact with acetaldehyde for 24 hours, and the concentration kept constant by adding acetaldehyde several times. In the experiment reported in this thesis, a single dose was used. Another study made with rats, in particularly rat astrocytes show that these cells are more resistant that the human. In that study (127) acetaldehyde started to show some toxic effects at concentrations above the 50 mM. These values are incredibly high compare to the normal levels fo acetaldehyde found in the human body, or in heavy drinkers. However these result are in agreement with a previous paper in which it was shown that only concentrations above 10 mM are able to affect the gluconeogenesis and concentrations above 20 mM are able to affect cell biosynthesis; this evidence was obtained from isolated rat samples (51).

ACETALDEHYDE EXPOSURE AND APOPTOSIS

Acetaldehyde appears slightly toxic to cells directly, but is known to cause significant damage in whole organisms. One mechanism that could explain these effects is via apoptotic processes (131). Apoptosis can be measured using several techniques but the main ones include looking at caspase-3 activation and also DNA fragmentation, both of which are well-characterized indicators of apoptosis. The purpose of this experiment was to see whether acetaldehyde was able to damage the cell by inducing apoptosis.

LEVELS OF ACTIVATED CASPASE-3

One of the proteins directly involved in apoptosis is caspase-3, which is activation during apoptosis and is often known as the executioner caspase (132). Caspase-3 is not the only caspase involve in the apoptosis and other caspases can be monitored, but caspase-3 tends to be the most commonly monitored. Hence this protein was used for evaluate the capacity of the acetaldehyde to induce apoptosis in the cells after an exposure to this acetaldehyde.

Different concentrations of acetaldehyde were added to the cells from 5 µM to 50 µM. The exposure time used was 24 hours. The highest concentration used was 50 µM because it is considered a concentration higher than that normally found in the human body. Cells were also treated with resveratrol as a control in the concentration range of 100µM. A concentration of 50 µM resveratrol is able to induce apoptosis in HepG2 cells lines (133). Following treatment of both the cell lines, extracts were prepared and the levels of activated caspase-3 were monitored using Western Blots and antibodies (Santa Cruz Biotechnology, caspase-3 H277) that are able to react with inactive pro-caspase-3 (30kDa) and the cleaved part of the protein the active part of capase p20 around 21kDa. Figure 12 shows the results for the detection of the caspase-3 in HepG2 cells.

Figure 12: Western Blotting with antibody against Caspase-3, cell line HepG2. C; control non treated cells 1; cells treated with 5µM acetaldehyde, 2; cells treated with 10 µM acetaldehyde, 3; cells treated with 25 µM acetaldehyde, 4; cells treated with 50 µM acetaldehyde, 5; resveratrol 100µM

In the Western Blot shown in Figure 12, is possible detect only the main protein, the pro-caspase-3. Under the main band it is not possible see bands that suggest caspase p20 fragment is present.

The strongest band corresponded to the control sample. The bands related to the acetaldehyde treatment the look a little fainter but don't show a real significant difference. The band relative to the resveratrol treatment looks really faint compared to the others. However, in this last band it is not possible see the activated form of the caspase-3 protein. In the paper previously quoted it was shown that the capacity to induce apoptosis utilized caspase-7 activity but the caspase-3 was not investigated. This Western Blot does not give too much information about the capacity of the acetaldehyde to induce apoptosis in the cells. The only conclusion that is possible is that there is no difference in the amount of inactive caspase-3 protein between the control and the treated samples. The same experiment was done carried out with the cell line 1321N1. As shown in the Figure 13 this cell line seems to be more influence by acetaldehyde treatment compared to the HepG2 in the long exposure time. The same caspase-3 antibodies were used for this experiment and the same amount of protein in each well. In Figure 13 is possible to see the result of the Western Blotting using the cells line 1321N1.

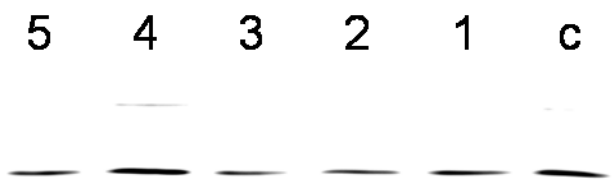


Figure 13: Western Blotting with antibody against Caspase-3, cell line 1321N1. C; control non treated cells 1; cells treated with 5µM acetaldehyde, 2; cells treated with 10 µM acetaldehyde, 3; cells treated with 25 µM acetaldehyde, 4; cells treated with 50 µM acetaldehyde, 5; resveratrol 100µM.

In Figure 13 the intensity of the bands is weaker compared to the Western Blotting made for the cell line HepG2 in Figure 12. Also in this Western Blot the control band is more intense, and lane 4 appears stronger than other bands. However this may reflect a slight variation in the amount of protein loaded on the Blot. In both samples, the pro-caspase-3 band corresponding to the sample treated with resveratrol looks weaker compared to control, but it is not possible detect the small fragment that should be generated during caspase-3 activation.

CASPASE-3 ACTIVITY

It appears that the Western Blots for both the cell lanes were not able to give a clear picture of the activity of the caspase-3 inside the cells treated with the acetaldehyde and resveratrol. For these reasons, the caspase-3 enzyme system was used to give an alternative way of measuring caspase-3. Caspase-3 activity is able to give a quantitative response and not just a qualitative response compared to the Western Blotting.

A Promega caspACE kit was used to measure caspase-3 activity. This is colorimetric enzyme assay, where the cell extract is the source of caspase-3 and the kit uses a special amino acid probe as a fluorophore substrate. The probe has an amino acid sequence recognized by caspase-3. When the probe is cleaved it releases a yellow dye (chromophore p-nitroaniline). The dye is quenched when linked to the probe, but is yellow after the cleavage. Monitoring the absorbance at 405 nm means it is possible to see how much dye is free at the end of the reaction. For this experiment, the cell line HepG2 was chosen because they are exposed to higher levels of acetaldehyde derived from ethanol metabolism. HepG2 cells were exposed to acetaldehyde at 5, 10, 25, 50 µM in the growth media for three hours. Resveratrol was also used as a control to induce apoptosis at a concentration of 100 µM (134).

Using the free dye (chromophore *p*-nitroaniline) it was possible to make a calibration curve, to determine the activity of the active caspase-3 activated. The first stock of dye was quantify by spectrophotometry using a extinction coefficient $\varepsilon = 10500\ M^{-1}\ cm^{-1}$.

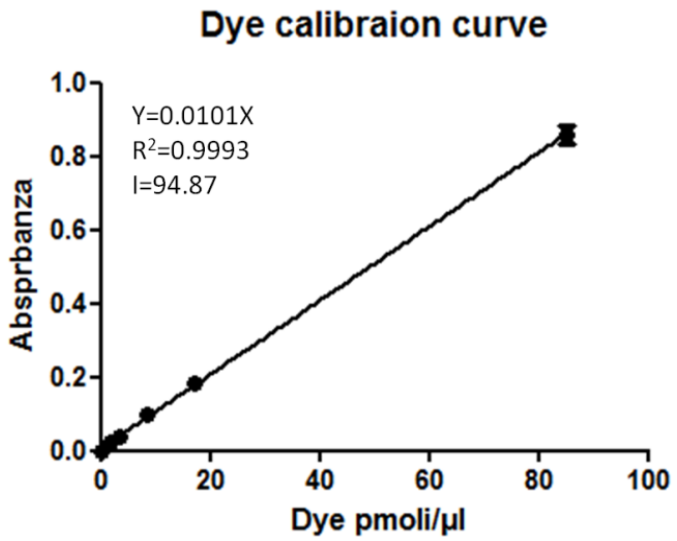


Figure 14: calibration curve of the dye (chromophore *p*-nitroaniline). N=3.

The good quality of the standard curve is expressed by the R^2 value. All the value were determined in triplicate.

In the Figure 15 is possible to see the response of the HepG2 cells treated with different concentration of acetaldehyde.

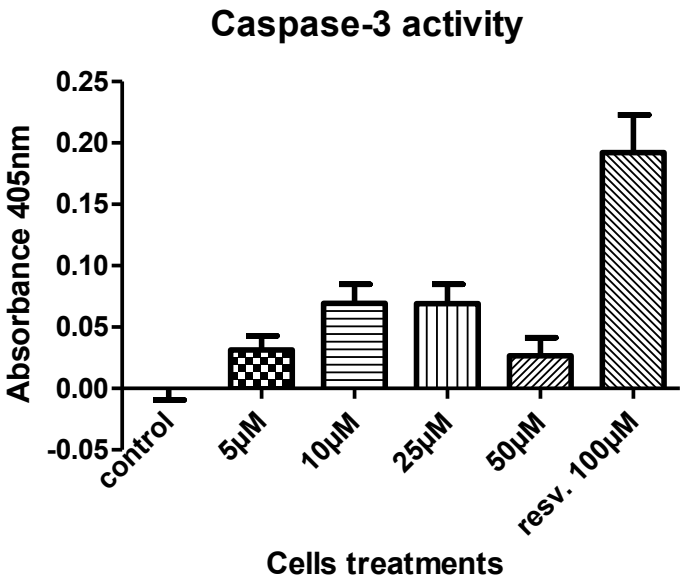


Figure 15: caspase-3 activity in cells HepG2 treated with difference concentration of acetaldehyde 5, 10, 25, 50µM and resveratrol as positive control. n=3.

In the data shown in Figure 15 the negative control value was subtracted and the graph shows the difference between the control value and the value recorded in different extracts. Between the control and the acetaldehyde-treated cells there are no statistically significant differences.

Cells treated with resveratrol shown a statistically significant difference compared to all the other samples ($p<0.05$). The results also show that caspase-3 activity is significantly increased by acetaldehyde at concentrations of 5, 10 and 25 µM. Treatment with 50 µM acetaldehyde shows less caspase-3 activity. This indicates that caspase-3 is activated by acetaldehyde treatment but not to the same extent as resveratrol.

With the formula expressed below it was possible calculate the amount of free dye generated.

$$Fres\ dye = \frac{A - (\ Y\ intercept\ of\ pNA\ std.curve)}{incubation\ time\ in\ hours} * \frac{sample\ voulme}{slope\ of\ pNA\ std.curve\ pmol/\mu l}$$

A= Absorbance 405nm (mean induced apoptosis sample)- (mean negative control sample)

$$Fres\ dye = \frac{0.192 - 0}{22} * \frac{100\mu l}{94.87}$$

Free Dye: $9.19x10^{-3}$ pmol/h

The calculated specific activity (SA) of the caspase-3 in HepG2 cells line activated during the resveratrol exposure.

$$SA = \frac{pmol\ pNA\ liberated\ per\ hour}{\mu g\ protein}$$

$$SA = \frac{9.19x10 - 3}{30}$$

Specific activity calculated (SA): $3.063X10^{-4}$ pmol/h/µg

The specific activity of the caspase-3 activated from the exposure to resveratrol is 3.063 X 10^{-4} pmol/h/µg.

EFFECT OF ACETALDEHYDE ON DNA FRAGMENTATION

Kitazumi and Tsukahara, (135) proposed that the specific role of the caspase-3 is the generation of DNA fragments by activating a nuclease. Genomic DNA fragmentation is a marker of the apoptosis (136), and this fragmentation can be easily detect by agarose electrophoresis (137). This method is independent of caspase-3 used to generate the DNA fragmentation. It gives less information than the Western Blotting with specific antibody but is still a good method for identifying apoptosis inside the cells.

This technique was used to investigate HepG2 cells after exposure to acetaldehyde at the same concentrations used previously. DNA is extracted and then split into Genomic DNA and Apoptotic DNA. In Figure 16 it is possible to see the result of the agarose gel for the detection of the fragmented DNA.

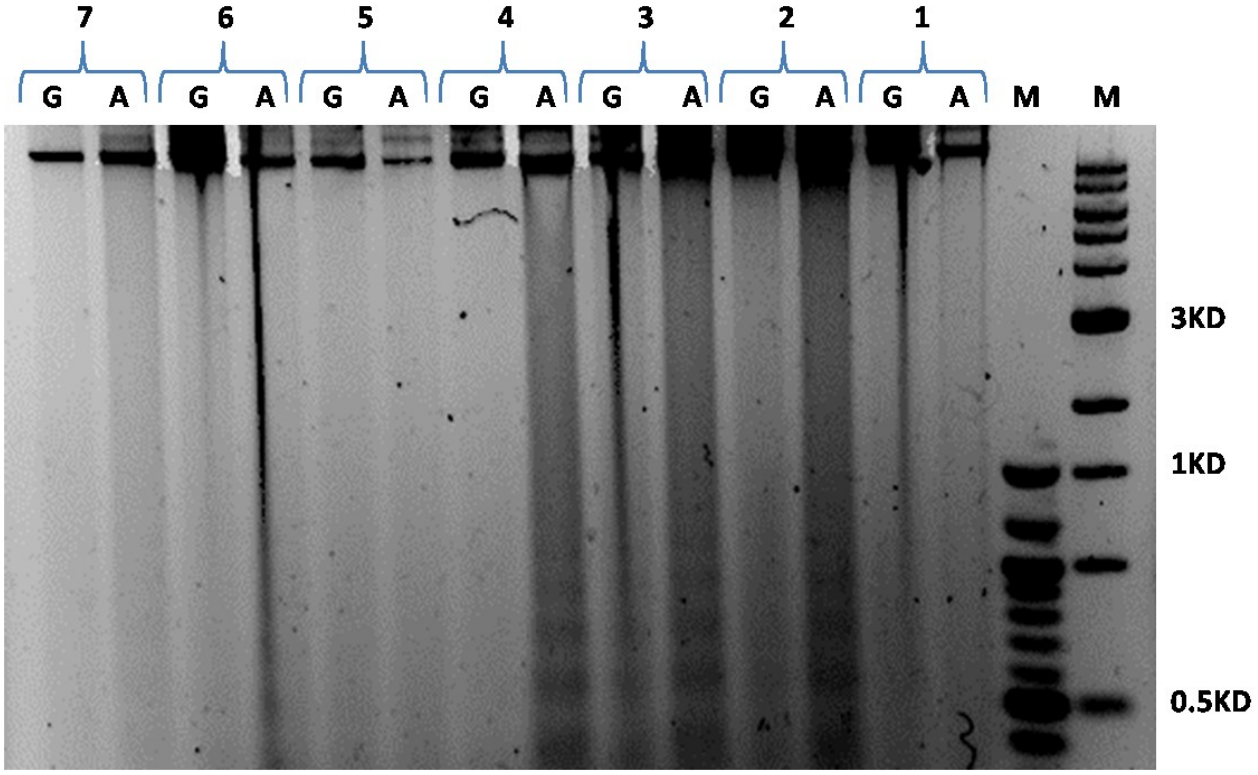


Figure 16: Apoptosis induced by acetaldehyde in cells line HepG2. A: the solution with the small fragment of DNA generated during apoptosis. G: the solution containing the genomic DNA as control of the DNA extraction. 1: control cells (un-treated). 2: cells exposed to 5µM of acetaldehyde. 3: cells exposed to 10µM of acetaldehyde. 4: cells exposed to 25µM of acetaldehyde. 5: cells exposed to 50µM of acetaldehyde. 6: cells exposed to 50µM of resveratrol. 7: cells exposed to 100µM of resveratrol. M: Marker.

The method chosen to detect DNA fragmentation appears to work well. In all the Genomic lanes it is possible see a strong band with a very high molecular weight. This band does not move too far from the loading well. The band is the intact genomic DNA. A small amount of contamination of genomic DNA is also present in the lanes that should contain only the fragmented (Apoptotic) DNA.

The control untreated sample (1) shows only genomic DNA in both lanes. No fragmented DNA is presented. The presence of the genomic DNA is a good indicator that the extraction procedure has worked. The situation is different for the samples treated with the acetaldehyde. Concentrations of 5, 10 and 25 µM are able to induce DNA fragmentation and presumably apoptosis in this cell line. In all the lanes corresponding to apoptotic DNA it is possible see the typical fragmentation with a molecular weight below 1kb. In samples 2 and 3 is possible see a little contamination in the genomic lanes. In these lanes it is possible to see a weak fragmented DNA that is clearer in the corresponding A lane. This data suggest that acetaldehyde is able to induce fragmentation at concentrations found in the human body after the ingestion of moderate consumption of alcohol. It is not clear why the higher concentration of acetaldehyde 50µM is not able to induce fragmentation, but it is possible that this high concentration reacts with the cell macromolecular and tissue causing direct damage and necrosis, which is an alternative cell death endpoint.

The sample treated with resveratrol does not shows the typical fragmentation or the minimum amount of fragmented DNA despite showing caspase-3 activation.

The same analysis was also carried out using the brain cells 1321N1. This cell line looks less resistant to the toxic aldehyde as confirmed by the MTT assay made with different concentrations of aldehydes for the different cells line studied.

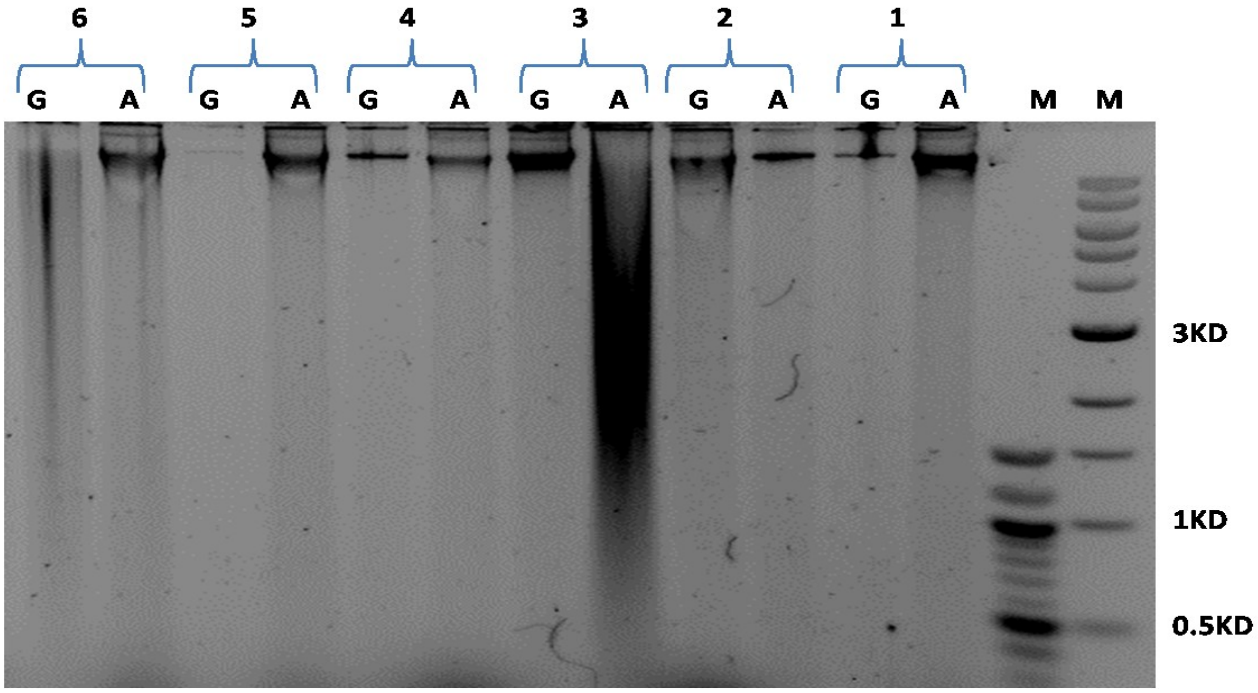


Figure 17: Apoptosis induced by acetaldehyde in cells line 1321N1. A: the solution with the small fragment of DNA generated during apoptosis. G: the solution containing the genomic DNA as control of the DNA extraction. 1: control cells (un-treated). 2: cells exposed to 5µM of acetaldehyde. 3: cells exposed to 10µM of acetaldehyde. 4: cells exposed to 25µM of acetaldehyde. 5: cells exposed to 50µM of acetaldehyde. 6: cells exposed to 100µM of resveratrol. M: Marker.

The agarose gel corresponding to the 1321N1 cells line is more easy to interpret. In every lane it is possible to see the bands corresponding to genomic DNA and consequently the quality of the extraction protocol. All the samples with the exclusion of the 3A, the lanes are totally clear with the minimal presence of fragmented DNA. Lane 3A looks degraded but it does not present the typical pattern of apoptosis. The sample 6 treated with resveratrol also did not show any fragmentation but this was not expected at this concentration. In conclusion the agarose gels had shown that acetaldehyde at low concentrations is not able to induce apoptosis in 1321N1 cells but it is in HepG2 cells.

CONCLUSION

With the data obtained in this thesis is possible to say that the acetaldehyde does not cause a significant reduction in cell viability in samples exposed to a high concentration of acetaldehyde higher than the physiological level found in the blood stream from 5 to 50μM. A 16 hours exposure does not generate a significant reduction of viability in HepG2 cells, but does show some decrease in viability in 1321N1 cells. A 3 hours exposure time did not show a difference in viability. The capacity of the acetaldehyde to induce apoptosis was also investigated. Apoptosis process was follow using the caspase-3 activity, which indicated that some apoptosis was occurring at medium doses of acetaldehyde. This was supported by the DNA fragmentation assay in HepG2 cells.

The next step will be to investigate the role of acetaldehyde in direct interactions with macromolecules. In particularly, it would be interesting to investigate the activity of different enzymes after cells were exposed to different concentrations of acetaldehyde. The main goal is to investigate whether there will a change in the substrate specificity or efficiency in the catalysis of different substrate. In this way it could be possible monitored the real interference made by the adduction between protein and acetaldehyde. Another analysis that will suggest is determining the expression of different genes after the exposure of the cells to acetaldehyde and evaluate the effect on the cell cycle. With this method it is possible to evaluate in the long term the effect on DNA adducts in a first exposure to acetaldehyde.

EVALUATING THE PROTECTIVE EFFECT OF PHENOLICS FOUND IN WHISKY AND WINE

In this Chapter, different concentrations of vanillin, gallic acid and resveratrol are investigated in order to evaluate the cells tolerability and response to these compounds. To investigate their role as chemoprotectants, 1321N1 and HepG2 cells are exposed to different concentrations of each compound follow by exposure to toxic aldehydes. The purpose is to evaluate the possible toxic effects of each compound, and then to determine the best concentrations that could give possible protective effects against different aldehydes such as acetaldehyde, acrolein and methylglyoxal . Acetaldehyde is directly related to alcohol consumption by its metabolism; acrolein is a marker of pollution; and methylglyoxal is an indicator of oxidative stress inside the cell.

TOXICITY OF VANILLIN, RESVERATROL AND GALLIC ACID

Initially an evaluation of the tolerability of the selected antioxidant by the two cell lines was carried out. In this part of the work, the normal amount of antioxidant ingested with the food was not considered for two main reasons. First it would be possible to supplement the diet using higher amounts of the antioxidant. The second and more important reason is that other studies would need to be carried out to evaluate to concentration present in the blood stream after the ingestion of the component. It was reported previously that even when the intake of resveratrol is quiet high, the resveratrol present inside the blood stream is very low, caused by the detoxification processes in the human body. The average intake of resveratrol with the ingestion of red wine is 25mg, and only 10 ng is the maximum amount found in the blood stream. This is equivalent to 2 μM in the blood stream (99). Resveratrol is mainly metabolized by conjugation to glucuronides and sulfates of resveratrol. The main organ responsible for this transformation is the liver and the bacteria present in the gut. Gallic acid seems to be well absorbed by the human body, and it is possible to obtain a concentration around 4 μM of gallic acid and its glucuronidated forms included the main metabolite 4-O-methylgallic acid after ingestion of a pure dose of 50 mg of this compound (138). Red wine is rich in gallic acid from 10 to 50mg/l (139).

1321 NI cells and the HepG2 cells used in this experiment were treated with a range of concentrations of antioxidants from 10 nM to 100 μM for 16 hours. The exact amount of compound entering the cells was not known but was assumed to be proportional to the amount added to the cells.

The first antioxidant tested was vanillin. In Figure 18 the viability of HepG2 and 1321N1 cells treated with different concentration of vanillin in the cell media is shown.

