Dicoumarol inhibits rat NAD(P)H:quinone oxidoreductase \textit{in vitro} and induces its expression \textit{in vivo}

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Abstract

OBJECTIVES: Dicoumarol is known to act as an inhibitor of NAD(P)H:quinone oxidoreductase (NQO1). This cytosolic reductase significantly contributes to the genotoxicity of the nephrotoxic and carcinogenic alkaloid aristolochic acid I (AAI). Aristolochic acid causes aristolochic acid nephropathy (AAN), and Balkan endemic nephropathy (BEN), as well as associated urothelial malignancies. NQO1 is the most efficient enzyme responsible for the reductive bioactivation of AAI to species forming covalent AAI-DNA adducts. However, it is still not known how dicoumarol influences the NQO1-mediated reductive bioactivation of AAI.

METHODS: AAI-DNA adduct formation was determined by \textsuperscript{32}P-postlabeling. Expression of NQO1 mRNA and NQO1 protein was determined by real-time polymerase chain reaction and Western blotting, respectively.

RESULTS: In this study, dicoumarol inhibited AAI bioactivation to form AAI-DNA adducts mediated by rat and human NQO1 \textit{in vitro} as expected. We however, demonstrated that dicoumarol acts as an inducer of NQO1 in kidney and lung of rats treated with this NQO1 inhibitor \textit{in vivo}, both at protein and activity levels. This NQO1 induction increased the potency of kidney cytosol to bioactivate AAI and elevated AAI-DNA adduct levels were found in \textit{ex-vivo} incubations of AAI with renal cytosols and DNA. NQO1 mRNA levels were induced in liver only by dicoumarol.

CONCLUSION: Our results indicate a dual role of dicoumarol in NQO1-mediated genotoxicity of AAI. It acts both as an NQO1 inhibitor mainly \textit{in vitro} and as an NQO1 inducer if administered to rats.
INTRODUCTION

Dicoumarol was found to function as a strong inhibitor of NAD(P)H:quinone oxidoreductase (NQO1) (Hosoda et al. 1974; Asher et al. 2006). Besides other functions, NQO1 plays an important role in the genotoxicity of the plant component aristolochic acid I (AAI) found in medicinal herbal remedies (Stiborova et al. 2003; 2008b; 2008c; 2011a; 2011b; 2012; 2013b; 2014a; 2014b; Schmeiser et al. 2009; Arlt et al. 2011; Martinek et al. 2011; Levova et al. 2011). The herbal drug aristolochic acid (AA) derived from Aristolochia species has been shown to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN) and its associated urothelial malignancies (Debele, et al. 2008; Schmeiser et al. 2009; 2014). The major component of AA, AAI, is the predominant compound responsible for this disease.

AAN is a rapidly progressive renal fibrosis that was initially observed in a group of Belgian women who had ingested weight loss pills containing Aristolochia fangchi (Vanherweghem et al. 1993; Nortier et al. 2000; Gökmen et al. 2013). Within a few years of taking the pills, AAN patients also showed a high risk (~50%) of upper urothelial tract carcinoma (UUC) and, subsequently, bladder urothelial carcinoma. Similar cases have been reported elsewhere in Europe and Asia (Schmeiser et al. 2009). Dietary exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer (Arlt et al. 2007; Grollman et al. 2007; Moriya et al. 2011; Schmeiser et al. 2012); this nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania.

Exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of AAN and BEN patients (Schmeiser et al. 1996; 2009; Arlt et al. 2002a; 2002b; 2007; Grollman et al. 2007; Jelakovíc et al. 2012; Yun et al. 2012). The most abundant DNA adduct detected in AAN patients is 7-(deoxyadenosin-N6-yl)-aristolactam I (dA-AAI) (Fig. 1) (Schmeiser et al. 2014), which causes characteristic AT→TA transversions. Such AT→TA mutations have been observed in the TP53 tumor suppressor gene and other genes in tumours from AAN and BEN patients (Lord et al. 2004; Grollman et al. 2007; Olivier et al. 2012; Hoang et al. 2013; Poon et al. 2013), indicating a probable molecular mechanism associated with AA-induced carcinogenesis (Arlt et al. 2007; Gökmen et al. 2013). More recently, AA exposure was discovered to contribute to the high incidence of UUC in Taiwan, where medicinal use of Aristolochia plants is widespread (Chen et al. 2012); again, the TP53 AT→TA transversion mutational signature in patients with UUC was predominant which are otherwise rare. AA has been classified as a Group I carcinogen in humans by the International Agency for Research on Cancer (Grosse et al. 2009).

The metabolic activation of AAI by nitroreduction is catalyzed by both cytosolic and microsomal enzymes and leads to formation of AAI-DNA adducts and in this process NQO1 is the most efficient cytosolic nitroreductase (Stiborova et al. 2003; 2005; 2008b; 2008c; 2011a; 2011b; 2012; 2013b; 2014a; 2014b; 2014c) (Fig. 1). Indeed, microsomal cytochrome P450 (CYP) 1A1/2 and NADPH:CYP oxidoreductase that also activate AAI are less efficient in AAI bioactivation than cytosolic NQO1 (Arlt et al. 2011; Stiborova et al. 2001a; 2011b; 2012; Jerabek et al. 2012). Results of our former studies also demonstrate that NQO1 plays an important role not only in AAI activation in vitro (Stiborova et al. 2003; 2008b; 2008c; 2011a; 2011b; 2012; 2013b; 2014a; 2014b; 2014c) (Fig. 1). Indeed, microsomal cytochrome P450 (CYP) 1A1/2 and NADPH:CYP oxidoreductase that also activate AAI were proven by Chen and collaborators (Chen et al. 2011), in one mouse model (male C57BL/6 mice). In their study, AAI metabolism in vivo was decreased by inhibitors of NQO1 such as dicoumarol (Hosoda et al. 1974; Asher et al. 2006). In contrast, we have recently found in Wistar rats, that dicoumarol treatment prior to AAI administration increased the reductive activation of AAI leading to enhanced genotoxicity (i.e. AAI-DNA adduct formation) in liver and kidney (Stiborova et al. 2014c). Under the experimental conditions used, higher NQO1 expression levels caused elevated levels of AAI-DNA adducts (Stiborova et al. 2014c).