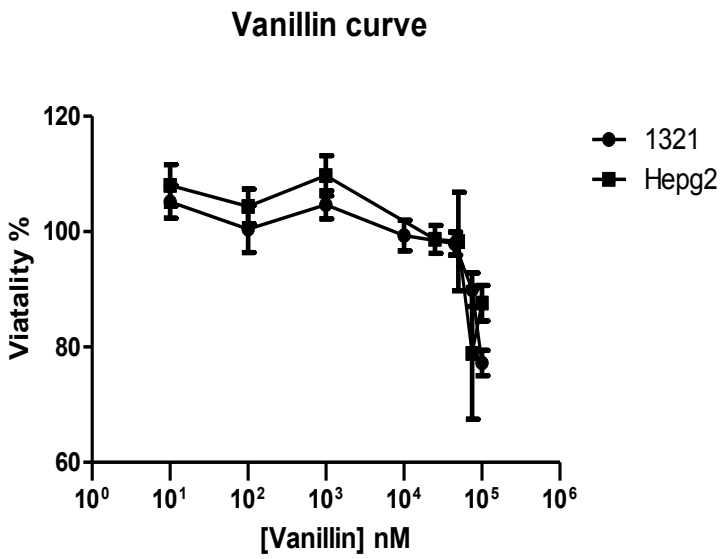


Figure 18: effect on the vitality of different concentration of Vanillin in cell 1321N1 and HepG2, n=4 for every sample.

Both curves looks very similar to each other. The 1321N1 cells show an increase in viability at the lower concentrations of vanillin from 10 nM to 10 µM. This increased is not statistically significant as judged by the ANOVA test with a confidence $p < 0,05$. The higher concentration of vanillin 100µM is able to significantly to reduce the cell's viability by 33% compared to untreated cells. The situation for HepG2 cells is different and the range of vanillin tested from 10nM to 100µM is not able to significantly change the viability of HepG2 cells. With these data it is possible say that the brain cells 1321N1 are more sensitive to vanillin at high dosage.

A similar viability curve was determined for resveratrol using the same range of concentrations in the media for16 hours. In this case, the curves made for the two different cells line are not similar.

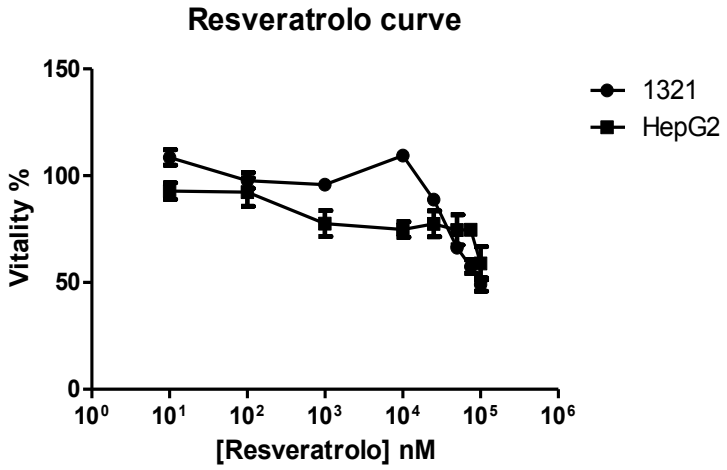


Figure 19: effect on the vitality of different concentration of Resveratrol in cell 1321N1 and HepG2, n=4 for every sample.

In particular, the 1321N1 seem to increase in viability up to 10μM resveratrol. This effect is not statistically significant. However there is a significant decrease in viability above 50 μM. A concentration of 75 μM of resveratrol is not able to reduce the viability of the cells compared to 50 μM of resveratrol. The highest concentration tested was able to reduce cell proliferation by around 50% compared to control.

In comparison, HepG2 cells are more sensitive to the amount of resveratrol present in the media than 1321N1 cells and 100 nM resveratrol is able to significantly reduce the viability of the cells ($p<0.05$). Higher concentrations of resveratrol are not able to significantly reduce the viability of the cells. The most noticeable difference was at 100 μM which reduced the cell's viability by around 40%. This value is not far from the 33% reduction in viability seen at a concentration of 1 μM. The two values between 1μM and 100μM are very close to each other and they are not able to generate a statistical difference. Statistically there is no difference between the reductions of viability obtained with 100μM of resveratrol in both cells line.

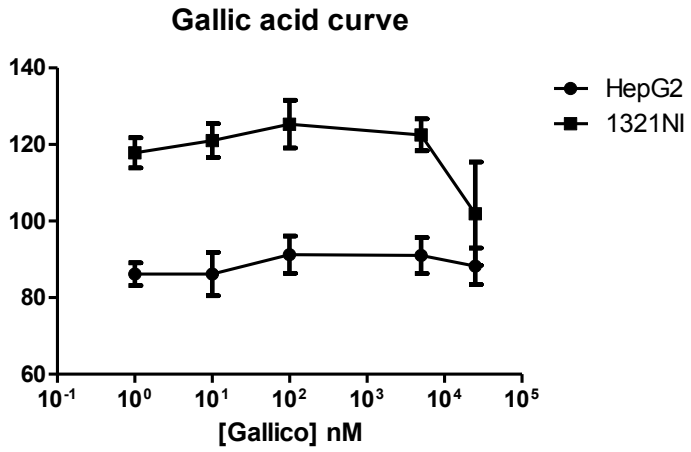


Figure 20: effect on the vitality of different concentration of gallic acid in cell 1321N1 and HepG2 n=3 for every sample. P <0.05.

Similarly, the two cells line 1321N1 and HepG2 were tested to evaluate their response after exposure to different concentration of gallic acid (Figure 20). HepG2 showed a minimal reduction in viability compared to the control cells which is not statistically significant. In the range tested from 1 nM to 25μM gallic acid is not able to reduce or increase cell viability. The cell line 1321N1 differed from HepG2 cells showing a slight increase in proliferation compared to the control, but this increase in viability isn't statistically significant. 1321N1 cells were not able to change the viability cells compared to the control.

To summarize, none of the antioxidants altered cell viability significantly at low concentrations. Only the high concentration 100 μM was able to cause a significant reduction in viability with exception of gallic acid which showed no effect.

As some loss of viability was observed at the highest concentration, each antioxidant was tested for its capacity to induce apoptosis in the cell. The capacity of 100 μM resveratrol to induce apoptosis in HepG2 cell lines after an exposure time of 24 hours has been reported previously (134).

The ability of 100 μM of the three antioxidants to induce apoptosis was investigated using a DNA fragmentation assay based on agarose gel electrophoresis. In addition to the antioxidants, 150 μM acrolein was used as a control. Cells were treated with compound for 24 hours and DNA extracted for analysis. DNA was separated into Genomic and Apoptotic DNA. In Figure 21 it is possible to see the agarose gel used to determine the DNA fragmentation. In all the samples, the DNA extraction was successful. All the Genomic (G) DNA samples show two different bands with a high molecular weight not able to migrate in the agarose gel, remaining close to the sample well.

For the Apoptotic (A) DNA, it is possible to see the same two bands but these much weaker compared to the G lane of each sample. The A lanes do not show any fragmented DNA with the notable exception of Sample 5 (100 μM resveratrol-treated cells) and to some extent Sample 4 (50 μM resveratrol).

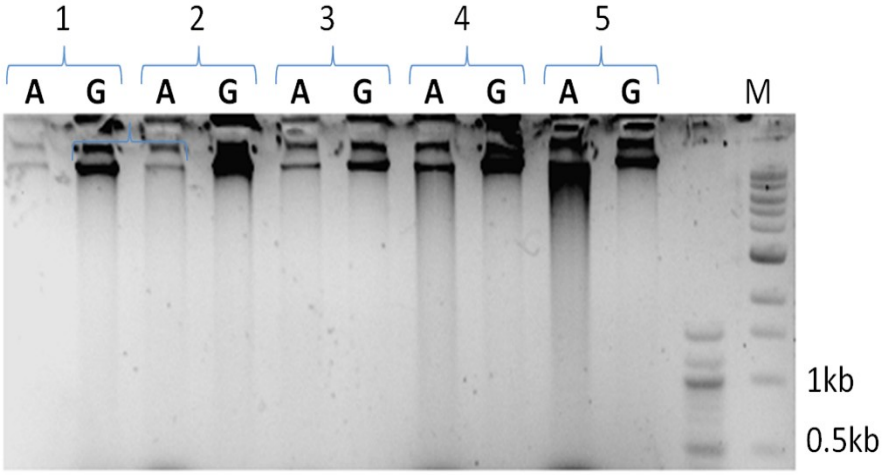


Figure 21: Agarose DNA gel 1% of human HepG2 cells treated with different antioxidants and toxic compounds. G: genomic DNA. A: apoptotic DNA. 1:sample treated with acrolein 150µM. 2: sample treated with 100µM gallic acid. 3: sample treated with vanillin 100µM. 4: sample treated with 50µM of resveratrol. 5: sample treated with 100µM of resveratrol. M: marker.

With this test is possible say that the high amount of vanillin (100 µM) is able to induce a reduction of viability but does not cause apoptosis. A similar situation is seen for gallic acid even though the high concentration used in the apoptotic test was not tested in the viability test. Resveratrol on the other hand leads to a reduction in viability and can cause apoptosis in HepG2 cells.

Analyzing the data present in this chapter indicates it is possible to say that the human cells are able to manage a different amount of antioxidant in the culture media and this could reflect the same tendency in the body. Only resveratrol was found able to induce DNA fragmentation supporting data reported by other authors (134).

With this result is possible to say that antioxidants in the diet at high concentrations may not always healthy. This data supports data presented in the paper (100) where it was reported that high doses of resveratrol causes nausea in individuals. This is not the first report that shows the toxicity of some antioxidants. β-carotene at a concentration of 1.5 µM able to reduce the vitality of the cells to around 30%(130). To make this data more significant, the amount of free resveratrol and its metabolite inside the cells needs to be calculated, and also the experiment needs to be repeated with different kind of cells.

EFFECT OF PHENOLICS IN PROTECTING AGAINST TOXIC ALDEHYDES

The phenolics vanillin, resveratrol and gallic acid were tested for their ability to protect cells against different aldehydes. Vanillin and gallic acid are present in whisky and resveratrol is present in red wine. Some phenolics found in the whisky have shown the capacity to interact and slow down with alcohol metabolism, resulting in a low amount of acetaldehyde in the blood stream (96). This study supports the idea that these phenolics and especially the vanillin are able to interact with ADH enzymes. That study does not investigate the role of these phenolics in the protection against acetaldehyde, and different aldehydes generated during the metabolism, especially ethanol.

The aim of this part of thesis is to evaluate the protection offer from these different phenolics (vanillin, resveratrol, and gallic acid) against the acetaldehyde, the direct product of the alcohol metabolism and some of the reactive aldehydes generate by the ROS such as methylglyoxyal and acrolein. A range of concentrations of antioxidant were used to treat the cells before being exposed to different concentrations of toxic aldehydes. The concentrations selected were based on the previous section, being those that did not cause significant toxicity to the cells. Toxicity was monitored using the MTT assay.

The first toxic aldehyde tested was acrolein. A range of concentrations of acrolein was used and a killing curve determined for both cell lines. In the Figure 22 is possible to see the reduction of viability caused in both the cells line by acrolein.

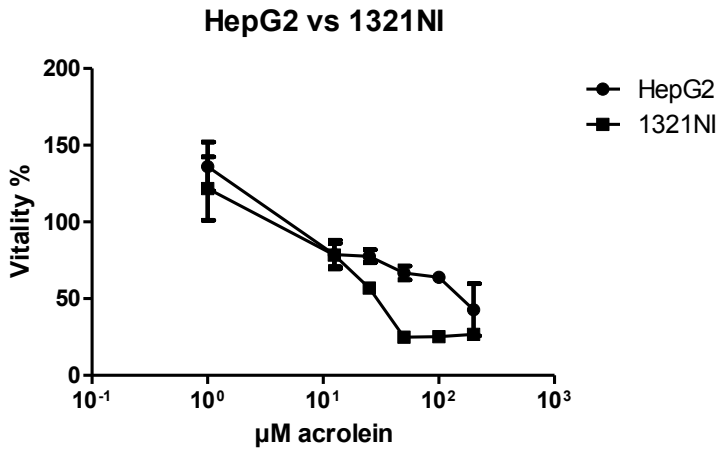


Figure 22: vitality of 1321NI and HepG2 cells line after acroelin exposure for 16 hours. N=3 p<0.05. exposure time 16 hours.

In both lines, treatment with acrolein led to a significant difference in viability compared to control cells. 200 µM acrolein is able to reduce cell viability around the 60%. In the 1321N1 cells line 25µM acrolein is able to cause a significant decrease in cell viability. Compared with control cells, the lowest concentration of acrolein able to generate a significant reduction of viability is 100µM for HepG2 cell line. Concentration of 150µM and 50µM of acrolein respectively for the HepG2 cells and 1321N1 were used. Other toxic aldehydes were also used – methyglyoxal and acetaldehyde.

The two cells line were pre-treated with different concentration of vanillin, resveratrol and gallic acid 10nM to 45µM as described in the previous section for 24 hours, and afterwards exposed to the toxic aldehyde. Cell viability was measured using the MTT assay.

Vanillin was the first antioxidant investigated in the protection against methylglyoxal, acetaldehyde and acrolein in the cells line 1321N1. In the Figure 23 below is possible to see the result of the MTT assays.

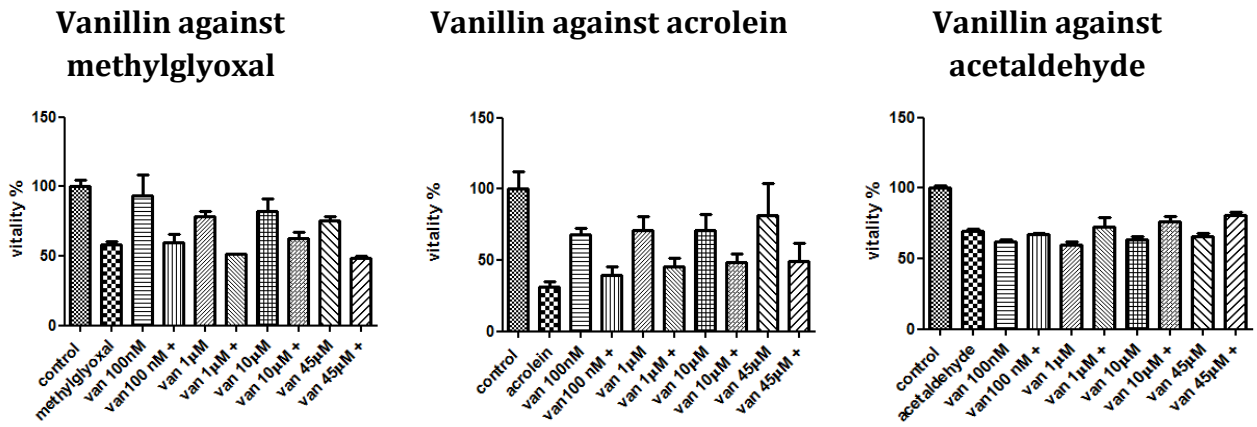


Figure 23: protection offer to cell line 1321N1 by vanillin. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Vanillin in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

The results show that there is no statistical difference between the control cells and those pre-treated with the vanillin only. However there is a significant difference between control cells and cells treated with methylglyoxal. The aldehyde causes a significant decrease in viability in 1321N1. However, none of the vanillin concentrations tested was able to significantly protect the cells against methylglyoxal toxicity. A similar result was observed with acrolein where pre-treatement with vanillin does not protect 1321N1 cells from acrolein toxicity. However with acetaldehyde, the cells pre-treated with vanillin show a significant reduction in viability compared to the control. The last plate treated with acetaldehyde showed the sample treated with the antioxidant very close to the sample treated with acetaldehyde and pre treated with antioxidant or with the acetaldehyde control.

Analyzing the effect of the pre-treated samples with different concentration of vanillin against the samples used as reference (treated only with the toxic) it is apparent that vanillin is not able to offer protection against the toxic aldehydes tested in this cell line.

The same analysis was made for the cell line HepG2. The result are shown in the Figure 24.

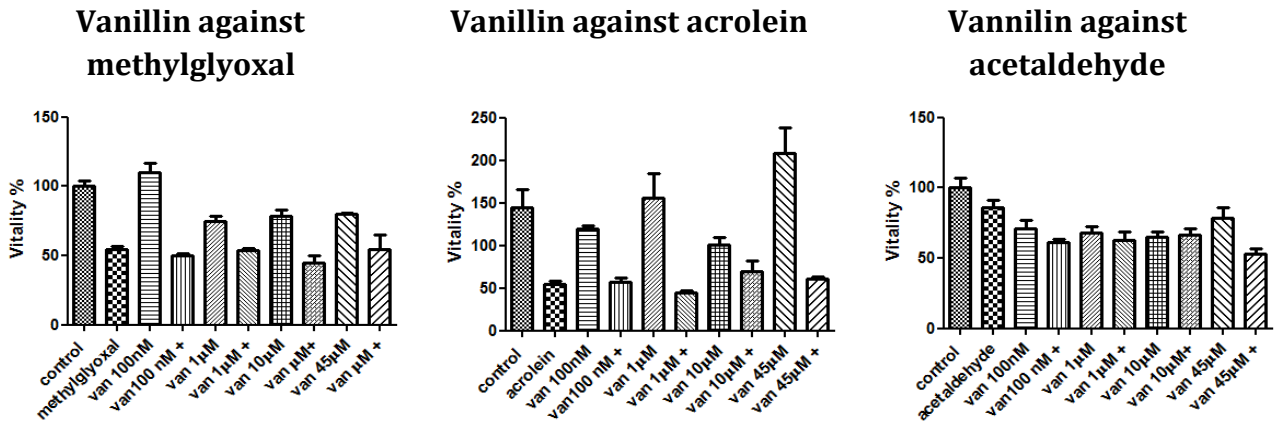


Figure 24: protection offer to cell line HepG2 by vanillin. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Vanillin in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

Treatment with 2 mM methylglyoxal led to a significant decrease in cell viability compared to control. Pre-treatment with vanillin was not able protect cells against methylglyoxal toxicity. This data is in agreement with the results for 1321N1 cells. Similarly, pre-treatment with vanillin could not protect cells against acrolein toxicity. The results for acetaldehyde show that the concentration used was not sufficient to cause significant loss in viability. Hence the effect of vanillin could not be fully evaluated. However it was noted that vanillin itself reduced the viability of cells, and that vanillin is not able to protect the cells against the acetaldehyde. The combination of vanillin and acetaldehyde is additive.

In conclusion vanillin is not able to protect the cells against the toxic aldehydes tested.

Resveratrol was the second anti-oxidant to be tested. In the Figure 25 is possible to see the response of the cells line 1321N1.

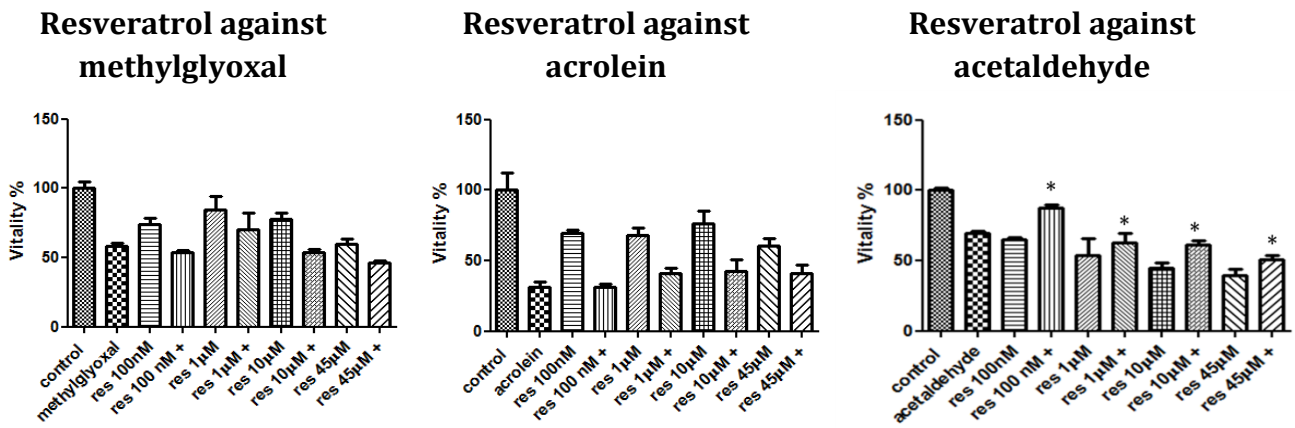


Figure 25: protection offer to cell line 1321N1 by resveratrol. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Resveratrol in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

Resveratrol concentrations of 45µM are able to reduce the viability of cells significantly. However, resveratrol is not able to protect the cells against methylglyoxal or acrolein toxicity. This is a different situation for the cells exposure to acetaldehyde. In this case, lower concentrations of resveratrol show a clear capacity to protect the cells from acetaldehyde toxicity. 45µM of resveratrol is able to induce a significant difference to the sample treated with acetaldehyde but the effect is not protective but increases the toxicity of the acetaldehyde reducing cell viability significantly.

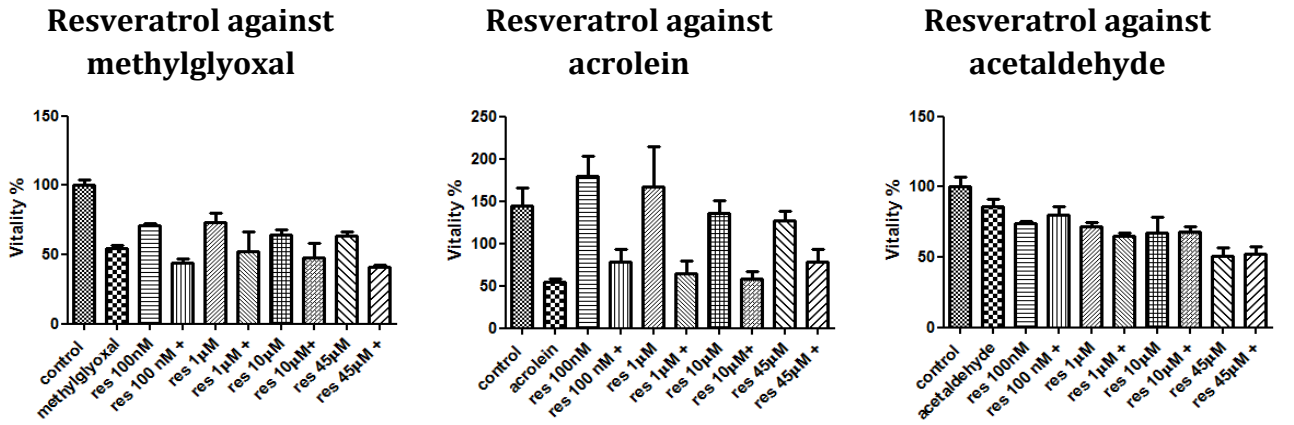


Figure 26: protection offer to cell line HepG2 by resveratrol. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Resveratrol in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

Resveratrol was also tested for its capacity to protect HepG2 cells (Figure 26) against the same aldehydes. The higher concentrations of resveratrol tested reduced the cells viability compared to control sample. This data are in agreement with previous results Figure 19. It can be seen that resveratrol does not protect cells against methylglyoxal. For cells exposed to acrolein however, the cells pre-treated with resveratrol did show protection against acrolein at higher concentrations of resveratrol. In the cells treated with acetaldehyde, acetaldehyde is not very toxic, and resveratrol is not able to protect the cells against the effects of acetaldehyde. The combination of resveratrol and acetaldehyde is toxic. The last anti oxidant tried is the gallic acid. The first cells line used for this experiment is the 1321N1.

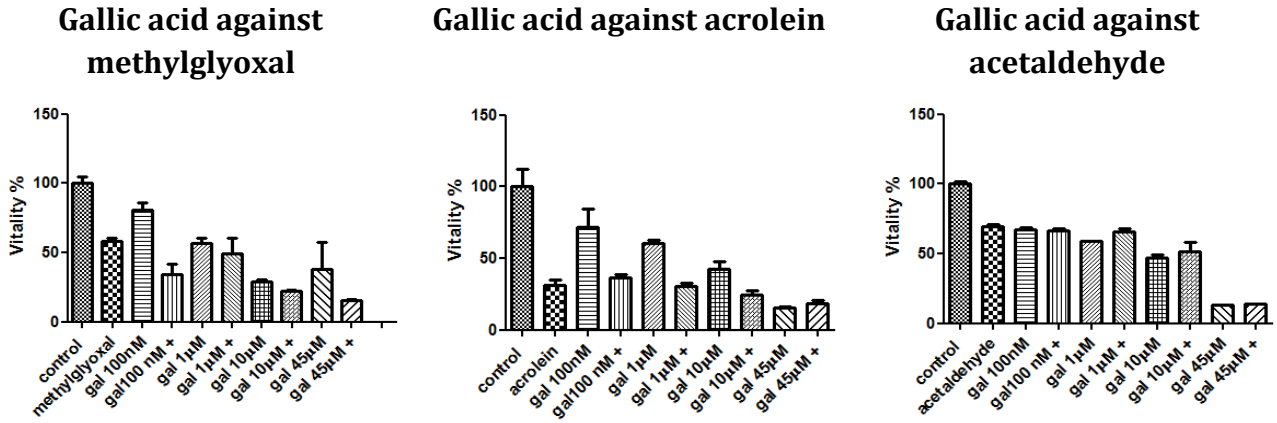


Figure 27: protection offer to cell line 1321N1 by gallic acid. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Gallic Acid in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

Gallic acid was also tested for its ability to protect cells. Treatment with gallic acid alone gave results that are in agreement with the curve shown at Figure 20. Pre-treatment with gallic acid did not protect cells against methylglyoxal toxicity, and in fact treatment with gallic acid followed by methylglyoxal led to significant toxicity. Pretreatment with gallic acid didn't protect cells against acrolein toxicity. For 1321N1 the gallic acid is not able to offer protection against acetaldehyde at the concentrations tested. In conclusion the gallic acid is not able to offer protection to the brain cells against the toxic aldehydes tried.

The possible role of the protection offer by gallic acid was also tested in the cell line HepG2 as show in the Figure 28 below.

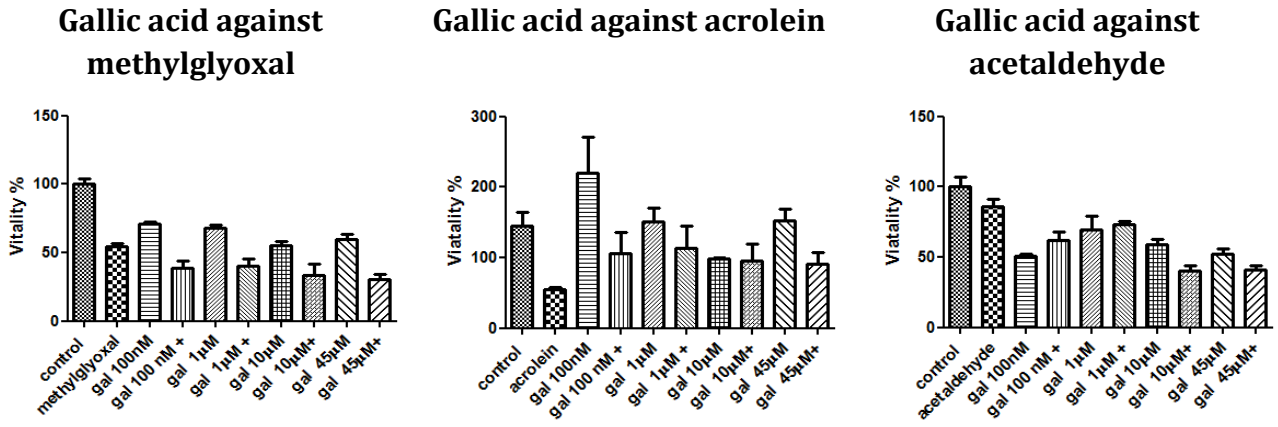


Figure 28: protection offer to cell line 1321N1 by gallic acid. Control is sample untreaded. Toxics used is the methylglyoxal, acrolein and acetaldehyde. Van in different concentration let in contact with the cells for 24 hours. The sample label with + were expose also to the toxic for 20 hours after the pre-treatment with the antioxidant.

In agreement with previous results, gallic acid treatment lowers cell viability in HepG2 cells. Liver cells pre-treated with gallic acid are not able to protect the cells against the methylglyoxal. None of the cells pre-treated with gallic acid were protected against acrolein toxicity. In the analysis of acetaldehyde toxicity there was no difference between the control sample and the sample only treated with acetaldehyde. Gallic acid appears to increase the toxicity of acetaldehyde and is not able to protect the cells against the different aldehydes tested.

CONCLUSION

Vanillin, resveratrol and gallic acid are compounds found in many alcoholic drinks. These compounds are intensively studied for their properties as antioxidants and for their interaction with the human body. In this study it was shown that when the cell lines 1321N1 and HepG2 are supplemented with these antioxidants in a range from 100nM to 45μM for 24 hours, resistance to reactive aldehydes methylglyoxal, acrolein and acetaldehyde is not improved.

It is apparent that the concentration of antioxidant in some instances is influencing the toxicity of the aldehyde. This could be investigated by using a wider range of concentrations of antioxidant. Further analysis needs to be carried out particularly to evaluate the actual amount of antioxidant present in the cells.

Another important part is to evaluate is the long term effect of the antioxidants and also their capacity to over or down regulated some genes.

In addition the aldehydes are all reactive and it is not clear the actual amount of aldehyde present inside the cells and for how long. Another problem to overcoming is the high volatility of the acetaldehyde that is incompatible with the incubator temperature (37°C). The cells can't be closed hermetically because they need a exchange of oxygen. For this reason another way is needed to add acetaldehyde to the media. A good solution would be to add the media supplemented with acetaldehyde every hour to keep the amount of acetaldehyde in contact with the cells constant.

With particular reference to alcohol metabolism, the antioxidants were not able to protect the cells against acetaldehyde, the first metabolite of the alcohol oxidation. In almost all cases, the cells pre-treated with antioxidant look less able to deal with aldehydes especially when the concentration of antioxidant is high at 45μM. This aspect needs more investigation especially as it has been shown that vanillin is able to decrease the alcohol metabolism and slow down the acetaldehyde release in the blood (91).

THE EFFECT OF VANILLIN ON THE ACTIVITY OF ALDEHYDE REDUCTASES

In 2007 two articles focused on vanillin and its interaction with alcohol kinetic metabolism in the mouse (91) and in human Hepatocarcinoma cells (109). The first paper showed the ability of vanillin to reduce ethanol metabolism in mice, the second showed the capacity of the vanillin to down regulate some of the genes related to the cell cycling and apoptosis. These down regulated genes were found in carcinoma cells and their down regulation indicates a protective role of vanillin against cancer. The data obtained during the MTT assay with the aim of investigating the protection offer by vanillin against different toxic aldehydes showed a reduction in viability of the cells pre-treated with this antioxidant. These papers and data provide ideas for better investigating the role of vanillin and its interference in the metabolism of aldehydes.

To test the effects of vanillin 1321N1 cells were treated with 45µM of vanillin in the media for 13 hours and total protein extracted used for enzyme assays. Two different substrates were used p-nitrobenzaldehyde and acrolein. The activity in mg/nmoli/minutes is expressed in the Table 4 and in the figure Figure 29.

Table 4: aldehyde reductase activity of protein extraction from cell 1321N1 treat with vanillin 45µM.

Substrate	NADPH µM	NADH µM	[substrate]	Control activity	Vanillin treated
pNBA	6.2	/	100 µM	8.97 ± 3.35	5.65 ± 2.45
pNBA	/	6.2	100 µM	1.75 ± 0.64	/
Acrolein	6.2	/	1mM	1.09 ± 0.51	1.28 ± 0.31

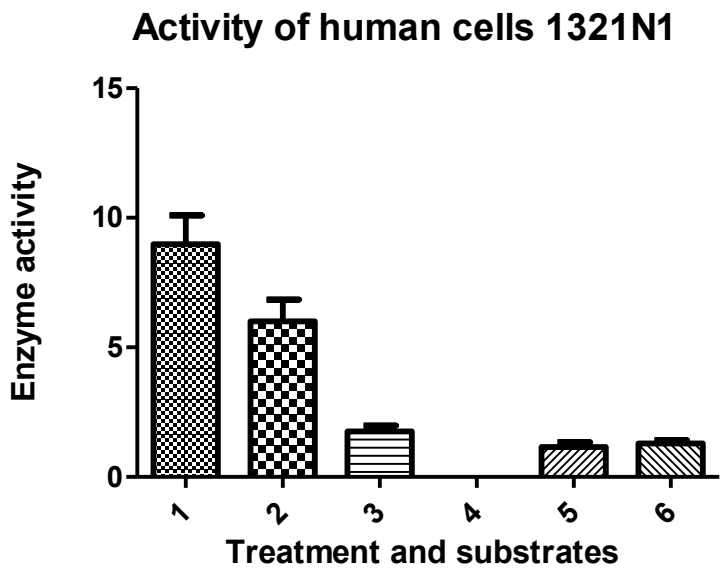


Figure 29: aldehyde reductase activity in mg/ml/minute of protein extract 1321N1 with different substrate (pNAB and acrolein) different cofactor (NADPH and NADH) and cells treated with vanillin 45μM and not treated used as control. 1 ,3 and 5 are the controls sample. 2,4 and 6 are the cells pre-treated with vanillin. Reactions 1 and 2 the substrate is pNAB and the cofactor is NADPH. In the reactions 3 and 4 the substrate is pNAB and the cofactor NADH, in the reactions 5 and 6 the substrate is the acrolein and the cofactor is NADPH. Each reaction is made with three sample from three different cells line.

Figure 29 shows that the pre-treatment with vanillin is able to lower the aldehyde reductase activity of the cell extracts by 37% when using pNBA as substrate. The reduction of activity is statistically significant. Changing the cofactor from NADPH to NADH made the activity of the treated samples so slow that was impossible to be measured. When acrolein was used as substrate there was no difference in activity. Within the cells it is likely that there are several different types of aldo-keto reductase. It's not possible to establish which of the enzymes has lower activity, but from the data it is possible to conclude that pNBA and acrolein are reduced by two different groups of enzymes, the first group being influenced by vanillin exposure and the second group is responsible for acrolein metabolism, and not influenced by the vanillin treatment.

It's possible that vanillin is interacting with a promoter and is down-regulating the expression of different enzymes involve in this reaction. A Q-PCR targeting specific mRNAs is needed to establish whether there is a change in gene expression at the mRNA level. Western Blots can be used to investigate changes in protein levels. Alternatively, the low activity of the vanillin groups could be also explained by an interaction of the vanillin with the catalytic site of the enzyme responsible as an inhibitor. This theory was also proposed to explain the low amount of acetaldehyde found in the blood stream of rats fed with ethanol and vanillin. It was also proposed that vanillin is able to interact with the active site of ADH (96). This data totally agrees with the previous analysis in which vanillin was not able to protect the cells against the toxic acrolein in the MTT assay.

VANILLIN AND EXPRESSION OF THE AKR7A2

Western Blotting was carried out to evaluate the possible change of expression of the aldo-keto reductase AKR7A2. The cells line investigated was 1321N1, because it was the same one used for the inhibition experiment previously discussed. Cells were exposure to 45 μM vanillin for 24 and the proteins extracted and used for Western Blotting. As a control cells not treated with vanillin were used. The samples didn't show any difference to each other in the expression of the AKR7A2 (data not shown). Other trials will be made using a quantitative PCR a technique more adequate for this purpose and other AKRs will be investigated.

INHIBITION OF ALDEHYDE REDUCTASE AND ADH BY PYRAZOL AND VANILLIN

The aim of this experiment was evaluate the total aldehyde reductase activity of the protein extract prepared from 1321N1 and test whether this activity was inhibit able by vanillin. The substrate used was pNBA 1mM, chosen because is able to give a good rate of reduction. Acetaldehyde and acrolein are not good enough substrates for whole crude cells extract. The cofactor used was NADPH and can be followed at 340nm. It is known that some enzymes of the family ADH are able to metabolize aldehydes and it is also known that pyrazole is a good inhibitor of this class of enzymes. The reduction of pNBA in the presence pyrazole and vanillin was investigated. This showed in Figure 30 that pyrazol at a concentration of 45μM is able to inhibit pNBA reductase activity by 24% compared to control, and the vanillin at the same concentration inhibited pNBA reductase activity by 50%. This pyrazole data suggests that ADH could play a role in the detoxification of aldehydes and has a quarter of the total pNBA activity. Vanillin is able to inhibit this activity more strongly than the pyrazole, probably is able to interact with enzymes other than ADH. This evidence is supported to the last experiment where the concentration of vanillin was 150μM and the inhibition of activity was the 72%. At the same concentration the pyrazole was not able to cause significant inhibition. The data also shows the power of the vanillin to inhibit the activity of the reaction. The vanillin is able to inhibit a wider range of enzymes compared to the pyrazole especially for the enzymes involve in aldehyde reduction.

Unfortunately, the data obtained with the MTT assay in which vanillin and acetaldehyde were involved were not able to give more information about the effect of the vanillin on the metabolism of the acetaldehyde. However the data collected suggests that the vanillin doesn't affect the viability of the cells treated with acetaldehyde and consequently its metabolism. This data is in contrast with the data obtained with the vanillin which is able to inhibit aldehydes metabolism.

A possible explanation could be the low capacity of the acetaldehyde to kill cells in vitro and consequently if the vanillin could inhibit acetaldehyde metabolism, this wouldn't be translated in a reduction of viability.

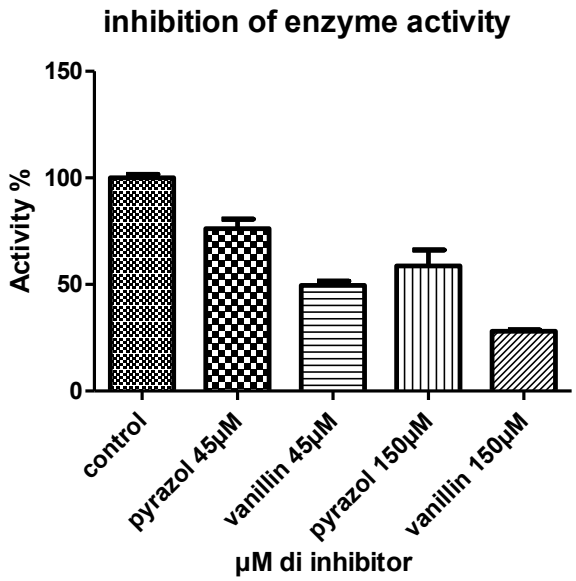


Figure 30: activity of protein extract 1321N1 with the presence of different inhibitors. Three samples for each measure. The reference value for the control is 9 nmoli/mg minute with a standard deviation +/- 0,26.

CONCLUSION

The effect of the vanillin to inhibit the total aldehyde reductase activity was evaluated in the cell line 1321N1. The vanillin shown can inhibit the reduction of the substrate pNBA by the cell extracts. The inhibition caused is able to block the enzymes involved in pNBA reduction, but not the enzymes involved in acrolein metabolism. With this evidence is possible to affirm that the enzymes involved in the pNBA reduction are not the same as those involved in acrolein reduction.

The comparison between proteins obtained from cells pre-treated with vanillin and control cells was useful to determinate whether the vanillin is able to change the expression of enzymes involved in pNBA metabolism. The absence of difference between the two samples suggests that the vanillin is not able to interact with the expression of these enzymes, but is able to inhibit their activity. The property of vanillin to inhibit pNBA activity was also compared with inhibitory power of pyrazole. Pyrazole is known to be selective for the ADH. The vanillin showed a major inhibition compared to the pyrazole in reducing the enzymatic activity on the substrate pNBA. Vanillin is able to interact with a wider spectrum of enzymes and this is demonstrated in its capacity to increase the inhibition of the enzyme assay when its concentration is increased, in contrast to the pyrazole.

CHARACTERIZATION OF THE HUMAN AKR7A2 AND AKR7A5

The purpose of this part of the thesis is add new data to the data present in literature about the two enzymes. This includes an evaluation of the preference for the two cofactors NADH and NADPH, as normally the AKR are able to use both substrates. In additional, further substrates of interest will be tested including acetaldehyde derived from alcohol metabolism and other aldehydes generated during oxidative stress. Oxidative stress is also generated during the ethanol metabolism. The main data obtained will be useful for better characterizing the physiological role of these enzymes with particularly focus on the human AKR7A2. Other than the role of these enzymes in vitro, particular attention will give to AKR7A2 and its physiological role inside the cell. The cell line 1321N1 will be used for generating transient cells that over express this enzyme. The transient cells will be used to test their resistance against different aldehydes recognized to be dangerous for humans, and the data obtained will be compared with a control cell line. The data obtained will be useful to explain the physiological role of AKR7A2 in protection against aldehyde and oxidative stress.

RECOMBINANT EXPRESSION OF THE ENZYMES AKR7A2 AND AKR7A5

The two aldo keto reductases were cloned previously (38), (44). The two proteins were cloned inside the pET-15b vector (Novagen) and transformed into *E.coli* BL21 pLysS to allow their expression. The proteins were expressed following the normal induction process treating with 0.05 mM IPTG (isopropyl-beta-thio galactopyranoside) at OD_{600} between 0.4-0.6. Using the His-tag the proteins were simply purified by automatic FPLC (Fast protein liquid chromatography). In Figure 31 , it is possible see the expression of both proteins in *E.coli* and the high purity of the protein obtained with the HIS-tag purification method. The AKR7A5 is expressed more strongly than AKR7A2.

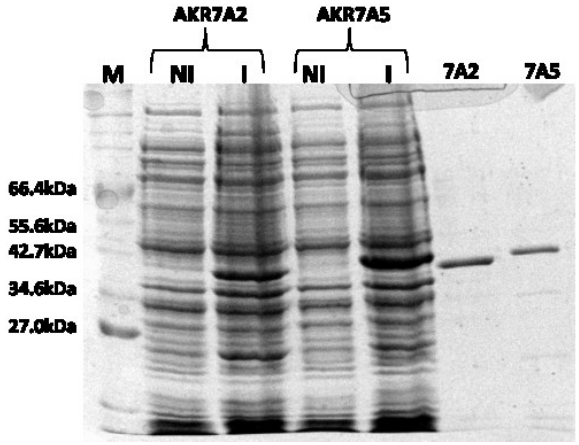


Figure 31: M: Marker, NI: not induced, I: induced bacteria, 7A2: purify , 7A5:purify.

Figure 32: Western Blotting using rabbit antibody against his tag present in the proteins expressed.

Antibodies against the HIS-tag were used to confirm the correct protein was expressed and the result is shown in Figure 32. Different fractions were eluted during purification by FPLC and analyzed by SDS-page to evaluate the purity of the single fraction. All the fractions to be used for other experiments were tested using an enzyme assay to evaluate the correct folding of the protein and consequently its correct catalytic activity. Normally, not more than three fractions for purification were kept. All the experiments were carried out not more than three days after the protein purification. These enzymes need to be used in a very short period of time after their purification because they tend to lose most of their activity in less than one week.

ACTIVITY OF THE TWO ALDO KETO REDUCTASE

In the first part of the study, the two aldo keto reductases were tested to evaluate the best cofactor for them. The test was made under saturated condition of different substrates and cofactors (NADH or NADPH). The first substrate tested was SSA known as a good substrate for these enzymes (46). Afterwards, other substrates were tested including acrolein and acetaldehyde. Acetaldehyde was of particular interest to evaluate the possibility of this enzyme to contribute to global ethanol metabolism with a focus on reducing the acetaldehyde to ethanol. All the experiments were carried out in triplicate and the values are expressed as the specific activity of the enzyme (nmol/mg/minute). As can be seen in Table 5 these enzymes show a preference for the cofactor NADPH regardless of the substrate used.

For the substrates in which this enzyme (AKR7A2) shows a high Vmax (SSA and p-nitrobenzaldehyde) the reaction is possible with both cofactors. When NADH was used as cofactor, the Vmax of the reaction is decreased by around four-fold.

When acrolein and acetaldehyde were used as substrates and NADPH as cofactor, the specific activity was too low to be calculated. Particularly when acetaldehyde was used as a substrate, the monitoring of the change in absorbance was very difficult, probably due to the high volatility of this chemical or because of its reaction with the enzyme and consequently a change in the UV spectra. During the reaction with acetaldehyde there were fluctuations in the absorbance at 340nm, which indicates some variability within the instrumentation. In a normal reaction, as seen for SSA, the saturated amount of co-factor and substrate generated a constant decrease in absorbance. In all the reactions the cofactor became oxidized and there was a decrease in absorbance at 340nm.

When acrolein was used as substrate, a small reduction in absorbance was detected. This reduction was very low compared to other substrates such as SSA. Therefore acrolein and acetaldehyde do not appear to be good substrates for this enzyme, especially acetaldehyde.

The results for mouse AKR7A5 showed a lower activity compared to AKR7A2 for the substrates SSA and p-nitrobenzaldehyde. Also, for this enzyme with these substrates, the activity was higher with the cofactor NADPH but still worked with NADH although at a slower rate. A difference was observed however between acrolein and acetaldehyde. With acrolein, AKR7A5 doesn't show a great preference for the co-factor. Acetaldehyde was still metabolized but with a very low rate. For both the enzymes studied it seems that acetaldehyde is not a good substrate for these enzymes, not only because of the low rate of reaction, but especially because of the high concentrations of substrate that need to be in the reaction.

Table 5: human AKR7A2 and mouse AKR7A5 and is activity with selected substrate and cofactor.

Substrate	Human AKR7A2			
	NADPH µM	**NADH µM**	**Substrate**	**Activity nmol/mg/minute**
SSA	5.6		1 mM	1056 ± 37.4
SSA	/	6.3	1 mM	220 ± 35.5
p-Nitrobenzaldehyde	5.6	/	100 µM	423.4 ± 2.9
p-Nitrobenzaldehyde	/	19	100 µM	56.4 ±1.3
Acrolein	11.2	/	100 mM	56.4 ± 1.3
Acrolein	/	25	100 mM	7.66 ± 2.9
Acetaldehyde	11.2	/	100 mM	7.6 ± 2.9
Acetaldehyde	/	12.6	100 mM	1 ± 0.4

Substrate	Mouse AKR7A5			
	NADPH µM	**NADH µM**	**Substrate**	**Activity nmol/mg/minute**
SSA	5.6	/	1mM	865.7 ± 20.9
SSA	/	6.3	1mM	74.5 ± 13.1
p-Nitrobenzaldehyde	5.6	/	100 µM	338.3 ± 32
p-Nitrobenzaldehyde	/	19	100 µM	42.3 ± 5.6
Acrolein	11.2	/	100mM	26.9 ± 2.5
Acrolein	/	25	100mM	32.54 ± 9.6
Acetaldehyde	11.2	/	100mM	13.1 ± 7.5
Acetaldehyde	/	12.6	100mM	/

With this experiment is possible to say that both the enzymes expressed show a strong preference for NADPH as cofactor, but are also able to utilize NADH, and this could be important inside the cells when the ratio between NADPH and NADH can change during metabolism or stress.

THE ROLE OF AKR7A2 AND AKR7A5 IN THE PROTECTION AGAINST TOXIC ALDEHYDES

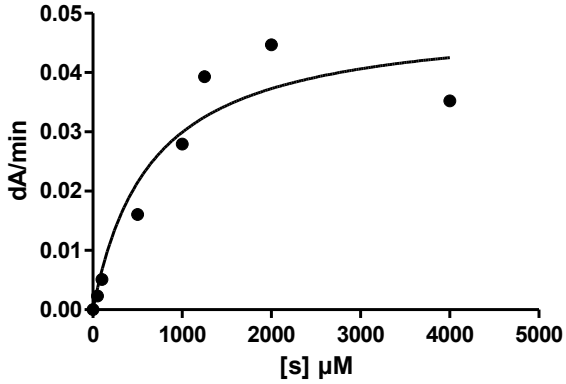
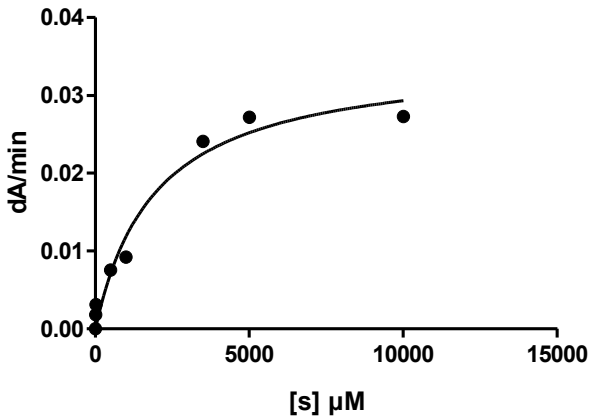
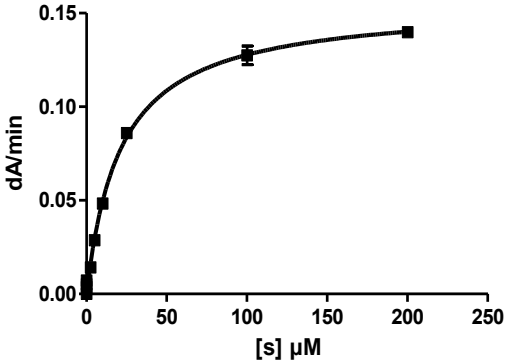
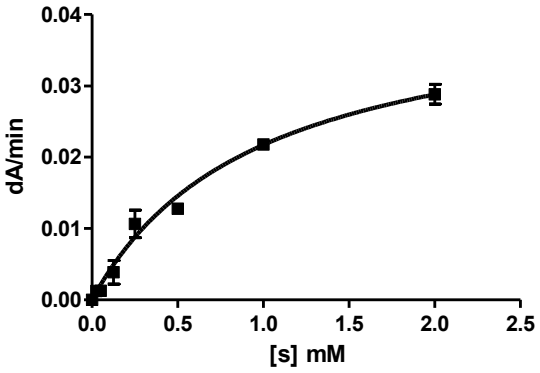
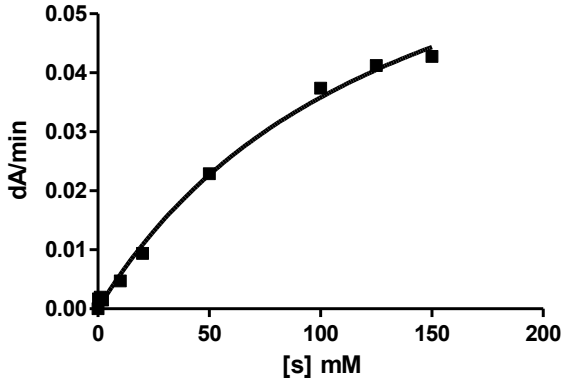
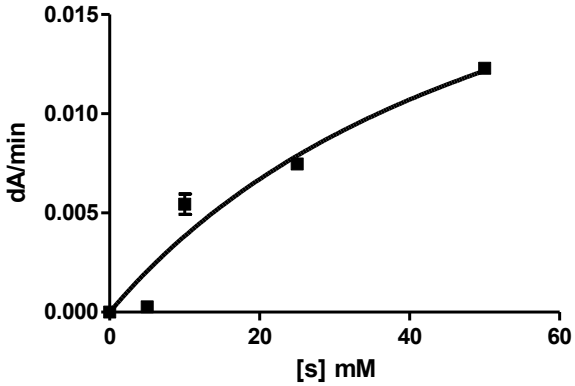
Ethanol metabolism is able to produce a stress inside the cell, in particular it is able to cause an imbalance in the normal ratio between NAD^+/NADH. The electron flow in the mitochondria is able to generate some ROS during the normal metabolism. ROS have the ability to react with different macromolecules such as proteins nucleic acids and lipids. In particular, the reaction with the lipids is able to generate some highly reactive aldehydes. These dangerous aldehydes can move throughout the human body or stay close to their source.

It is thought that the aging process begins when the human body is not able to detoxify the aldehydes present in the human body that have arisen from endogenous or exogenous sources. The aldo keto reductase family has a known ability to metabolize a wide range of aldehydes and ketones.

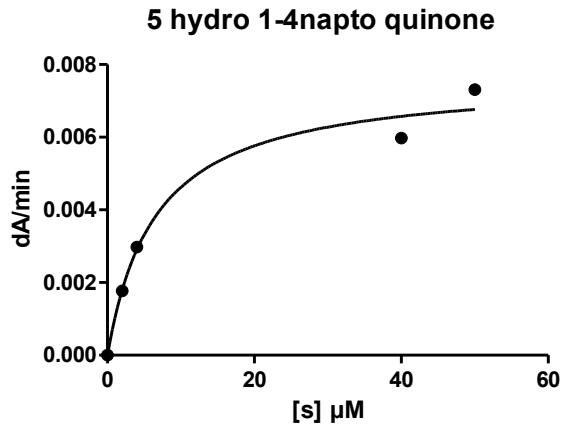
To better understand the physiological role of the two AKR7A enzymes, especially the human AKR7A2, they were characterized in vitro using different toxic aldehydes, and physiological molecules such as SSA. In the Table 6 are shown the Michaelis-Menten curves obtained with different substrates. The apparent K_m and Vmax values were estimated from the initial velocities measured over a range of substrate concentrations. The cofactor NADPH was used at high concentration (0.1 mM) in order to consider it saturating during all the reactions, as describe in the Material and Methods. The Michaelis-Menten curves were also used to the determinate the K_m and Vmax. In the study of the kinetic parameters it was assumed that these enzymes are dimeric (with two catalytic sites), so the molecular weight used is the double of a single subunit of the enzyme. The same considerations were used for the experiment involving the human AKR7A2 and for the mouse AKR7A5, and the Michaelis-Menten curves are shown in the Table 7.

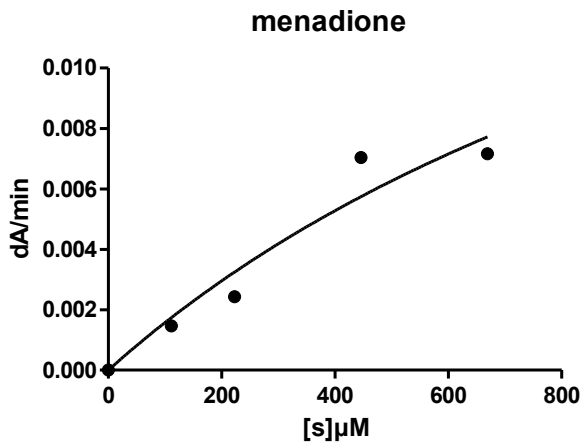
The enzymes used were freshly prepared and kept on ice and they are not more than three days old.

Table 6: characterization of the human AKR7A2 with different substrate made during the lipid peroxidation or present physiology in the human body.

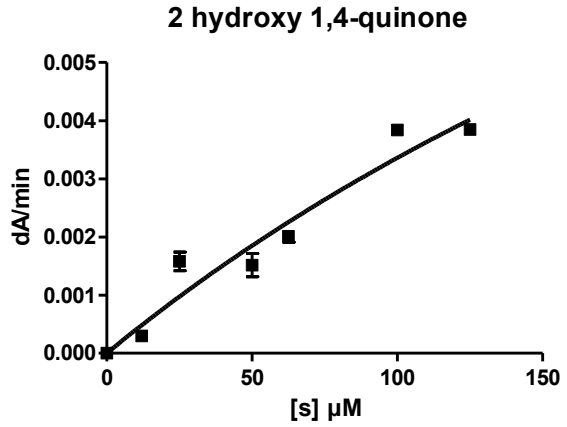
<table>
<tr><td colspan="2"></td><td colspan="2">AKR7A2</td></tr>
<tr><td>Vmax 0,99 µM/mg/min</td><td>K_m 654,2 µM</td><td>Vmax 0,93 µM/mg/min</td><td>K_m 1,948mM</td></tr>
<tr><td colspan="2">4p-nitrobenzaldehyde
 
</td><td colspan="2">Methylglyoxal
 
</td></tr>
<tr><td>Vmax 1,34 µM/mg/min</td><td>K_m 21,38 µM</td><td>Vmax 0,379 µM/mg/min</td><td>K_m 966 µM</td></tr>
<tr><td colspan="2">SSA
 
</td><td colspan="2">Muconaldehyde
 
</td></tr>
<tr><td>Vmax 0,072 µM/mg/min</td><td>K_m 23,86mM</td><td>Vmax 0,237 µM/mg/min</td><td>K_m 59,65 mM</td></tr>
<tr><td colspan="2">Acrolein
 
</td><td colspan="2">Crotonaldehyde
 
</td></tr>
</table>

Vmax 0,062 µM/mg/min	K_m 6,55µM	Vmax 0,08 µM/mg/min	K_m 1,481mM





Vmax 0,149 µM/mg/min	K_m 449,2µM

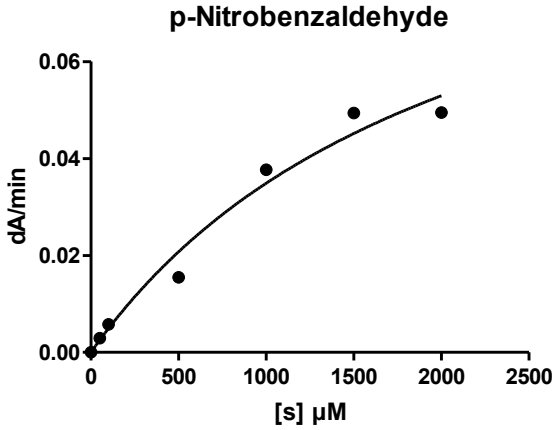
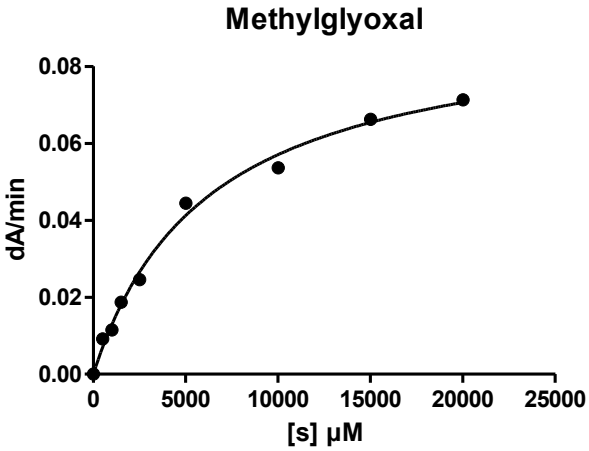
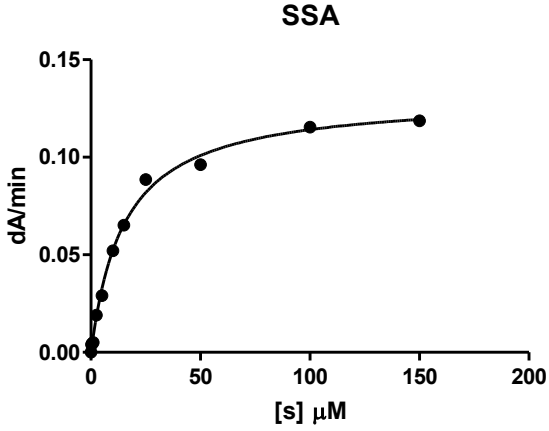
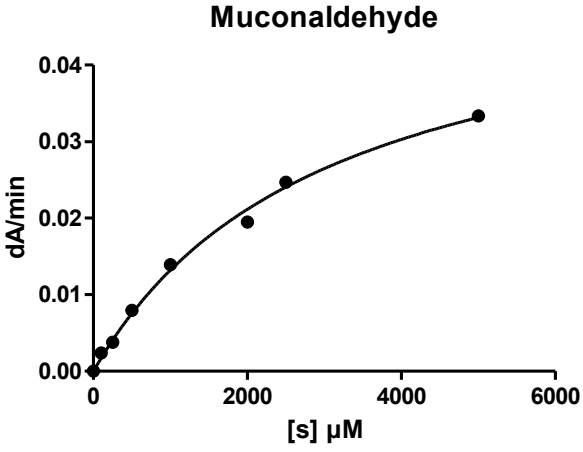


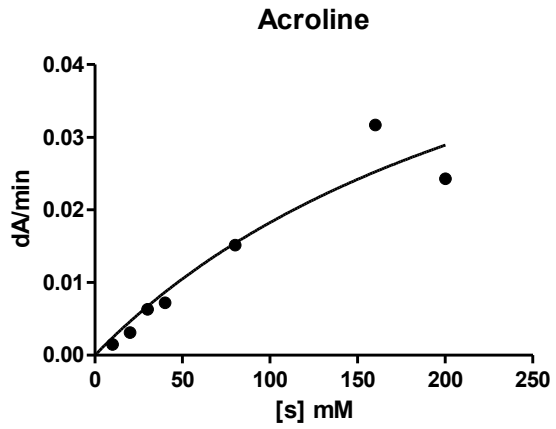
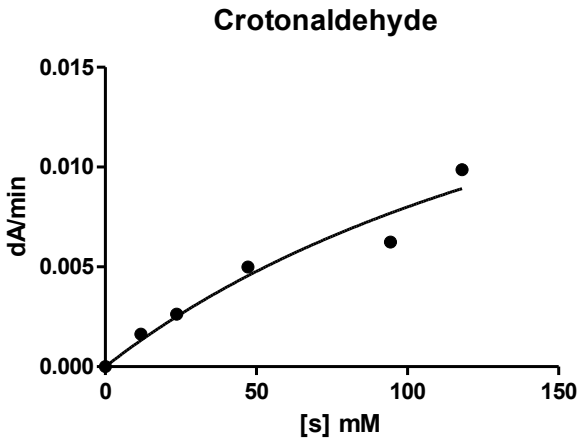
Some of the graphs show ideal Michaelis-Menten curves, for example SSA, 4-nitrobenzaldehyde, methylglyoxal and 5 hydro 1-4 napto quinone. The K_m calculated for SSA is around 21 µM, and this is the only K_m with a real low value, providing more supporting evidence for the role of this enzyme inside the human body in the metabolism of the succinic semialdehyde. Some curves show a poor fit to Michaelis-Menten, such as like the 2-hydroxy 1,4-quinone. These substrates were very difficult to study particularly because is very difficult to get reproducible data. The reduction in absorbance at 340nm was not constant in all the reactions and sometimes there were fluctuations in the absorbance. For this reason, this reactions were carried out several times and only the reproducible data collected was used to generate the curves. There is no real explanation for these fluctuations in absorbance, which could be made for a chemical reaction between the aldehydes used and the cofactor or the enzyme itself. These substrates for this reason were considered poor and the high K_m calculated led to the assumption that these substrates are not suitable for enzyme study. The same consideration can be made for the AKR7A5 enzyme.

This kinetic study was also difficult because most of the aldehydes studied are liquid so they change the density of the solution inside the reaction tube.

With some substrates, the enzymes did not work well and is possible see this from the graphs presented - crotonaldehyde and acrolein are good examples of this. This is different from the situation for SSA, where the Michaelis-Menten curve is very close to the ideal one.

Table 7: characterization of the mouse AKR7A5 with different substrate made during the lipid peroxidation or present physiology in the mouse body.

<table>
<tr><td></td><td colspan="2">AKR7A5</td><td></td></tr>
<tr><td>Vmax 3,9 µM/mg/min</td><td>K_m 2,14mM</td><td>Vmax 1,90 µM/mg/min</td><td>K_m 6.24mM</td></tr>
<tr><td colspan="2"> 
</td><td colspan="2"> 
</td></tr>
<tr><td>Vmax 2,7 µM/mg/min</td><td>K_m 15,17 µM</td><td>Vmax 1,09 µM/mg/min</td><td>K_m 3,04mM</td></tr>
<tr><td colspan="2"> 
</td><td colspan="2"> 
</td></tr>
</table>

Vmax 0,736 µM/mg/min	K_m 280mM	Vmax 0,253 µM/mg/min	K_m 209mM
			

As described earlier, as the sequence identity between the enzymes is 78% with a similarity of 87% (40) it is interesting to compare the values obtained for the same substrate. This data can be also interpreted and can be helpful to structural biologists to increase the knowledge about the structure and function of the active site of the enzymes, that can be combined with data obtained using software used to predict the tertiary structure from the amino acid sequence.

The data obtained from these two different enzymes is summarized in Table 8. The physiological substrate succinic semialdehyde appears to be a better substrate for the mouse than for the human enzyme. The turnover for this substrate is around twice as high for the mouse, respectively 1.73 and 0.88; the Km is very low for the enzymes in the range of 20 µM. These data confirm that probably in the mouse that SSA is really the native substrate for AKR7A5 but this may not be the case for the human enzyme as the turnover is lower. The model substrate for the aldo-keto reductase 4-NBA interacts in a different way with these two enzymes. The kcat for the mouse is 2.51 s^{-1} and the kcat for the human is four time less. The mouse enzyme is also more difficult to be saturated by 4-NBA as indicated by its higher K_m of 2.14 mM. In any event, the global efficiency of the enzyme expressed as kcat/ K_m is not very different.

However noticeable differences were observed when other aldehydes were used as substrates. The aldehydes tested can be generated inside the human body by the peroxidation of the lipids. Both the enzymes show a very low Vmax with the substrates (methylglyoxal, muconaldehyde, acrolein and crotonaldehyde), and normally a high K_m which suggests that these substrates are not ideal for these enzymes. The K_m for all these substrates is in the millimolar range, increasing to 205mM for crotonaldehyde for the mouse AKR7A5.

The corresponding K_m for the human enzyme is four times lower at 59mM for crotonaldehyde. These values being so high for the K_m suggest that these enzymes are not involved in the detoxification of this reactive aldehyde under physiological conditions.

The K_m calculated for muconaldehyde is 0.96 mM for the human enzyme, indicating that this enzyme could play a role in the detoxification of muconaldehyde during exposure to high levels.

Using the efficiency parameter for the enzyme, the kcat/ K_m, it is possible to see that this parameter is around hundred times less for muconaldehyde and methylglyoxal, compared to succinic semialdehyde. Succinic semialdehyde was proposed as physiological substrate for this enzymes (80), and the data obtained in this thesis indicate that AKR7A2 could play a key role in the metabolism of this substrate.

The situation is not the same for two other aldehydes tried, that is acrolein and crotonaldehyde with a K_m of 23.8 mM and 55.9 mM respectively, and the kcat/ K_m is a thousand times less than for succinic semialdehyde. For these two last substrates, the mouse enzyme shows a higher K_m compared to the human enzymes. A comparison of the substrates studied and the values obtained, in particular the kcat/ K_m shows that AKR7A5 prefers longer chain aldehydes. This looks clear when comparing the shorter acrolein with the longer crotonaldehyde, which has two more carbon atoms. Also as has been shown previously, the presence of an alpha-beta unsaturated double bound between the carbon atoms makes the turnover slower.

The considerations made for AKR7A5 can also be seen for human AKR7A2. Analyzing the situation for the AKR7A2 and comparing it with AKR7A5 it is possible see that the mouse enzyme has a better efficiency for the metabolism of SSA and pNBA.

The other substrates tested are better substrates for the human enzyme compared to the mouse but they are still very poor compared to the SSA and 4-NBA. These data can be used to better understand the mechanism of interaction between the enzyme and substrate, and the importance of particular amino acid residue differences between the two enzymes utilized. These data can be also utilized to predict how other enzymes of the aldo keto reductase family might function. In Table 8 it is possible see all the data generated with the different substrates tried and the comparison between the two enzymes.

Table 8: comparison of the main data obtained with AKR7A2 and AKR7A5 with different substrates.

Substrate	Enzyme	Vmax µmoles/mg enzyme/min	K_m mM	$V_{max}/_{km}$ $Min^{1}*mg*ml$	Kcat s^{-1}	K_{cat}/k_m Min^{-1}/M
Succinic semialdehyde	AKR7A5	2.7	0.015	178	1.73	6.88×10^6
	AKR7A2	1.34	0.021	62	0.88	2.49×10^6
pNBA	AKR7A5	3.90	2.148	1.81	2.51	7.01×10^4
	AKR7A2	0.99	0.65	1.52	0.65	6.03×10^4
Methylglyoxal	AKR7A5	1.90	6.23	0.30	1.22	1.18×10^4
	AKR7A2	0.93	1.94	0.47	0.616	1.9×10^4
Muconaldehyde	AKR7A5	1.09	3.04	0.36	0.70	1.39×10^4
	AKR7A2	0.379	0.966	0.39	0.25	1.56×10^4
Acrolein	AKR7A5	0.736	280	2.63×10^{-3}	0.47	1.02×10^2
	AKR7A2	0.072	23.86	3.5×10^{-3}	0.048	1.21×10^2
Crotonaldehyde	AKR7A5	0.253	209	1.21×10^{-3}	0.16	4.69×10^1
	AKR7A2	0.237	55.9	3.9×10^{-3}	0.156	1.57×10^2

The values obtained for the enzymes expressed and purify during this thesis were compared with the data present in the scientific lecture. In Table 9 is shown the main values obtained for the mouse AKR7A5 during this thesis and a comparison with other value present in the science literature.

Table 9: comparison of the main value obtained from the mouse AKR7A5 during this thesis works and the data present in the literature.

Substrate	Source	Vmax µmoles/mg enzyme/min	K_m mM	Vmax/k_m Min^{1}*mg*ml	Kcat s^{-1}	Kcat/k_m Min^{-1}/M	Reference
Succinic semi aldehyde	Mouse	2,70	0,015	178	1,73	$6,88X10^{6}$	This thesis
	Rat	1.46	0.14	10,43	0,93	$4,02X10^{4}$	(39)
	Rat	Not given	0,184	Not given	1,15	$3,8X10^{5}$	(40)
	Mouse	Not given	0,02	Not given	1,33	$4,2X10^{6}$	(45)
pNBA	Mouse	3,90	2,148	1,81	2,51	$7,01X10^{4}$	This thesis
	Rat	Not given	3,6		4,1	$6,9X10^{4}$	(40)
	Rat	2,58	0.72	3.58	1,40	$1,16X10^{5}$	(39)
	Mouse	Not given	0.87	Not given	0,76	$5,34X10^{4}$	(45)
Methylglyoxal	Mouse	1,90	6,23	0,30	1,22	$1,18X10^{4}$	This thesis
	Rat	1,13	7,22	0,16	0,71	$5,9X10^{3}$	(39)
	Mouse	Not given	3,98	Not given	0,78	$1,18X10^{4}$	(45)
Muconaldehyde	Mouse	1,09	3,04	0,36	0,70	$1,39X10^{4}$	This thesis
	Mouse	Not given	7,1 +/- 6	Not given	1,56	$1,31X10^{4}$	(45)
Acrolein	Mouse	0,736	280	$2,63X10^{-3}$	0,47	$1,02X10^{2}$	This thesis
Crotonaldehyde	Mouse	0,253	209	$1,21X10^{-3}$	0,16	$4,69X10^{1}$	This thesis

The data obtained in this thesis are close with the data published in the 2002 (45) especially for the value of the Kcat/k_m that is an important indicator of the enzyme efficiency, and is also able to give an indication as to which substrate is preferred by an enzyme. The mouse AKR7A5 shows a higher preference for the substrate tested than the rat characterized previously (39). This data became particularly more evident for the physiological substrate succinic semialdehyde where the mouse enzyme showed a preference of a hundred times for this substrate compared to the rat. The reactive aldehydes made during lipid peroxidation can also be part of the substrates metabolized by this enzyme. Methylglyoxal and muconaldehyde have a good kcat/K_m ratio. AKR7A5 may play a secondary role in detoxification because for both substrates the K_m calculated is very high, in the range of mM. Other than comparing the data the mouse AKR7A5 was also characterized with two new substrates, acrolein and crotonaldehyde.

These two substrates are very poor for this enzyme as shown by the kcat/k_m value. The other two important parameters are the Vmax and the K_m, and together these values reveal how poor these substrates are. The Vmax values are very low compared to the succinic semialdehyde, around four times for acrolein and ten times for crotonaldehyde with a very high K_m for both indicating low affinity. This enzyme is very difficult to saturate with these two substrates.

A similar comparison was also made for the human AKR7A2 to better understand its role in human physiology. The data obtained during this thesis were compared with the data present in the literature and summarized in Table 10.

Table 10: comparison of the main value obtained from the mouse AKR7A2 during this thesis works and the data present in the literature.

Substrate	Source	Vmax µmoles/mg enzyme/min	K_m mM	Vmax/k_m Min^{1}*mg*ml	Kcat s^{-1}	Kcat/K_m Min^{-1}/M	Reference
Succinic semi aldehyde	Human	1,34	0,021	62	0,88	$2,49X10^6$	This thesis
	Human	Not given	0,02	Not given	1, 88	$5,7X10^6$	(140)
	Human	0,512	0,0154	33,2	1,53	$6X10^6$	(40)
pNBA	Human	0,99	0,65	1,52	0,65	$6,03X10^4$	This thesis
	Human	Not given	6,1	Not given	1,56	$1,5X10^4$	(40)
	Human	Not given	2,74	Not given	0,78	$2X10^4$	(140)
Methylglyoxal	Human	0,93	1,94	0,47	0,616	$1,9X10^4$	This thesis
	Human	0,096	Not given	Not given	0,063		(40)
Muconaldehyde	Human	0,379	0,966	0,39	0,25	$1,56X10^4$	This thesis
	Human	0,168	Not given	Not given	0,11	Not given	(40)
Crotonaldehyde	Human	0,237	55.9	$3,9X10^{-3}$	0,156	$1,57X10^2$	This thesis
	Human	0,018	Not given	Not given	0,011	Not given	(40)
2-OH-1,4-NQ	Human	0,149	0,44	0,33	0,098	$1,32X10^4$	This thesis
	Human	0,061	Not given	Not given	0,04	Not given	(40)
5-CH_3-1,4-NQ	Human	0,062	$6,56X10^3$	9,45	0,04	$3,75X10^5$	This thesis
	Human	0,05	Not given	Not given	0,033	Not given	(40)
Menadione	Human	0,08	1,48	0,054	0,053	$2,15X10^3$	This thesis
	Human	0,018	Not	Not given	0.019	Not	(40)

			given			given	
Acrolein	Human	0,072	23,86	$3{,}5X10^{-3}$	0,048	$1{,}21X10^{2}$	This thesis
	Human	0,013	Not given	Not given	0,0085	Not given	(40)

The data presented in this work has added significantly to the previous characterization of the enzyme AKR7A2. In particularly a good affinity for the substrate succinic seimialdehyde is confirmed, showing with a very low K_m and high turnover value indicating a great efficiency with this substrate. Its does not appear to be particularly involved in the detoxification of other aldehydes generated during the lipid peroxidation. The data obtained were also compared with other data present in the science literature wherever possible. The data obtained in this experiment especially for the SSA are very close to the data presented previously with the K_ms reported being almost identical as is the efficiency calculated as kcat/K_m. A difference is for the ratio Vmax/k_m, as the value obtained in this experiment is around twice the value of earlier data (40) and (140) as well the kcat s^{-1}. When pNBA was used as a substrate, the difference in K_m was more evident, and the K_m calculated in this thesis is four to ten times less than others. However, the turnover values are very close to each other in particularly when compared to the data obtained from (140).

When methylglyoxal is used as substrate the enzyme expressed in this study is saturated more readily and shows a higher kcat, around ten times more compared to (40). The same situation is seen for crotonaldehyde, 2-OH-1,4-NQ and menadione, but the data for the 5-CH3-1,4-NQ are very close to each other. The data obtained for acrolein though are quite different to each other, especially for the turnover ratio.

In conclusion it is possible to affirm the role of the AKR7A2 in SSA metabolism especially because of its low K_m value and the data are in agreement with other authors. The data obtained for other substrates and analyzed and compared with other authors also showed similar differences in the K_m, and the turnover ratios indicate the low affinity of this enzyme for these substrates, and normally the K_m value is higher than 1 mM.

TRANSFECTION OF THE GENE ENCODING AKR7A2 INTO HUMAN CELLS 1321N1

Other than the characterization of the purified enzymes with different substrates, their role inside human cells was investigated. 1321N1 cells were used to make transient transfectants that were able to overexpress the human protein AKR7A2. The enzyme was cloned in the mammalian expression vector pCI-Neo (Promega), and the plasmid DNA was transfected into the cells. Different conditions were tested to identify the best ratio of pCI neo vector / lipofectamine that was able to give high expression of the enzyme studied. Different ratios of DNA and lipofectamine were evaluated in their capacity to generate an efficient expression of the exogenous protein. The ratios tried were 0.33, 0.25, 0.2 and 0.16 µg DNA / µl of lipofetamine. The efficiency of the transfection was also evaluated quantitatively by making extracts of the cells and analyzing with Western Blotting.

Using antibodies against AKR7A2 on the Western Blot, it was possible evaluate the efficiency of the transfection. 70 µg of protein was loaded for every sample, in order to detect the AKR protein as it is only a small fraction of the whole protein extraction. In the Figure 33 the Western Blotting confirmed that the cells had been transfected and were expressing AKR7A2. The AKR7A2 band protein is weak and it was confirmed by comparison to markers and a previous Western Blot using the AKR7A2 purified from bacteria as a marker. The nonspecific band on the blot is due to the use of polyclonal antibodies that are able to react with other proteins present in the whole cell extract.

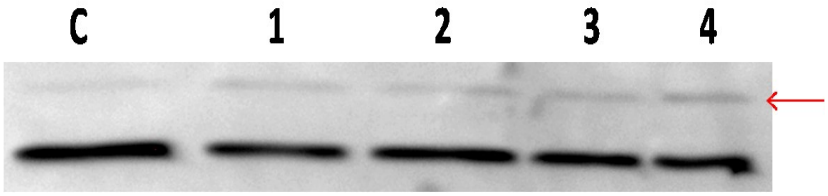


Figure 33: different ratio DNA/lipofectamine for generate transfected cells line 1321N1. C; control, 1; ratio DNA/lipofectamine 0.33, 2; ratio DNA/lipofectamine 0.25, 3; ratio DNA/lipofectamine 0.2, 4; DNA/lipofectamine 0.16. The red arrow indicates the AKR7A2 used for the transfection.

The bands present in this Western Blotting are weak. This is because the target protein is present in a relatively low amount in the whole extract. It was not possible increase the exposure time to give stronger bands, because the nonspecific band would saturate the image and make the protein target totally obscured. The target protein is present in all the samples tested including the control, this is not an expected result because the AKR7A2 is a physiological protein present in most cells. The target band present in the control is weaker compared to the other samples, especially compared to Lane four. This data suggests that the transfection protocol worked in the cell line 1321N1.

Another experiment was carried out to evaluate the efficiency of the transfection. The second experiment was made by transfecting the cells with another gene, the *Renilla luciferase.* This gene is able to produce a protein that can oxidize the coelenterate-luciferin (coelenterazine) and produce light. The presence of the light in the well reaction confirmed the good method used for the transfection. Figure 34 shows the result of this experiment with the cells 1321N1. The amount of *Renilla luciferase DNA* used for transfection is the same amount that was used for the experiment when the AKR7A2 DNA was used to transfect the cells.

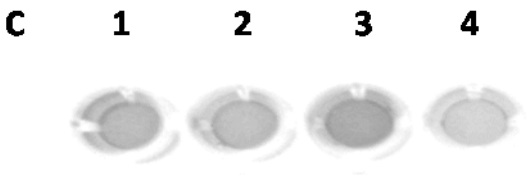


Figure 34: different ratios of *Renilla luciferase* /lipofectamine used to transfect 1321N1. C; control, 1; ratio *Renilla luciferase* /lipofectamine 0.33, 2; ratio *Renilla luciferase* /lipofectamine 0.25, 3; ratio *Renilla luciferase* /lipofectamine 0.2, 4; *Renilla luciferase* /lipofectamine 0.16.

This luciferase assay is more specific than the Western Blotting using the AKR7A2 because the *Renilla luciferase* is a exogenous gene and is not normally present in 1321N1 cells. Figure 34 shows very clearly that the control sample does not show any light in its well. All the other wells present gave a different intensity of light particularly in the third and fourth well. The result of this experiment made it clear that the method used for the cells transfection was efficient and confirmed that it is likely that the AKR7A2 gene was also transfected efficiently inside this cell line. After the result of the two previous experiments a large number of cells were transfected and used to evaluate the role of the AKR7A2 in the protection against toxic aldehydes, namely methylglyoxal, muconaldehyde and crotonaldehyde. The DNA / lipofectamine used were 0.2. The cells were kept in contact with the toxic aldehyde for 20 hours. The method used to evaluate the role of this enzyme in preventing cell toxicity is the MTT assay. The protective role of this enzyme is evaluated by the viability of the transfected cells that over-express AKR7A2 compared to control cells in response to exposure of different toxic aldehdyes. The first aldehyde tested is methylglyoxal. The enzyme kinetic study gave an apparent K_m 1,948mM for methylglyoxal. The concentration of methylglyoxal used for this experiment start from 55µM to 2,75mM which covers the concentration at which the AKR7A2 would be working at half the maximum rate.

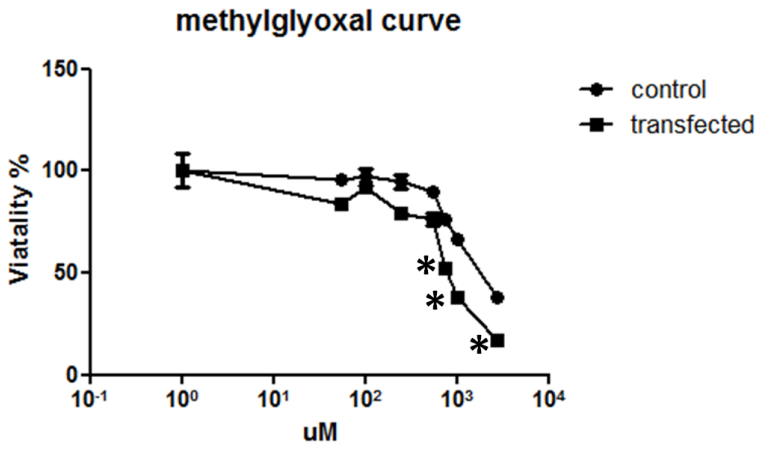


Figure 35: comparison of viability between the cell line 1321N1 (control) and cells 1321N1 transfected with the gene *AKR7A2*. The methylglyoxal used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. N=4.

Treatment of cells with 550 µM methylglyoxal significantly reduced the cells viability. The situation is different for the cells transfected with AKR7A2, and the lowest concentration able to reduce the cells viability is 55µM. All the concentration tested are able to reduce the cells viability with exclusion of 100 µM. The standard error associated with the calculated viability for the concentration at 100µM is very low in both cells line and the vehicle used to treat the cells is the same for both the cells line. This indicates that AKR7A2 is increasing the sensitivity of the cells to methylglyoxal.

The data is not easy to explain. It looks that the enzyme AKR7A2 is involved in the methylglyoxal metabolism, because the difference between the control sample and the transfected sample starts to be significant after 750µM which is very close to the K_m calculated for this substrate. More difficult is to understand why there is a significant reduction of viability in the transfect cells compared to the control. The explanation could be in the molecules generated from reduction of the methylglyoxal to its relative alcohol the 1,2 propanediol which may be more toxic to these cells than methylglyoxal. Another possible explanation could be that other metabolic pathways contribute to detoxification of methylglyoxal, creating an abundance of $NAD(P)^+$ which leads to increase in the exposure time of high levels of methylglyoxal inside the cell. These are only hypothesis because the capacity of this enzyme to convert the 1,2 propanediol to methylglyoxal has not been investigated.

A similar analysis was made to evaluate the protective effect against muconaldehyde, a very reactive aldehyde (Figure 36). It dangerous propriety are evident in the low amount that require for reduce cell viability compared to the other aldehydes tested in all this dissertation.

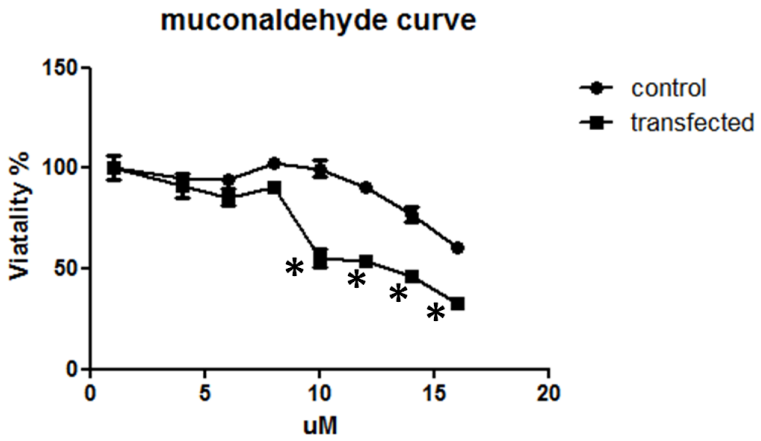


Figure 36: comparison of vitality between the cell line 1321N1 (control) and cells 1321N1 transfected with the gene *AKR7A2*. The muconaldehyde used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. N=3.

Muconaldehyde is able to induce a significant (50%) reduction in viability in the transfected cells 1321N1 at concentrations of 10 µM, which is much lower than for the control cells where the concentration required is 14 µM for have a reduction of vitality. The two curves are very close to each other up to the concentration of 8 µM. After this concentration the transfected cells reduce their viability drastically compared to the control. It seems that 8 to 10 µM is the threshold value indicating that the transfected cells have a major tendency to succumb to muconaldehyde exposure.

It is not clear how the over expression of this enzyme leads to the effects observed, in particularly because the apparent K_m (966µM) calculated for this substrate is very different to the value used for generating the curve. However, similar to the situation seen with methylglyoxal, it is possible that over expression of AKR7A2 leads to an imbalance of cofactor levels which could reduce cell viability in the presence of aldehyde.

The last substrate tested was crotonaldehyde. The range of crotonaldehyde tested is in the range between 0.59 µM to 118 µM, so this aldehyde is looks less toxic compared to muconaldehyde which an IC_{50} between 100 and 118 µM. In the Figure 37 it is possible see the curve generated when the cells are expose to this toxic aldehyde.

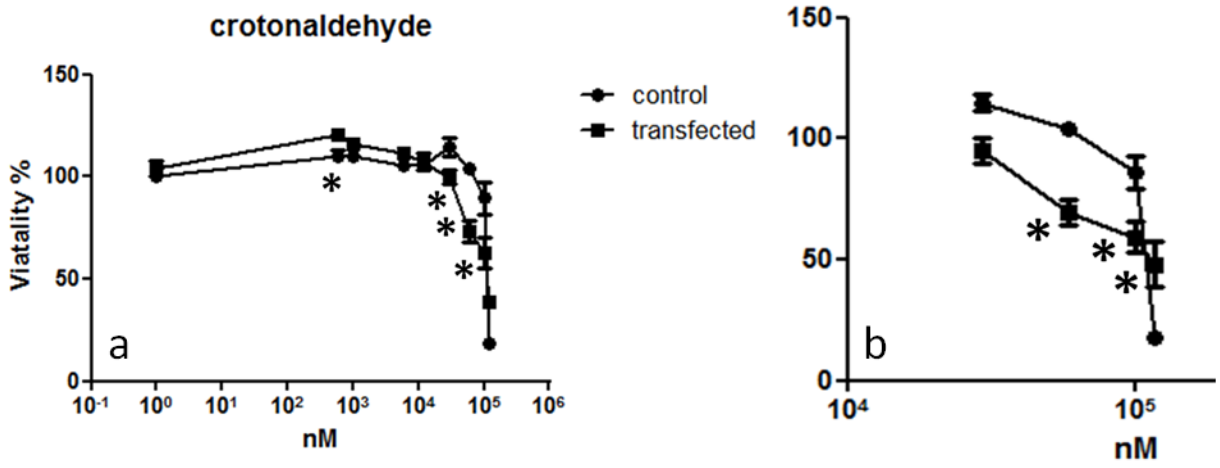


Figure 37: comparison of viability between the cell line 1321N1 (control) and cells 1321N1 transfected with the gene *AKR7A2*. The crotonaldehyde used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. A; the full curves generated, B; the focusing on the high concentration of crotonaldehyde. N=4.

The analysis of the data shows that only the higher concentrations of crotonaldehyde (118 µM) are able to reduce the cells viability in the control sample in a significant manner. The other concentrations show no real different compared to untreated cells. The transfected cells show a low resistance to crotonaldehyde compared to the control cells. The first concentration able to reduce significantly the cells viability is 59µM. Comparing the two curves shows that the transfected cells are more sensitive at lower concentrations of crotonaldehyde but this difference disappears at concentrations of 1 µM. However at the higher concentrations tested the transfected cells show a higher vitality compared to the control. This is the only situation found in which the transfected cells have more resistance then the control.

The apparent K_m calculated for this substrate is 55.9mM. With this data it is possible hypothesize that the aldo-keto reductase AKR7A2 could play role in protecting cells against crotonaldehyde at higher concentrations. AKR7A2 is not able to offer protection to 1321N1 cells against muconaldehyde but is able to protect the cells against crotonaldehyde. This enzyme shows a higher K_m for crotonaldehyde than the K_m calculated for muconaldehyde. Also the turnover rate calculated for the muconaldehyde is hundred times higher that the one calculated for the crotonaldehyde. Hence it appears that a low level of activity towards a substrate may allow the enzyme to function more usefully in the cell that a high level of activity which appears to to lead to increased toxicity.

This experimental data show that in cells, AKR7A2 plays a more significant role in the detoxification of crotonaldehyde than muconaldehyde which is opposite the data expected from the enzyme characterization.

TRANSFECTION OF THE GENE ENCODING AKR7A2 INTO HUMAN CELLS HEPG2

The cell line HepG2 was also transfected with the human enzyme AKR7A2. The HepG2 cell line being derived from liver should be richer in metabolizing enzymes compared to 1321N1 cell line. For the transfection of the cell line HepG2 only one ratio of DNA/ lipofectamine was tried because the method has already been optimized for this cell line by Invitrogen (141). The ratio DNA / lipofectamine used was 0.25. The efficiency of transfection method was evaluated as for the transfection of the cell line 1321N1, and the level of expression of AKR7A2 evaluated using Western blotting.

The toxic aldehydes methylglyoxal, muconaldehyde and crotonaldehyde were used to compare the different responses of the two cells line. The first toxic aldehydes tested was methylglyoxal and the viability curve is show in Figure 38 below.

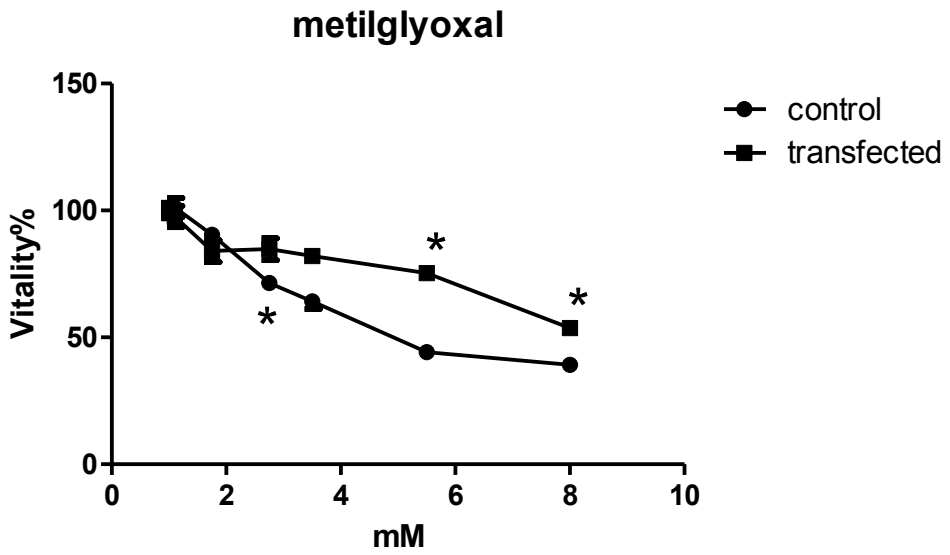


Figure 38: comparison of viability between the cell line HepG2 (control) and cells HepG2 transfected with the gene *AKR7A2*. The methylglyoxal used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. N=4.

In contrast to the viability curve made with the same aldehyde in the cells line 1321N1 show in Figure 35, the HepG2 cells have a greater resistance to MG. In addition, the transfected HepG2 cells appear to show higher viability than the control cells after exposure to MG. The first concentration able to significantly affect the control cells line is 2.75 mM. The HepG2 transfected cells show their capacity to protect the cells against methylglyoxal at concentrations of 5.5mM.

Control cells show a viability of 45%; transfected cells around the 75%, indicating that the AKR7A2 has a major role in the metabolism of this aldehyde when it is present at high concentrations. There is no evidence that the enzyme offers any protection at concentrations below 5.5 mM.

In the 1321N1 transfected cells, a reduction in viability in the transfected cells was observed. As discussed this may be due to the inability of the 1321N1 cells to deal with the product of the reaction or redox/cofactor imbalance. This is not the case with HepG2 cells, which seem capable of handling the product of the reaction or the supply of cofactor.

The second aldehyde tested was muconaldehyde. This toxic aldehyde was shown to be more dangerous for the cells, able to reduce their viability in concentrations in μM range. In Figure 39 it is possible see the response of the HepG2 cells.

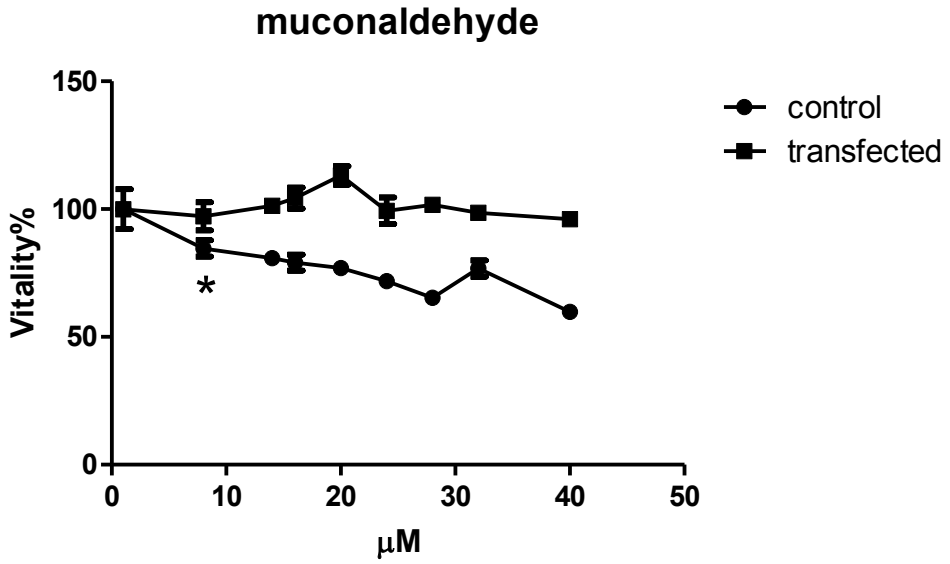


Figure 39: comparison of viability between the cell line HepG2 (control) and cells HepG2 transfected with the gene AKR7A2. The muconaldehyde used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. N=4.

8μM of muconaldehyde are able to generate a significant reduction in viability in the control cells compared to the untreated cells. The reduction of viability is around the 15%. The transfected cells in comparison do not seem to be affected by muconaldehyde treatment at any of the concentrations tested. A comparison between the two curves generated shows that above the concentration of 8μM exists a real significant difference between the two curves, show that the AKR7A2 is important in protect the cells against this aldehyde. This indicates that AKR7A2 enzyme can play a key role in the metabolism of muconaldehyde or one of its metabolites and is able to protect the cells against this compound.

The role of the AKR7A2 was also tested in protect the cells line HepG2 against the toxic aldehyde crotonaldehyde. Figure 40 shows the response of the control and transfected cell lines.

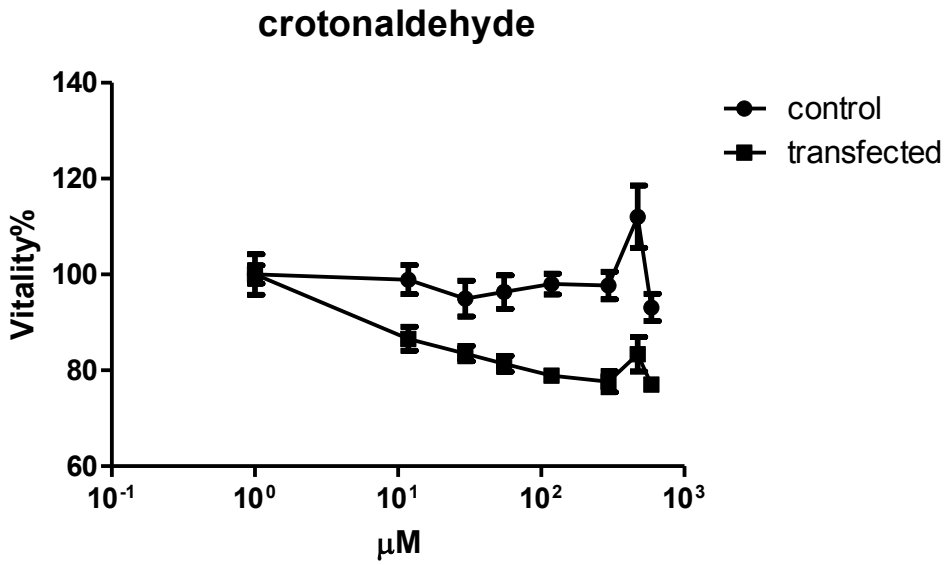


Figure 40: comparison of viability between the cell line HepG2 (control) and cells HepG2 transfected with the gene *AKR7A2*. The crotonaldehyde used for generate the cells reduction was kept in contact with cells for twenty hours. * the data in the transfected cells line significantly different to the control cells line. A; the full curves generated, B; the focusing on the high concentration of crotonaldehyde. N=4.

In these curves the control HepG2 cells show no decrease in viability when exposed to crontoaldehyde. This is in contrast to the 1321N1 cells where high concentrations of crotonaldehyde showed a decrease in viability. Surprisingly the transfected HepG2 cells were much less resistant to crotonaldehyde that the control cells at all the concentrations tested. This indicates that expression of AKR7A2 increases the sensitivity of HepG2 cells to crotonaldehyde suggesting that AKR7A2 is metabolizing crotonaldehyde to a more toxic compound or depleting cofactor reserves. It is clear that the AKR7A2 is not involved in the protection of the cells line HepG2 against the crotonaldehyde.

These results are in contrast with those obtained for 1321N1 cells, where the expression of AKR7A2 was able to offer some protection to the cells at the higher concentrations of crotonaldehyde.

CONCLUSION

The evaluation of the favorite substrates for the enzymes AKR7A2 and AKR7A5 was made by calculating the Vmax of these two enzymes with different substrates under saturating conditions and using different cofactors. The enzymes studied show a strong preference for the cofactor NADPH, but they are also able to catalyze same reaction with NADH but with a real slow Vmax compared to the same reaction made with NADPH.

The characterization of the two enzymes was made using different substrates, especially aldehydes that are known to be dangerous for the cells, and that are generated in different ways including normal eating or smoking. Different aldehydes were tested including the physiological substrate succinic semialdehyde, acrolein present in cigarette smoking and acetaldehyde, the first metabolite of alcohol metabolism.

The first enzyme tested was the human AKR7A2. The experiments carried out *in vitro* showed that succinic semialdehyde is the best substrate compared to all the ather substrates tried. This data is confirmed by the high Kcat/k_m 2,49X10^6 and the relative low value of the Km 21μM. All the other reactive aldehydes tested show a higher k_m value, but more importantly show a slower turnover number. The data obtained were compared with other data present in literature. With some substrates the data obtained are very close to other published data especially for substrates that better fit this enzyme (SSA and pNBA). The other substrates show value of kcat/K_m very close to the value obtained in this thesis.

A similar set of experiments were carried out for the mouse AKR7A5. For this enzyme, the NADPH is the favorite cofactor in accordance with previous work (45). For AKK7A5 the SSA is also shown be the best substrate and this enzyme was discovered as succinic semialdehyde reductase, the ratio kcat/k_m (6,88X10^6) is higher than the human enzyme. The data obtained were compared with the data found in the literature which showed that the data are very close to each other. In general both enzymes are not particularly good at reducing toxic aldehydes tested. In particular, acetaldehyde does not appear to be a good substrate for either of the enzymes tested.

The role of AKR7A2 was also investigated by transiently transfecting the human cells line 1321N1. The transfected cells did not show an increase in resistance compared to a control cells when treated with different aldehydes, in almost all cases. The transfected cells did show a protection against crotonaldehyde when the chemical is used in high concentrations 118μM. In fact the transfected cells show a reduction in viability compared to control cells.

This may be because the enzyme leads to the production of toxic metabolites and/or influences the NADPH/NADP ratio that causes a loss of viability.

The cells line HepG2 was also transfected with the enzyme AKR7A2 to evaluate the role of this enzyme against the same compounds tested to the cells line 1321N1. In general HepG2 is more resistant to the toxic aldehydes compared 1321N. The concentration of muconaldehyde used was increased in an attempt to generate a significant reduction of viability. In cells that over express AKR7A2 it is evident that the enzyme provides significant protection against methylglyoxal and muconaldehyde indicating that this enzyme can play a significant role in the metabolism of these two compounds directly or indirectly. However the situation is different with crotonaldehyde. With this aldehyde, AKR7A2 expression did not offer any protection, compared to the control cells and it appears that the enzyme increases the toxicity of crotonaldehyde.

In the future it will be important to investigate the role of these enzymes using a wider range of toxic aldehydes. The data obtained with the transient cells need to be confirmed with data generated using stable cells line that overexpress AKR7A2. The full metabolism of the aldehydes needs to be evaluated using labeled carbon atoms in order to evaluate which compounds are generated inside the cells and obtain more data about the metabolism of these toxic aldehydes.

V MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Sigma-Aldrich UK unless otherwise stated.

PLASMIDS

Plasmids were obtained from lab stocks as follows (Table 11):

Table 11: list of the plasmids used in this thesis

Plasmid	Purpose	Description	Source/Reference
pAH	Bacterial expression of AKR7A5	Based on pET15b. AKR7A5 cloned into the Nde1-BamHI site	Hinshelwood et al., 2002
pLI16	Bacterial expression of AKR7A2	Based on pET15b. AKR7A2 cloned into the Nde1-BamHI site	Ireland et al., 1998
pCI-neo Vector	Mammalian expression of AKR7A5	Based on pCI-Neo	

CELL LINES

1321N1 human astrocytoma cells were obtained from Dr Eve Lutz, University of Strathclyde (142). HepG2 human hepatoma cells were obtained from American Type Culture Collection (143).

CELL CULTURE GROWTH

Cells were grown in flasks and dishes pre-treated for cells culture use. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) bought from Sigma-Aldrich. To the media was added:

1321N1: 10% bovine calf serum, 1% penicillin/streptomycin

HepG2: 10% bovine calf serum, 1% penicillin/streptomycin1% non essential amino acid 1% sodiumpyruvate.

The cells were kept in CO_2 5% incubator at 37°C saturated of humidity.

CELL COUNTING

Cells were washed twice with cold PBS (Sigma) then treated for five minutes with a solution of trypsin to lift the cells from. Once released, fresh medium was added to the cells to block the action of the trypsin. The cells were harvested in a sterilized tube at 1000rpm for 5 minutes and re-suspended in a reasonable amount of media (normally 1ml for a 75cm ^{2} flask). A mix of 10µl medium and 10µl cell suspension was added to the haematocytometer. The cells deposited on the glass surface were counted using a microscope. Only the cells in the four squares present in the haemocytometer were counted. Every square present in the haemocytometer has a surface of 1mm^2 and deepness of 0.1mm. The average value of the cells counted was determined according to the formula:

$[c] = \frac{cells\ counted}{4} *$ dilution factor * 10^4

MTT ASSAY

The MTT assay is a cell viability assay that depends on the ability of metabolically active cells to reduce MTT (yellow) to the purple formazan derivate. It can be used to measure loss of viability due to treatment with toxic agent, or growth inhibition, as well as proliferative effects and increases in cell number. The amount of purple formed will depend on the number of viable cells present and the time of incubation with the dye. The assay is linear only within a certain range and it is important that various preliminary experiments were included to demonstrate the assays validity.

Protocol:

Cells were seeded in triplicate in 96-well plates at a density that gives around 60% confluence within 24 hours. This needed to be experimentally determined for each cell line used. After 24 hours, cells were treated with different concentration of toxic agent or proliferating agent for a specified amount of time. A treatment with a known effect on the cells was used a control. For cell death, Triton X-100 was used. After the required period of time the media was changed.

20µl of a 4mg/ml MTT solution (made up in PBS and filter sterilized) was added to the media in each well and incubated for up to four hours under normal condition. Three control wells that have no cells in it were included (just MTT in media).

The formation of the purple dye was monitored every 30 minutes. Once sufficient purple had formed, all the media was carefully removed without disturbing the attached cells. To stop the assay 150µl of isopropanol or DMSO was added to each well and pipette up and down to solublize the crystals and left at 37°C for 5 minutes.

The purple colour was read at 540nm using a plate-reader. The background value (no cells) was substracted and the % viability was calculated as the absorbance for treated cells / absorbance for control cells.

PREPARATION OF CELL EXTRACTS:

Media was removed and cells were washed with HBSS. Cells were collected by centrifugation and the pellet frozen at -80 °C. The pellet was re-suspended in prewarmed 100 -150 ul TE buffer (250 mM Tris-Cl pH 7.5) and kept at 37oC for 5 mins. Cells were frozen at -80oC and the freeze-thaw cycle repeated. The cells suspension was centrifuged at 13000 for 5 minutes and the supernatant store at -80^{o}C.

TRANSFECTION OF HUMAN CELLS

Transfection is the process used to introduce exogenous DNA inside the mammalian cells. Different methods were considered but the most effective uses Lipofectamine reagent (Invitrogen). The cells were transfected when the confluence was between the 50-80%. The amount of DNA to be transfected into the cells was diluted in Opti-MEM® I Reduced Serum Medium (invitrogen). The Opti-MEM® I Reduced Serum Medium is a serum-free media.

A range of amount of Lipofetamine reagent was added to the diluted DNA and mixed as shown in Table 12

Table 12: amount of reagents need for generate transient cells using lipofectamine from invitrogen.

Culture vessel	Surface area per well	Volume plating medium	Cells per well	Volume dilution medium	DNA	Lipofectamine™ LTX Reagent
96-well	0.3 cm^2	100µl	8X10^3	20 µl	100ng	0.35 - 0.55 µl
48-well	1 cm^2	200 µl	2 X10^4	40 µl	200 ng	0.7 - 1.1 µl
24- well	2 cm^2	500 µl	5 X10^4	100 µl	500 ng	1.75 - 2.75 µl
12- well	4 cm^2	1ml	8 X10^4	200 µl	1µg	3.5 - 5.5 µl
6- well	10 cm^2	2ml	2 X10^5	500 µl	2.5 µg	8.75 - 13.75 µl

After 25 minutes of incubation time at room temperature the cell growth media was replaced with fresh media and the DNA-lipofectamine complex was added drop-wise. The plate was rocked back and forth during the drop-wise process. The transfected cells were grown from 12 to 48 hours in a CO_2 incubator at 37°C before being tested for transgene expression.

The optimum ratio between lipofectamine and DNA needed to be tested experimentally to identify the best conditions to give efficient transfection and a high level of expression. It was important also to evaluate the viability of the cells after lipofectamine exposure because lipofectamine is toxic for the cells.

DNA MIDI PREP

This method yields about 20-100µg of plasmid DNA, depending on the copy number of the plasmid. The method was is based on that of Birnboim and Dolly (144). *E. coli* NM522 containing the vector pCI-Neo (Invitrogen) or pCI-Neo with the gene of interested inserted was used. The *E.coli* was grow overnight in LB broth 40ml at 37°C in selective conditions ampicillin. Cells were harvested in a benchtop centrifuge at 5000 rpm for 5 minutes. Cells were resuspended in 1 ml TEG and vortexed thoroughly. 1 ml 4mg/ml lysozyme was added made up in TEG. Cells were transferred to a 15ml corex tube and incubated on ice for 20 minutes. Cells were vortexed, and 4ml lysis solution was added the tube inverted several time. The mixture was kept on ice for 5 minutes. 3M NaAcetate pH5, was added, and mixed by inversion then left on ice for 45 minutes. The lysed cells were centrifuged at 9000 rpm for 30 minutes. The supernatant was carefully removed to a 30ml corex tube, and 16ml of cold ethanol was added and the tube incubated for 15 minutes on ice. To collect the DNA, cells were centrifuged at 9000rpm for 10 minutes. The DNA pellet was dissolved in 2ml of Low Salt Buffer. 2ml of phenol/chloroform/isoamylalcohol (50:49:1) was added, mixed and centrifuged for 10 minutes at 3000 rpm in benchtop centrifuge. The aqueous phase was transferred to a 15ml corex tube. To the phenol layer, 2ml Low Salt Buffer mix was added and centrifuged at 3000 rpm for 10 minutes. Both the aqueous phases were combined and 15ml of cold ethanol was added. The solution was incubated at -20°C for 15 minutes, and then centrifuged for 10 minutes at 9000rpm. The pellet was dissolved in 0.4ml TE and transferred to an eppendorf tube. 20µl 4M NaCl was added along with 1 ml cold ethanol and kept at -20°C for 20 mins, then centrifuged at 12000rpm for 5 minutes at 4°C. The pellet was dissolved in 0.2ml TE, 20µl pancreatic Ribonuclease A was added and incubated for 1 hour at 37°C. 20µl 4M NaCl, 0.2ml water and 0.4ml phenol/chloroform/isoamylalcohol (50/49/1) was added, the mixture vortexed and centrifuged for 1 minute. The aqueous phase was removed 0.4 ml chloroform/isoamylalcohol was added and the solution vortexed and centrifuged. The aqueous phase was removed and 0.8 ml cold ethanol was added. The DNA was kept at -20°C for 10 minutes and centrifuged for 5 minutes. The pellet was dissolved in 0.3ml TE.

Solutions needed:

TEG: 25mM Tris-HCl, 10mM EDTA 50mM glucose pH 8

Lysis buffer: 0,2M NaOH, 1% SDS

Low Salt Buffer: 0,1M Na acetate, 1mM EDTA, 0,1% SDS, 40mM Tris.HCl pH 8

TE: 10mM Tris-HCl, 1mM EDTA pH8

Ribonuclease A solution 1mg/ml: 10mg Bovine Pancreatic Ribonuclease A, made up in 10ml 10mM Tris HCl, 15mM NaCl, pH 7.5 Heat to 100°C for 15 minutes to remove DNAse. Store at -20°C in 0.5ml aliquots.

EXPRESSION OF RECOMBINANT PROTEIN AND PURIFICATION

The expression of AKR7A2 and AKR7A5 was induced for 4 hours at 37 °C by adding 0.5 mM IPTG and cells were lysed by sonication. Purification of this enzyme from bacterial cultures was carried out using HiTrap affinity column (1 ml) (Amersham pharmacia biotech) with Pharmacia FPLC (Fast Protein Liquid Chromatography) system. Enzyme activities of purified protein were measured and the most active fractions of protein were combined and frozen at -80 °C until required. Purified protein was also separated by SDS–PAGE to confirm purity.

SDS-POLY ACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

This technique is able to separate proteins according to their molecular weight. The proteins interact with the Sodium dodecyl sulphate present in the loading buffer and in the gel. The proteins have a negative charge proportionate to the length of the protein chain. The proteins with their negative charge are exposed to a electric field, that causes them move inside the gel. The gel has two components; the stacking gel which compacts all the proteins in the sample in a unique band. The second part - the resolving gel - separates the protein.

The solution and the concentration used are shown briefly in this thesis because the SDS-page is a very common technique (145).

Bottom gel (resolving)	**10% acrylamide**
30% acrylamide/bisacrylamide solution	6.6ml
4X resolving buffer	5ml
Distilled water	8.2ml
Ammonium persulfate 100mg/ml	100µl
TEMED	10µl

Top gel (stacking)	**5% acrylamide**
30% acrylamide/bisacrylamide solution	1.64ml
4X stacking buffer	2.5ml
Distilled water	5.86ml
Ammonium persulfate 100mg/ml	60µl
TEMED	10µl

Resolving buffer: Tris 1.5M, SDS 0,014M pH 8.8

Stacking buffer: Tris 0.5M, SDS 14mM, pH 6.8

Runnig buffer: Tris 0.25M, glycine 1.91, SDS 35mM,

Loading buffer: Tris 66mM, SDS 0.14M, 18% glicerolo, 10% β-mercaptoethanol, 0.001 bromophemo blue.

WESTERN BLOTTING

Western blots in this thesis works were made on Polyvinyl fluoride (PVF) membrane. The antibodies used were bought from Santa Cruz (Caspase-3 H227). Polyclonal antibodies against AKR7 proteins were made in the Ellis lab in Strathclyde. Goat anti-rabbit secondary antibodies were from Biorad.

Proteins were separated by SDS-Page, the stacking gel discarded and soaked briefly in Transfer Buffer along with the Whatman 3MM paper, PVF membrane and sponges

A sandwich was set up as shown in the scheme, carefully removing all air bubbles between layers.

----------	Plastic holder (+)
>>>>>>>>>>>	Sponge
==========	2 layers of whatman
**********	PVF
.....	Gel
==========	2 layers of whatman
>>>>>>>>>>>	Sponge
----------	Plastic holder (-)

The sandwich was placed in the Transfer Tank with the black face of the plastic holder next to the black terminal, and the clear face next to the red terminal. The transfer was run for 1 hour at 300mA. The sandwich was disassembled and the membrane placed into blocking solution for 1-16 hours.

ANTIBODY DETECTION

The primary antibody was mixed with the blocking solution at a 1:3000 ratio (10µl per 30ml blocking solution). The blot was incubated with antibody for 1-16 hours with gentle mixing. The blot was washed 4 times, each time for 25 minutes with 100ml of TBST. The secondary antibody was added at the same ratio mixed in blocking solution and incubated for 4 hours with gentle agitation. The blot was washed 3 times in TBST for 25 minutes and once in TBS for 25 minutes. The blot was developed by Enhanced Chemiluminescence (ECL) and visualized using a Fuji Imager.

ENHANCED CHEMILUMINESCENCE

Kit ECL^{tm} Prime Western Blotting Detection Reagent (GE healthcare).

The secondary anty bofy are from Bio RAD:
Goat Anti Rabbit IgG (H+L)-HRP Conjugate.

Solutions required:

Transfer Buffer Solution: 0,19M glycine, 25mM Tris, 20% methanol.

TBS: 0.1 M Tris-HCl, 0,137M NaCl, pH 7.6

TBST: TBS solution 0,1 Tween 20.

Blocking solution: 5% skimmed milk in TBST solution.

ISOLATION OF FRAGMENTED DNA

The method used for the isolation of the fragmented DNA was derived from (137) and some modifications were added to the protein extraction to make the method more rapid. Briefly, the cells were collected from a in a 25cm^2 flask and were washed twice in ice cold PBS before collecting by centrifugation in an Eppendfor tube. Cells were resuspended in 50 ul lysis buffer and centrifuged for 5 minutes at 1600 x g. The supernatant was collected and the above step repeated. The two supernatants were combined. The supernatant was assumed to contain the fragmented (apoptotic) DNA. The pellet was resuspended in 100 ul lysis buffer. The pellet contains the intact (genomic) DNA. Both samples were brought up to 1% SDS and 5µg/µl of RNase A, and were incubated at 56^{o}C for 2 hours. An equal volume of phenol/chloroform was added to the samples and mixed by vortexing. The sample was centrifuged for 5 minutes at 5000 rpm. The supernatant was collected and the phenol:chloroform extraction repeated. The supernatants for each fraction of DNA were combined and the DNA precipitated using a 1/10 volume of NaAcetate 3M pH 5.2 and 3 volumes of ETOH, incubating overnight at -80°C. The DNA was collected by centrifugation for 30 minutes at 4°C, and the supernatant discarded. The pellet was suspended in appropriate amount of TE buffer.

Lysis buffer: 1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5

TE buffer: 10 mM Tris-Cl, pH 7.5. 1 mM EDTA.

AGAROSE ELECTROPHORESIS

All gels were made in TBE buffer using high grade agarose (1%). The electrophoresis solution was TBE.

TBE: 0.089M Tris base, 0.089M boric acid, 0.002M EDTA, disodium sale, dihydrate, final pH 8.3.

ALDEHYDE REDUCTASE ASSAYS

All the reactions were carried out in sodium phosphate buffer 0.1M pH 6.6 in 1ml reaction volume. The concentration of the cofactors used was 50µM in the cell reaction. Different substrates at different concentrations were used to determinate the apparent Km and Vmax values. The reaction was followed by monitoring the change in the cofactor concentration by measuring absorbance at 340nm. The reaction time was followed from 1 to 5 minutes.

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Chemico-Biological Interactions 195 (2012) 25–34

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Human aldo–keto reductase AKR7A2 protects against the cytotoxicity and mutagenicity of reactive aldehydes and lowers intracellular reactive oxygen species in hamster V79-4 cells

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ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form 26 September 2011
Accepted 27 September 2011
Available online 5 October 2011

Keywords:
Aldo–keto reductase
Oxidative stress
Aldehydes
Menadione

ABSTRACT

Aldo–keto reductase (AKR) enzymes are critical for the detoxication of endogenous and exogenous aldehydes. Previous studies have shown that the AKR7A2 enzyme is catalytically active toward aldehydes arising from lipid peroxidation, suggesting a potential role against the consequences of oxidative stress, and representing an important detoxication route in mammalian cells. The aim of this study was to determine the ability of AKR7A2 to protect cells against aldehyde cytotoxicity and genotoxicity and elucidate its potential role in providing resistance to oxidative stress. A transgenic mammalian cell model was developed in which AKR7A2 was overexpressed in V79-4 cells and used to evaluate the ability of AKR7A2 to provide resistance against toxic aldehydes. Results show that AKR7A2 provides increased resistance to the cytotoxicity of 4-hydroxynonenal (HNE) and modest resistance to the cytotoxicity of *trans*, *trans*-muconaldehyde (MUC) and methyglyoxal, but provided no protection against crotonaldehyde and acrolein. Cells expressing AKR7A2 were also found to be less susceptible to DNA damage, showing a decrease in mutation rate cause by 4-HNE compared to control cells. Furthermore, the role of the AKR7A2 enzyme on the cellular capability to cope with oxidative stress was assessed. V79 cells expressing AKR7A2 were more resistant to the redox-cycler menadione and were able to lower menadione-induced ROS levels in both a time and dose dependent manner. In addition, AKR7A2 was able to maintain intracellular GSH levels in the presence of menadione. Together these findings indicate that AKR7A2 is involved in cellular detoxication pathways and may play a defensive role against oxidative stress *in vivo*.

1. Introduction

Aldehydes and ketones are found in a range of compounds including drugs, food and environmental pollutants, and are also produced endogenously. Some aldehyde and ketones such as acrolein and 4-hydroxy *trans* 2-nonenal (4-HNE) are extremely reactive, and can cause damages to protein, DNA and lipids, leading to pathophysiological consequences [1]. For example, 4-HNE has been reported to cause cytotoxicity in several cell lines, and is the most toxic aldehyde produced during the peroxidation of ω-6-polyunsaturated fatty acids [2]. In addition, several reactive aldehydes have been confirmed as mutagenic through their interactions with DNA [3], and have been reported to induce mutations in mammalian cell lines in a dose-dependent manner [4]. These damaging events may play a significant role in the progression of diseases where elevation of oxidants is known to be high [5].

Other reactive aldehydes such as the benzene metabolite *trans*, *trans*-muconaldehyde (MUC) can cause DNA strand breaks leading to induce bone marrow depression and inhibition of erythropoiesis in mice [6]. Another reactive aldehyde, methyl glyoxal, is an active intermediate in the Maillard reaction, cross-linking with proteins. It is elevated in uncontrolled diabetes, and is associated with diabetic complications [7].

In order to counter the potentially lethal effects of toxic aldehydes and ketones, several metabolizing enzyme systems exist that can render aldehydes either more readily excreted or less biologically active. Aldo–keto reductases (AKR) are a superfamily of NADP(H)-dependent enzymes that reduce aldehydes and ketones to alcohols [8]. The AKR superfamily includes enzymes from bacteria, yeast, plants and mammals that are capable of reducing

Abbreviations: AKR, aldo–keto reductase; SSA, succinic semialdehyde; CBA, carboxybenzaldehyde; IC_{50}, inhibitory concentration of 50% the cells; HNE, 4-hydroxynonenal; MTT, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; NQ, naphthoquinone; HGPRT, hypoxanthine guanine phosphoribosyl transferase; DCFH/DA, 2′7′-dichlorodihydrofluorescein diacetate; PQ, phenanthrenequinone; ROS, reactive oxygen species; MUC, *trans*, *trans*-muconaldehyde.

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0009-2797/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2011.09.007

carbonyls present in a diverse range of natural and synthetic compounds [8]. Several human AKR enzymes have been identified as capable of reducing reactive aldehydes such as 4-HNE, acrolein and methyl glyoxal. These include aldose reductase (AKR1B1) [9,10]; 20α(3α-hydroxysteroid dehydrogenase (AKR1C1) [11]; aldehyde reductase (AKR1A1) [12]; and the aflatoxin B1 aldehyde reductases (AFAR/AKR7A2 and AKR7A3) [13,14]. Of these, the human AKR7A subfamily members are of interest, because they are related to a rat enzyme (AKR7A1) that is inducible by dietary antioxidants [15]. This inducibility has the potential to lead to valuable therapeutic approaches for diseases in which reactive carbonyls have been implicated [5,15].

We have shown previously that mammalian cells expressing rat AKR7A1 are protected from the mutagenicity of acrolein [16]. Rat AKR7A1 has also been shown to be associated with protection against aflatoxin toxicity *in vitro* [15,17], and more recently, using transgenic rats, AKR7A1 has been shown to protect animals against aflatoxin toxicity but not mutagenicity [18,19]. In the mouse, there appears to be only one AKR7A enzyme, AKR7A5, and we have shown previously that V79 cells transfected with mouse AKR7A5 are relatively more resistant to 4-HNE-induced apoptosis [20,21]. Taken together this information suggests that the AKR7A subfamily of enzymes generally serves a cytoprotective function. However little is known of the function of the human members of the AKR7A subfamily. Two human AKR7A enzymes have been isolated, human AKR7A2 and human AKR7A3 [13,14]. Whether the human AKR7A enzymes are similarly involved in detoxication pathways has not been explored previously.

The human AKR7A2 was identified in brain as a succinic semialdehyde reductase [22], and we have shown previously that it is the main enzyme responsible for the reduction of succinic semialdehyde (SSA) to γ-hydroxybutyrate (GHB) in human neuroblastoma cells [23]. AKR7A2 is known to be present in a range of tissues, including liver, kidney as well as brain [12]. However, it appears to accumulate in specific regions of the brain in Alzheimer's Disease [24].

The aim of this study was to test whether the human enzyme AKR7A2 also plays a role in detoxication, as is the case with rat AKR7A1 and mouse AKR7A5. In order to investigate this, we have stably expressed AKR7A2 in V79-4 Chinese hamster lung cells, and have tested the ability of expressed AKR7A2 to protect against the cytotoxicity and mutagenicity of several toxic aldehydes. Furthermore, the role of the AKR7A2 enzyme in enhancing the cell's ability to cope with elevated ROS is also assessed.

2. Methods

2.1. Chemicals

All chemicals were obtained from Sigma except for 4-hydroxynonenal, purchased from Alexis Caymen Corporation – Europe (Nottingham, UK).

2.2. Cell culture

V79-4 Chinese hamster fibroblasts cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Media (DMEM) with L-glutamate, 1% (v/v) penicillin and streptomycin, and 5% (v/v) fetal bovine serum.

2.3. Generation V79-4 cell lines stably transfected with AKR7A2

The full length human AKR7A2 cDNA was amplified from an AKR7A2 expression construct [13], a gift from Professor John D. Hayes, and subcloned into the pCI-Neo expression plasmid to give plasmid pCI-Neo-AKR7A2. V79-4 cells were seeded in 24 well plates and pCI-Neo-AKR7A2 was transfected into V79-4 cells using Fugene 6 reagent. Several stable clones were selected as geneticin (G418) resistant. AKR7A2 expression levels in each clone were analyzed by Western blotting. One clone demonstrating strong AKR7A2 expression was maintained for further study.

2.4. Preparation of cell extracts from cell lines

For detection of AKR7A2 expression, whole cell extracts from V79 cells were prepared from 75 cm^2 flasks using a "Freeze–thaw" protocol that does not affect enzyme activity [23]. Briefly, after cells were washed three times in ice-old PBS, 3 ml ice-cold TEN (40 mM Tris–Cl, PH 7.5, 1 mM EDTA, PH 8.0, 150 mM NaCl) solution was added to each flask. Cells were harvested and resuspended in 0.2 M phosphate buffer pH 7.5. The cell suspension was frozen in dry ice for 5 min and then transferred to 37 °C for 5 min. The freeze–thaw procedure was repeated twice. The cell lysate was centrifuged at 12,000*g* for 5 min at 4 °C, and the supernatant was ready for further analysis. Protein concentrations were determined using the method of Bradford against bovine serum albumen standards [25].

2.5. Protein gels and Western blots

Portions of cell extract were separated by SDS–PAGE gel (12%) [26] and transferred onto nitrocellulose membrane (Bio-Rad Inc.). Antisera to AKR7A2 was raised in this lab as previously described [20] and used at a dilution of 1:2000 and goat anti-rabbit IgG-Horse radish peroxidase conjugate secondary antibody (1:10000 dilution). Western blots were developed with enhanced chemiluminescence. (ECL; Amersham) and images were analyzed by LAS-3000 luminescent image analyzer (Fuji). Quantification of band intensities was performed using Kodak one-dimensional image analysis software. A standard curve allowing quantitation of AKR7A5 was achieved by using serial dilutions of purified AKR7A2 recombinant enzyme and plotting concentration versus pixel density of each band determined using NIH Image.

2.6. Expression and purification of recombinant human AKR7A2

Bacterial expression vector pLI19 containing an N-terminal polyhistidine tagged AKR7A2 was a gift from Professor John D. Hayes, University of Dundee [13]. Purification of this enzyme from bacterial cultures was carried out using HiTrap affinity column (5 ml) (Amersham pharmacia biotech) with Pharmacia FPLC (Fast Protein Liquid Chromatography) system. Enzyme activities of purified protein were measured and the most active fractions of protein were combined and frozen at −80 °C until required. Purified protein was also separated by SDS–PAGE to confirm purity.

2.7. Aldo–keto reductase (AKR) assays

Aldehyde- and ketone-reducing activity was determined using an enzyme assay described previously [27], and routinely measured with a Beckman DU650 UV single-beam recording spectrophotometer by following the initial rate of oxidation of NADPH at 340 nm ($\varepsilon_{340} = 6270\ M^{-1}\ cm^{-1}$). The assays were performed at 25 °C in reaction mixtures of 1 ml containing 100 mM sodium phosphate buffer, pH 6.8, and 0.05 mM NADPH. The concentration of the substrate in the reaction mixture was between 10 μM and 10 mM (depending on solubility) with a maximum concentration of 2% (v/v) methanol or 4% (v/v) acetonitrile as carrier; neither methanol nor acetonitrile was found to interfere with the assay or enzyme activity under these conditions. Approximately 1 μg of enzyme or 100 μg cell extract was added to the cuvette to initiate

the reaction and the reaction rate was measured against an identical blank with no enzyme added. Activity was measured for 5 min. Any background rate of decrease in NADPH arising in the absence of substrate was subtracted from the rate determined in the presence of substrate. K_m and V_{max} values for purified AKR7A2 were determined by altering the substrate concentration while maintaining NAPDH at 0.05 mM. Fitting to the Michelis–Menten equation was carried out using Graph-Pad Prism software. The k_{cat} was calculated assuming the molecular weight of the AKR7A2 dimer as 79,198 Da, and 2 active sites per molecule of dimer.

2.8. Cytotoxicity assay (MTT assay)

Cell viability assays were performed using a modified method that uses 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [28]. Briefly, cells were seeded in a 24 well plate and incubated until a confluent monolayer formed. On the day of assay, media was aspirated and fresh media (1 ml) containing a range of various concentrations of aldehydes was added. Cells were exposed with chemicals for certain time. Media containing 0.2 mg/ml MTT solution was added to each well and plates were incubated at 37 °C for 4 h. The medium was discarded and the reaction was stopped by the addition of 200 μl acidified DMSO (0.04 M HCl/DMSO). With 2 h incubation at 37 °C, the solubilized formazan solution was transferred to a 96-well plate. The absorbance was read at 540 nm using a 96-well Labsystems iEMS plate-reader and plotted as % of untreated control. The half maximal inhibitory concentration (IC_{50}) values were determined as the concentration which reduced the viability to 50%.

2.9. Mutagenicity (HGPRT) assay

Mutagenicity assays were performed essentially using the modified hypoxanthine guanine phosphoribosyl transferase (HGPRT) method [29]. Briefly, cells were seeded at 5×10^5 in 75 cm^2 flasks and incubated for 24 h before the treatment. Aldehydes were dissolved in DMSO and added to the flasks. After 4 or 8 h exposure, fresh media was added. After a further 72 h incubation, cells were harvested and re-seeded at 10^6 cells/100 mm dish for the detection of 6-thioguanine-resistant colonies (5 dishes/per cell line). For cloning efficiency 200 cells/60 mm dishes were seeded (5 dishes/per cell line or treatment). 5 ml media containing 7 μg/ml (30 μM) 6-thioguanine was added to 100 mm dishes and media only was added to 60 mm dishes for cloning efficiency. The cloning efficiency was obtained by counting the number of colonies after incubation for 7–8 days. The cloning efficiency of both treated and untreated cells was comparable and considered as 100% in the determination of the survivors. The selection of 6-thioguanine resistant mutants required about 13 or 14 days incubation. For colony counting, 1 ml methanol was added and left for 5 min to fix the cells, and 0.4% Giemsa (dissolved in methanol) was used to stain the colonies.

2.10. Measurement of cellular ROS level

Intracellular ROS level was determined by measuring the fluorescence of the oxidized form of the probe 2′7′-dichlorodihydrofluorescein diacetate (DCFH/DA) using a modified method of [30]. Cells were seeded in 100 × 20 mm dishes and incubated in 2 ml serum-free DMEM containing 100 μM DCFH/DA (48.7 g/ml media) at 37 °C for 1 h. Cells were then washed with PBS and pretreated with menadione for required conditions in a serum-free media. Cells treated with DCFH/DA only were used as a control and duplicate dishes were measured for each sample. After treatment, cells were washed with ice-old PBS and 2% TritonX-100/PBS was added to the cells and incubated for 5 min. Lysate cells were sonicated for 5 s twice and centrifuged for 10 min at 3000*g*. DCF fluorescence in the supernatant was measured using a Bio-Tek FL600™ fluorescent plate-reader (Bio-Tek Instruments, Inc.). Ninety-six-well black solid plates were used and sensitivity of the photomultiplier was set up to 75. KC4 System software was used to create the protocol and analyze the data.

2.11. Measurement of intracellular glutathione levels

Intracellular glutathione (GSH) levels were determined using a fluorimetric method described previously [31]. Cells were seeded in 100 × 20 mm dishes and treated with menadione at the specified conditions in 5 ml media. DMSO (0.1%) was used as a solvent control. Following treatment, media was aspirated and cells were washed twice with 1× PBS. 1 ml of 15% trichloroacetic acid was added to cells at room temperature for 15 min. The supernatant was collected and centrifuged at 16,000*g* for 10 min and was kept at −80 °C until GSH measurements were carried out. The precipitated protein remaining in the dish was dissolved in 1 ml 0.5 M NaOH and 10 μl of this solution was used to measure the protein content by standard Bradford reagent. For GSH measurement, 50 μl of GSH standard solution or the acidified supernatant was added to 2.3 ml phosphate–EDTA buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0) and 100 μl of 1 mg/ml *o*-phthalaldehyde. The mixture was mixed well and kept at room temperature in the dark for 15 min and fluorescence was measured in 4.5 ml polymethyl methacrylates cuvette at room temperature at 350 nm excitation and 420 nm emission using a RF-5301PC fluorimeter (Shimadzu), with excitation and emission slit widths both set at 1.5 nm. Experiments were set up in duplicate dishes for each condition ($n = 2$), and two parallel determinations per dish were carried out for each GSH assay. All GSH values were normalized and calculated as nmol GSH/mg total cell protein.

2.12. Caspase-3 activity assay and subunit detection

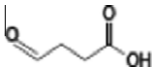
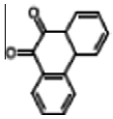
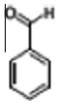
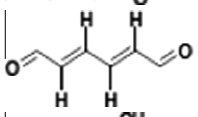
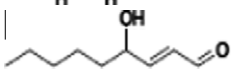
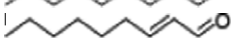
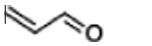
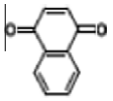
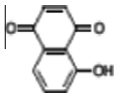
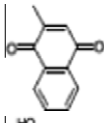
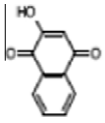
Caspase-3 activity was determined using the CaspACETM assay-kit (Promega) as described previously [20]. Briefly, cells were seeded in 75 cm^2 flasks and treated with various concentrations of menadione for 4 h. Cells were then collected and resuspended in Caspase Lysis Buffer. After centrifugation, the supernatant was used to determine caspase 3 activity according to the manufacturer's instruction. In the assay system, specific cleavage of the substrate (Ac-DEVD-pNA) by caspase-3 releases free *p*-nitroanilide (pNA) producing a yellow color that can be monitored spectrophotometrically at 405 nm. Data was normalized to protein content of each sample.

3. Results and discussion

3.1. Identification of new AKR7A2 substrates

AKR7A2 has been characterized previously with regards to its ability to reduce a range of toxic aldehydes and ketones [12,13]. To gain a clearer view of the function of AKR7A2, we tested additional aldehyde substrates that have not been previously investigated. The results in Table 1 show that purified recombinant AKR7A2 displays modest activity towards the benzene metabolite MUC, and has reductase activity towards benzaldehyde, hexanal and *trans*-2-nonenal. AKR7A2 appears to have no activity towards 1,4-naphthoquinone (NQ), but was able to reduce 1,4-NQ derivatives, including menadione, with a substituted group on either benzene ring (Table 1). The apparent K_m and k_{cat} values were determined for some of these substrates, and the turnover number calculated. The data in Table 2 reveals that of the substrates tested,

Table 1
Enzymatic activity of recombinant human AKR7A2 with aldehydes and dicarbonyls.

Substrate	Structure	Concentration (mM)	Specific activity (nmol min^{-1} mg^{-1})
Succinic semialdehyde		1	512 ± 21
2-Carboxybenzaldehyde		1	726 ± 5
9,10-PQ		0.05	1061 ± 5
Benzaldehyde		1	65 ± 3
Methylgloxal		1	96 ± 11
Hexanal		1	14 ± 2.5
***t,t*-Muconaldehyde**		0.1	168 ± 20
4-Hydroxynonenal		1	18 ± 1.8
***trans*-2-Nonenal**		1	9.5 ± 1.2
Acrolein		1	13 ± 1.9
Crotonaldehyde		1	18 ± 2.2
1,4-NQ		0.1	<5.0
5-OH-1,4-NQ		0.1	50 ± 9.1
5-CH3-1,4-NQ (Menadione)		0.1	18 ± 2.8
2-OH-1,4-NQ		0.1	61 ± 11

Enzyme assays were performed at 25 °C in reaction mixtures of 1 ml containing 100 mM sodium phosphate buffer, pH 6.8 and 0.05 mM NADPH. Substrate concentrations were measured as presented in the table (**bold** highlights substrates not tested in previous studies with human AKR7A2). Data is presented as mean ± S.E.M. (n = 3) from triplicate independent experiments.

Table 2
Apparent catalytic constants for human AKR7A2 with selected aldehydes and dicarbonyls.

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})
Succinic semialdehyde	21 ± 1.4	54.59 ± 1.05	2.55×10^6
Methyl glyoxal	1948 ± 302	37.03 ± 3.74	1.90×10^4
2-OH-1,4-NQ	449 ± 39.8	7.47 ± 0.553	1.66×10^4
t,t-Muconaldehyde	966 ± 177	15.03 ± 1.27	1.56×10^4
4-Hydroxynonenal	1940 ± 320	13.85 ± 2.77	7.14×10^3
5-CH_3-1,4-NQ (Menadione)	1481 ± 207	6.12 ± 3.06	2.06×10^3
Acrolein	136,000 ± 23,000	29.83 ± 2.9	2.18×10^2
Crotonaldehyde	59,650 ± 4500	9.39 ± 4.2	1.66×10^2

K_m and k_{cat} values for recombinant AKR7A2 determined by altering the substrate concentration while maintaining NAPDH at 0.05 mM (saturating). Fitting to the Michaelis–Menten equation was carried out using Graph-Pad Prism software. The k_{cat} was calculated assuming the molecular weight of the AKR7A2 dimer as 79,198 Da, and 2 active sites per molecule of dimer.

recombinant AKR7A2 has very high affinity towards SSA as previously demonstrated, with a K_m of 21 μM [12,13]. The k_{cat} for SSA, calculated per active site of the dimer, is also similar to that previously reported [12,13]. The results also show that AKR7A2 has relatively high affinity for MUC and modest affinity for the NQ derivatives, including menadione. AKR7A2 displayed lowest affinity towards acrolein and crotonaldehyde. However, despite this, the enzyme was able to catalyze the reduction of acrolein reasonably efficiently. This indicates that at higher concentrations of these aldehydes, AKR7A2 may play a useful role in their detoxication.

3.2. Characterization of V79 cells stably expressing human AKR7A2

V79-4 Chinese hamster lung fibroblast cells were used for this study as we have previously shown that they are a useful model for studying toxicity and do not appear to express AKR7A2 protein [20]. V79-4 cells were transfected with the pCI-Neo-AKR7A2 construct in which AKR7A2 has been placed under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter, and stable clones were selected in the presence of G418. The expression of AKR7A2 in stably-transfected V79-4 cells was confirmed by Western blots (Fig. 1). A novel band of approximately 38 kDa was detected in cells transfected with AKR7A2 corresponding to the size of purified recombinant AKR7A2, but was not detected in control cells transfected with the empty plasmid pCI-Neo only (V79-pCINeo). The expression levels of AKR7A2 in V79-AKR7A2 cells was quantified using a standard curve derived from purified recombinant AKR7A2 and calculated as approximately 20.1 μg per mg of cell extract. One transfectant (V79-AKR7A2) that expressed AKR7A2 at levels comparable to human kidney and liver tissues (approximately 3 μg per mg cell extract, [13]) was selected for further study (Fig. 1).

In order to establish that the human AKR7A2 enzyme expressed in V79-AKR7A2 cells was functionally active, cell extracts were prepared from V79-AKR7A2 cells and V79-pCI-Neo (vector only control) cells. Aldo–keto reductase activity was measured in total cell extracts using the model substrates 2-carboxybenzaldehyde (2-CBA), SSA and a selection of toxic aldehyde or ketone substrates, including MUC, 4-HNE, menadione, acrolein and crotonaldehyde. V79-AKR7A2 cells showed a significant increase in 2-CBA and MUC reductase activity (Fig. 2A and B) and a small but significant increase in crotonaldehyde, 4-HNE and acrolein reductase activity compared to control V79-pCINeo (Fig. 2C, F and G). There was no significant increase in reductase activity towards menadione which is not unexpected, given the low activity of AKR7A2 towards this substrate (Fig. 2D). Surprisingly, though there was no significant increase in SSA reductase activity (Fig. 2E), which would be expected as SSA is such a good substrate for AKR7A2. It is possible however that the presence of competing enzymes in the cell extract (for example SSA dehydrogenase) are contributing to the reduction of any NADP formed. Together, these results demonstrate that stably-expressed AKR7A2 enzyme in V79-AKR7A2 cells

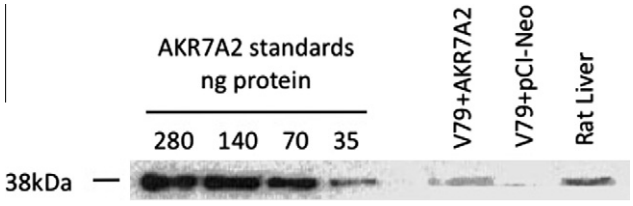


Fig. 1. Western blotting analysis of overexpressing human AKR7A2 in V79 cells. V79 cells were stably transfected with a construct containing human AKR7A2 or transfected with empty vector pCI-Neo as control. 10 μg protein extract was separated by SDS–PAGE and analyzed by Western blots using AKR7A2 antibodies. Purified recombinant AKR7A2 protein was serially-diluted and 1 μl loaded as standards to quantify the level of overexpressed AKR7A2, and analysis carried out by comparing pixel densities using ImageJ software. 10 μg protein extract from rat liver was also included to compare expression with rat AKR7A1 expression *in vivo*.

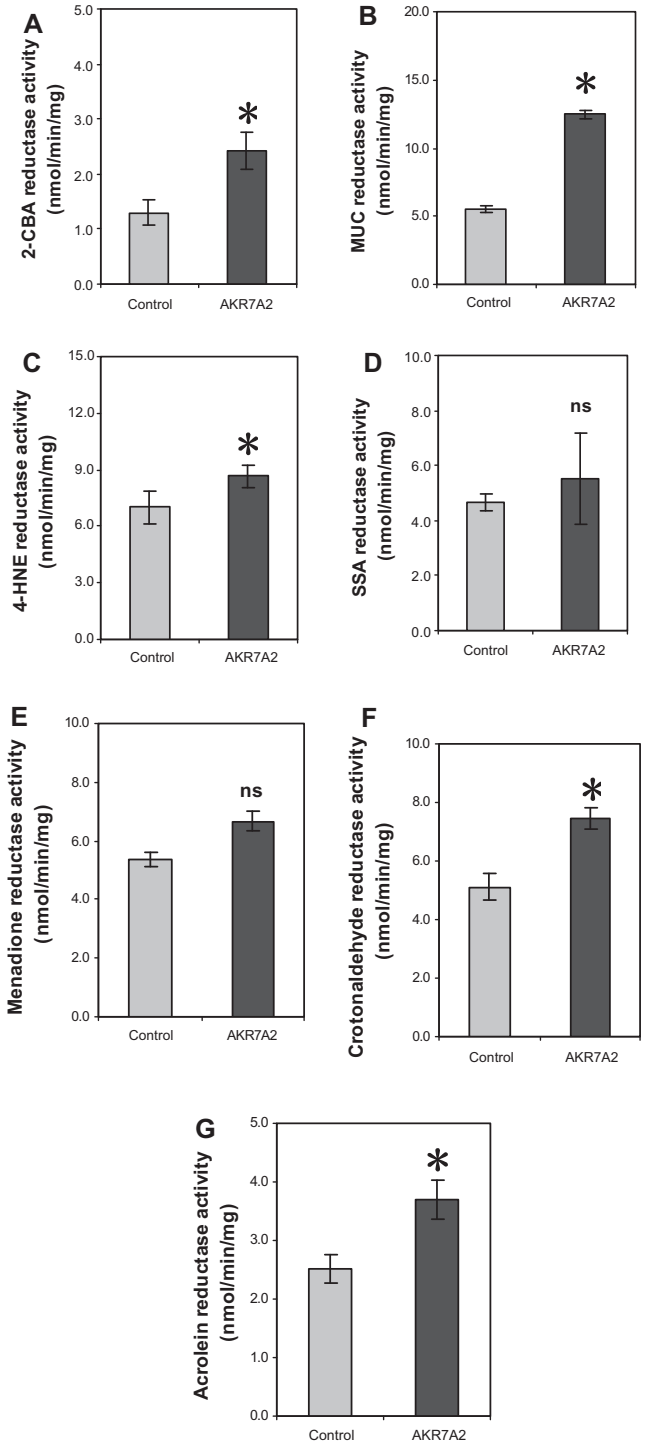


Fig. 2. Aldo–keto reductase activity in V79 cells overexpressing AKR7A2. Enzyme assays were carried out on cell extracts prepared from control and AKR7A2 transfected V79 cells using substrates A, 2-CBA; B, MUC; C, 4-HNE; D, SSA; E, Menadione; F, Crotonaldehyde; G, acrolein at a standard concentration of 1 mM (except for MUC and menadione, 0.1 mM). AKR activity was calculated as nmol/min/mg protein. Values were from three independent experiments ($n = 3$) and presented as mean ± S.E.M. ($^{**}p < 0.01$ indicating statistically significant difference, ns; no significant difference.)

is active and contributes to the reduction of aldehydes and ketones in a cell environment.

3.3. Protective role of AKR7A2 against aldehyde cytotoxicity

To study the ability of AKR7A2 to detoxify aldehydes in cells, the sensitivity of cells lines to the cytotoxicity of some of the reactive aldehydes was measured using a modified MTT cytotoxicity assay. The results in Fig. 3 show that expressing AKR7A2 confers significant protection against 4-HNE, a major product of lipid peroxidation (Fig. 3B). With a 4 h exposure to 4-HNE, only 20% of V79-pCINeo survived at 40 μM 4-HNE; in contrast, 60% of V79-AKR7A2 cells survived (Fig. 3B), and the IC_{50} increased significantly from 28.5 ± 2.7 μM in vector-only transfected cells to over 40 μM in cells expressing AKR7A2.

Similarly, AKR7A2 was also able to protect against another reactive aldehyde, MUC, Only 50% of V79 control cells survived after treatment with 20 μM MUC for 24 h, but cells expressing AKR7A2 showed significantly increased resistance to 20 μM MUC, with 80% surviving (Fig. 3A), giving rise to an increase in IC_{50}. Cells

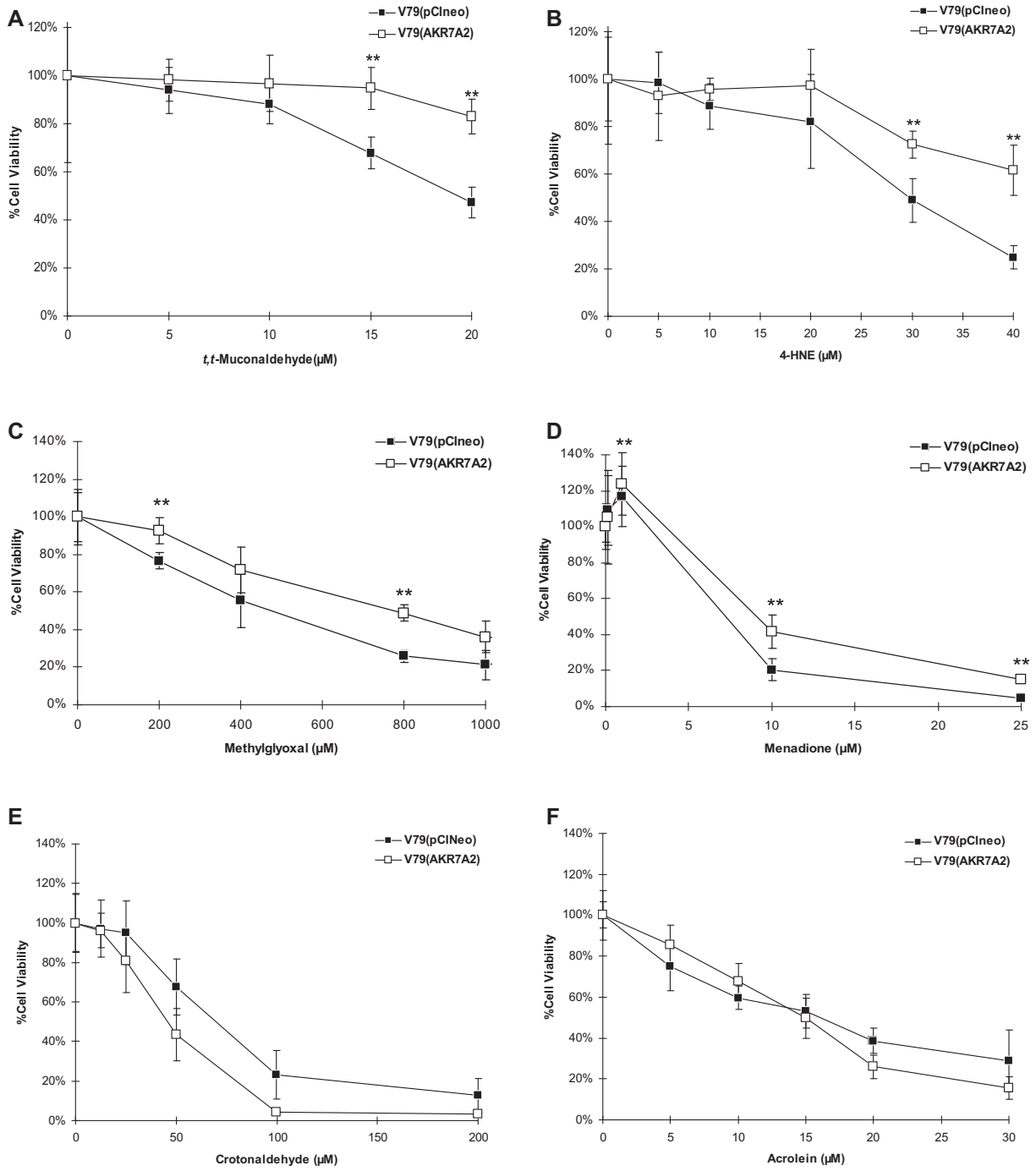


Fig. 3. Effect of overexpressing AKR7A2 on the resistance of V79 cells to aldehyde and ketone cytotoxicity. V79 cells transfected with AKR7A2 or pCI-Neo control were treated with various concentrations of aldehydes for 24 h or menadione for 4 h and cell viability was measured using the MTT assay. A, MUC; B, 4-HNE; C, methylglyoxal; D, menadione; E, crotonaldehyde; F, acrolein. Values represent the mean of four experiments ($n = 4$) ± S.E.M. Statistically significant differences relative to control were determined using two-way ANOVA: $^{**}p < 0.01$, statistically significant difference between V79 pCI-Neo control and V79-AKR7A2 cells.

expressing AKR7A2 also showed a modest increase in resistance to methylglyoxal (Fig. 3C), with IC_{50} values increasing approximately 2-fold compared with control cells.

Interestingly, expression of AKR7A2 was able to increase resistance against the cytotoxic effects of menadione (Fig. 3D), a compound that is known to give rise to increased ROS within the cell through redox-cycling, but which does not appear to be a particularly good substrate for AKR7A2 (Table 2).

Overexpression of AKR7A2 did not appear to provide any significant increase in resistance against the cytotoxicity of crotonaldehyde or acrolein (Fig. 3E and F) at tested concentrations, despite the increase in crotonaldehyde and acrolein reductase activity observed earlier (Fig. 2). This could be due to several factors including the low efficiency of the enzyme for these substrates (Table 2), and the fact that the concentrations used in the cytotoxicity assays were lower than the K_m and also lower than the concentrations used for the enzyme assays. In addition, other metabolizing enzymes are known to contribute to the detoxification of these two aldehydes [32,33], and their presence at high levels in V79-4 cells would considerably influence the relative contribution of AKR7A2 towards their metabolism.

Overall, however, the resistance to toxicity conferred by AKR7A2 is reasonably well correlated with the activity measured towards these aldehydes indicated in Table 1, with the notable exception of menadione, which does not appear to be a good substrate of AKR7A2.

To investigate the mechanism by which aldehydes and ketones cause cell death, the activity of the executioner caspase 3 was investigated following exposure to HNE or menadione. The results in Fig. 4 show that treatment of cells with reactive carbonyls leads to an increase in caspase 3 activity at low concentrations, indicating that cells are undergoing apoptosis. At higher concentrations, caspase 3 activation is lower indicating that cells are undergoing necrosis. In cells that are expressing AKR7A2, there is significantly less activation of caspase 3, indicating that this enzyme protects cells from apoptosis at the lower concentrations of both 4-HNE and menadione.

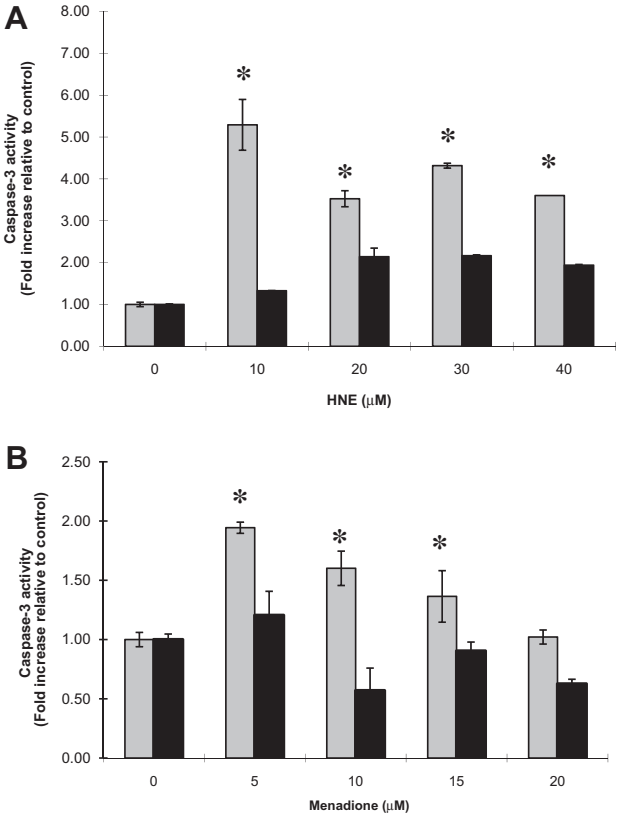


Fig. 4. Effect of AKR7A2 on protection of V79 cells against aldehyde and ketone-induced apoptosis. V79 pCI-Neo cells (light shading) or V79-AKR7A2 cells (dark shading) were treated with various concentrations of A, HNE or B, menadione for 4 h and caspase-3 activity was evaluated in cell extracts using DEVD-pNA as substrate, calculated using a pNA standard calibration curve. Results are presented as mean ± S.E.M. from three independent experiments. Statistically significant differences relative to control were determined using two-way ANOVA, $^{*}p < 0.01$, statistically significant difference between V79 control and V79-AKR7A2 cells.

3.4. Effect of AKR7A2 on 4-HNE – induced mutagenicity

Given the ability of ARK7A2 to protect cells from the cytotoxic effects of 4-HNE, an aldehyde that is known to damage DNA, the ability of AKR7A2 to protect against the mutagenicity of 4-HNE was evaluated. The HGPRT assay was used to identify mutations generated by 4-HNE [34]. Cells expressing AKR7A2 or vector-only transfected control cells were treated with 4-HNE or DMSO for 4 h prior to sub-culturing and reseeding with the selection agent (7 μg/ml 6-thioguanine). A subtoxic concentration of 4-HNE (10 μM) was utilized to avoid the overlapping of cytotoxic and mutagenic concentrations; this concentration is in agreement with that previously used by others [35]. There was no significant difference in mutation rate between V79 control cells and V79-AKR7A2 cells. Exposure to 4-HNE (10 μM) significantly increased the mutation frequency in V79 control cells by approximately 1.5-fold compared to untreated cells (Table 3). However, the mutation frequency in HNE-treated V79 cells expressing AKR7A2 was significantly lower than HNE-treated control cells. The genotoxic effect of 4-HNE has been explained previously through it forming DNA adducts with the guanine group of DNA or by the oxidation of 4-HNE to its epoxide which then forms an adduct with the guanine group of DNA. The resulting etheno adducts have been detected in human lung tissue [36]. It appears that the expression of AKR7A2 contributes to the removal of 4-HNE that effectively prevents the formation of adducts in the cell, leading to the observed decrease in mutation rate.

We also tested the ability of AKR7A2 to protect against the mutagenicity of crotonaldehyde. At low sub-lethal concentrations of crotonaldehyde, cells expressing AKR7A2 were significantly protected against crotonaldehyde mutagenicity, despite the previously observed inability to protect against crotonaldehyde cytotoxicity at higher concentrations. It is likely that AKR7A2 has some protective effect at lower sub-lethal, yet genotoxic concentrations of crotonaldehyde, but little effect at higher concentrations of this aldehyde.

Table 3
Effect of overexpressing AKR7A2 on resistance against aldehyde mutagenicity.

Relative mutation rate		
	V79-control	V79-AKR7A2
Control (DMSO)	1.5 ± 0.1	1.0 ± 0.2
4-HNE (10 μM)	2.3 ± 0.3*	1.5 ± 0.2[a]
Crotonaldehyde (10 μM)	3.2 ± 0.6*	2.1 ± 0.2[a]

Cells expressing AKR7A2 or empty vector were exposed to 10 μM 4-hydroxynonenal or 10 μM crotonaldehyde for 4 h prior to sub-culturing and reseeding into medium containing 7 μg/ml 6-thioguanine. DMSO was used as a control. Data are presented as mutation frequency relative to cloning efficiency and values represent means ± S.E.M. ($n = 5$). The mutation frequency represents the number of mutant colonies per 10^6 survivors. The cloning efficiencies were comparable for untreated and treated cells. Values were compared using one-way ANOVA followed by Dunnett's Multiple Comparison post test.

* $p < 0.01$, treated cells are significantly different from V79 control cells.

[a] $p < 0.01$, treated V79-AKR7A2 cells are significantly different from treated V79 control cells.

3.5. Protective role of AKR7A2 against menadione toxicity

It was surprising that V79-4 cells that expressed AKR7A2 were protected from the cytotoxic and apoptotic effects of menadione (Figs. 3 and 4), given that purified recombinant AKR7A2 exhibited low menadione reductase activity (Tables 1 and 2), and also that the overexpression of AKR7A2 did not significantly increase the total menadione reductase activity of V79 cells (Fig. 2E). This indicated that the observed protection against menadione is likely to be due to a mechanism other than the direct metabolism of menadione by AKR7A2.

Menadione is a quinone-containing chemotherapeutic drug that can generate ROS, and has been extensively used as a model compound for the study of cellular oxidative stress [37]. Most of the menadione-induced cytotoxicity is thought to be a consequence of the oxidative damage induced by ROS [38]. We hypothesized that AKR7A2 is able to defend against menadione toxicity by preventing an increase in intracellular ROS levels. To examine this hypothesis, ROS levels were measured in control and AKR7A2-expressing cells following exposure to menadione.

Following 4 h treatment with 5 μM menadione, ROS levels in control cells transfected with vector only were significantly increased, up to 1.5-fold relative to untreated control (Fig. 5A). In contrast, in cells expressing AKR7A2, ROS levels were not significantly increased 4 h after menadione treatment. This difference was also seen 6 and 8 h after treatment with 5 μM menadione. In the dose response curve, cells expressing AKR7A2 had significantly lower ROS levels than vector-only transfected cells when exposed to between 5 and 20 μM menadione for 4 h, but not at 30 μM menadione, a dose that is highly cytotoxic (Fig. 5B). The capability of the cells to deal with menadione-induced ROS appeared to be exceeded at this higher concentration of the redox cycler.

How might AKR7A2 contribute to the lowering of menadione-dependent intracellular ROS? It is known that menadione exposure leads to increased ROS production, which in turns gives rise to the depletion of GSH levels. To test the effect of AKR7A2, we measured GSH levels in menadione-treated V79 cells. The results in Fig. 6 show that menadione treatment leads to GSH depletion, indicating a significant alteration in redox balance. It is also apparent that

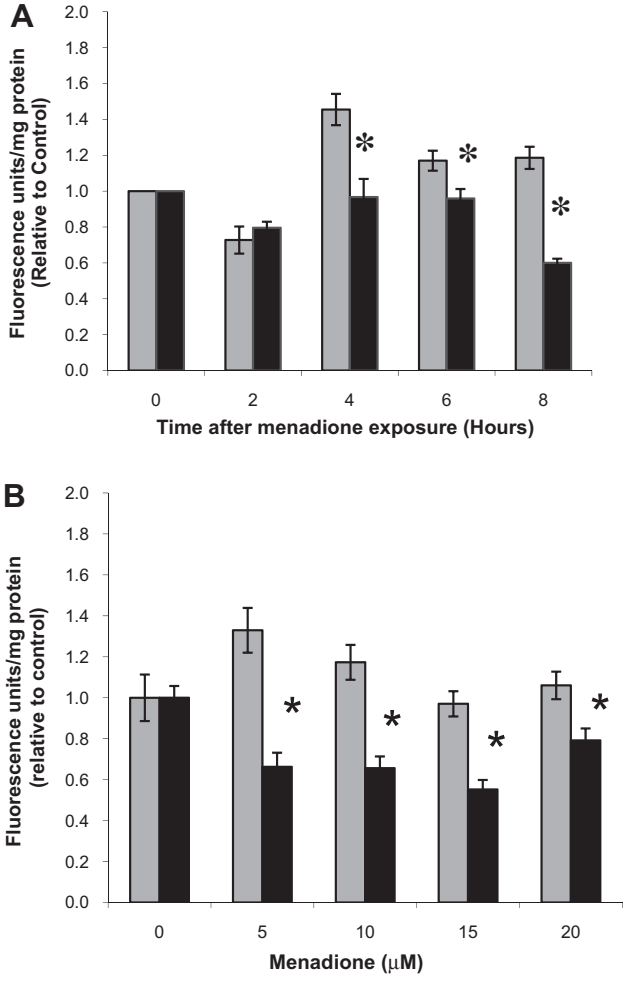


Fig. 5. Effect of AKR7A2 in preventing menadione-induced oxidative stress. V79 pCI-Neo cells (light shading) or V79-AKR7A2 cells (dark shading) were seeded on 100 mm dishes and incubated until 80% confluent. Cells were preincubated with 100 μM DCFH/DA for 1 h and exposed to (A) 5 μM menadione for increasing time or (B) increased concentrations of menadione for 4 h. Duplicate dishes were used for each condition and each sample was measured in triplicate. Protein content was measured using the standard Bio-Rad Bradford reagent. Cellular ROS level was calculated as fluorescence units/mg protein. Values represent mean ± S.E.M. from two independent experiments and calculated as percentage of untreated cells. Statistically significant differences relative to control were determined using two-way ANOVA. $^{*}p < 0.01$ indicating statistically significant difference.

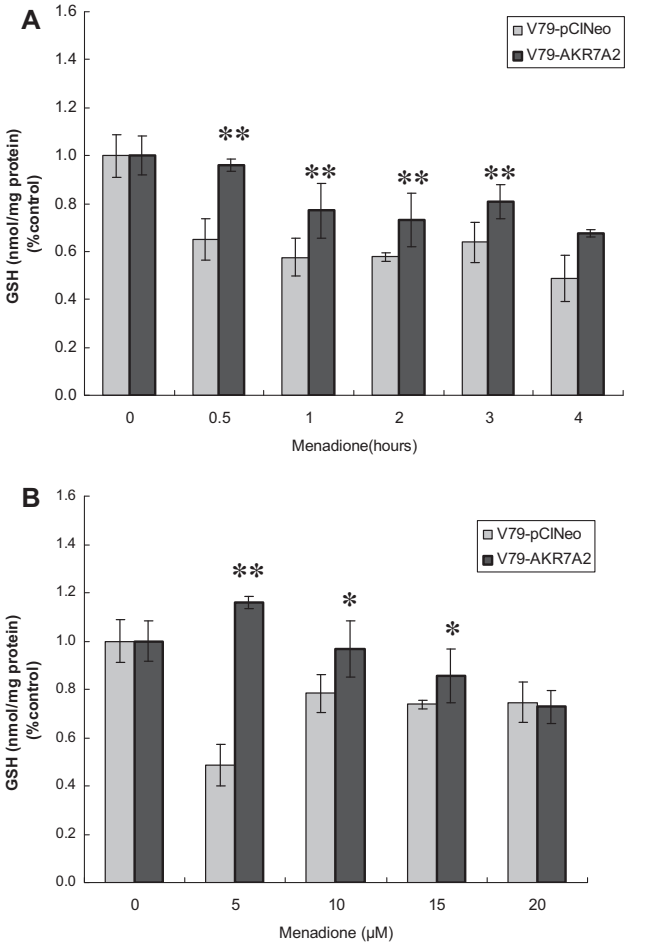


Fig. 6. Effect of AKR7A2 in preventing menadione-dependent GSH depletion. V79 pCI-Neo cells (light shading) or V79-AKR7A2 cells (dark shading) were seeded in 100 mm dishes and pretreated with (A) 15 μM menadione for increasing time or (B) increased concentrations of menadione for 4 h. DMSO was used as a solvent control. Each condition was performed in duplicate dishes and two parallel determinations per dish were measured for GSH assay. GSH values was normalized to mg protein and calculated as nmol GSH/mg protein. Results are presented as mean ± S.E.M. from three independent experiments. Statistically significant differences relative to control were determined using two-way ANOVA. $^{**}p < 0.01$ or $^{*}p < 0.05$ indicating statistically significant difference.

AKR7A2 expression is able to restore GSH levels within 4 h of exposure. We propose that AKR7A2 is able to prevent GSH depletion caused by menadione and thereby maintain redox status, preventing further ROS production and contributing to the resistance observed. This indicates AKR7A2 may serve an important physiological role in preventing the further production of ROS in oxidant exposed cells.

In terms of defining a role for human AKR7A2, this enzyme is known to be expressed in a range of tissues including liver, kidney as well as brain [12]. In human brain, immunohistochemistry has revealed that in the cortex and hippocampus, AKR7A2 is localized to glial cells, astrocytes and microglia [24]. Subcellularly, AKR7A2 localization appears to be perinuclear and cytoplasmic in neurons and astrocytes. If the only role of AKR7A2 was the synthesis of GHB, it is unclear why it would be located in perinuclear regions, given that it is likely that SSA is synthesized from GABA in the mitochondria. Elevated levels of AKR7A2 are also observed in the brains of patients with Alzheimer's Disease (AD) [24]. In AD, a range of biochemical changes are known to occur, including an association with elevated ROS and production of reactive aldehydes such as 4-HNE, and 4-HNE and acrolein protein-adducts are elevated in these same regions of brains from AD patients [39,40]. Given its ability to protect cells from the products of lipid peroxidation as well as contributing to lowering ROS levels, AKR7A2 may therefore play a significant role in protecting the brain from oxidative stress.

4. Conclusions

In this study, we have shown that human AKR7A2 can protect cells from the cytotoxicity of reactive aldehydes, and also against DNA damage caused by two of these aldehydes. In addition, we have shown that the level of menadione-induced ROS in V79-4 cells expressing AKR7A2 is significantly reduced compared with vector-transfected cells in both a time and dose dependent manner, and that this correlates with enhanced GSH levels. This is the first demonstration of the role of AKR7A2 in detoxication in a cell environment. Overall, the results presented in this study support the role of AKR7A2 as a detoxifying enzyme and indicate that it has a potential role in the defense against ROS, an important factor in disease states that have an oxidative stress component.

Conflict of interest statement

None.

Acknowledgements

Dan Li was originally funded by an Overseas Research Scholarship Award and a University of Strathclyde scholarship.

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