Therefore, in the present study, the effect of dicoumarol upon NQO1-mediated reductive activation of AAI ex vivo, and its effect upon NQO1 gene expression, NQO1 protein levels and NQO1 enzyme activity in vivo, was investigated in several organs (liver, kidney and lung) of rats treated with this compound. Expression of NQO1 mRNA and NQO1 protein in rats was determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting, respectively, while the activity of NQO1 was measured with menadione as a marker substrate and by AAI-DNA adduct formation in ex vivo incubations with AA and DNA.

Abbreviations:
AA - aristolochic acid
AAI - 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid
AAN - aristolochic acid nephropathy
ARE - antioxidant response element
BEN - Balkan endemic nephropathy
bw - body weight
CHN - Chinese herbs nephropathy
CYP - cytochrome P450
dA-AAI - 7-(deoxyadenosin-N6-yl)aristolactam I
dA-AAII - 7-(deoxyadenosin-N6-yl) aristolactam II
KEAP1 - Kelch-like ECH-associating protein 1
NADPH - nicotinamide adenine dinucleotide phosphate (reduced)
NQO1 - NAD(P)H:quinone oxidoreductase
NRF2 - nuclear factor-erythroid 2-related factor 2
PEI - polyethylenimine
RAL - relative adduct labeling
ROS - reactive oxygen species
qRT-PCR - quantitative real-time polymerase chain reaction
TLC - thin layer chromatography
UUC - upper urinary tract urothelial carcinoma

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MATERIALS AND METHODS

Animal experiments
The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Groups of male Wistar rats (150 g, n=3/group) were treated with dicoumarol in a suspension in corn oil. Dicoumarol was administered by gavage to rats twice at either doses of 30 or 60 mg/kg body weight (bw), once in the afternoon (3 p.m.) and then again the next day (8 a.m.) (total doses of 60 and 120 mg/kg bw, respectively). Animals in the control groups received vehicle, corn oil, only. Animals were sacrificed 24 h after the last treatment. Livers, kidneys and lungs were removed immediately after sacrifice, frozen in liquid nitrogen and stored at −80°C until isolation of cytosolic fractions.

Preparation of cytosolic samples
Hepatic, renal and pulmonary cytosolic fractions from untreated and dicoumarol-pretreated rats were isolated by differential centrifugation as previously described (Stiborova et al. 2003; 2011a; 2012). Pooled cytosolic fractions (n=3 rat/group) were used for further analyses.

Determination of NQO1 protein levels by Western blotting
The chicken anti-rat NQO1 antibodies were prepared as described previously (Stiborova et al. 2006). Immunooquantification of cytosolic NQO1 was carried out on proteins transferred to a polyvinylidene fluoride membrane after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Stiborova et al. 2006). Human recombinant NQO1 (Sigma) was used to identify the NQO1 band from rat cytosols. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (Stiborova et al. 2006). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by commercial antibody (1:750, Millipore; MA, USA).

Measurement of NQO1 enzyme activity
NQO1 activity was determined using menadione as a substrate as described (Stiborova et al. 2003); improved measurement was achieved by addition of cytochrome c (Mizerovska et al. 2011).

Cytoplasmic incubations of AA with DNA to analyze AAI-DNA adduct formation
The de-aerated and nitrogen-purged incubation mixtures, in a final volume of 750 µl, included 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 1 mM NADPH, 1 mg rat cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI. Incubations were carried out at 37°C for 60 min; AAI-derived DNA adduct formation in cytosols is known to be linear up to 2 h (Stiborova et al. 2003). Control incubations were carried out (i) without cytosol, (ii) without NADPH, (iii) without DNA, or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the standard phenol/chloroform extraction method (Stiborova et al. 2007b; 2013a).

Incubations with rat and human NQO1
The incubations used to evaluate DNA adduct formation by AAI with rat NQO1 isolated from rat liver (Stiborova et al. 2001b) and human recombinant NQO1 (Sigma) were done as described (Stiborova et al. 2002; 2003). To study the effect of dicoumarol on AAI-DNA adduct formation, incubations in a final volume of 750 µl consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 1 mM NADPH, 0.1 mM AAI (dissolved in water), 0.5 mg of calf thymus DNA (2 mM dNp) and 20 µg (0.06 units) of rat or human NQO1 in the absence or presence of 10 µM dicoumarol (dicoumarol dissolved in 7.5 µl of ethanol). One unit of NQO1 reduces 1 µmol of cytochrome c per min in the presence of menadione as substrate at 37°C. The reaction was initiated by adding NADPH. All reaction mixtures were incubated at 37°C for 60 min; NQO1-mediated AAI-derived DNA adduct formation was found to be linear up to 90 min (Stiborova et al. 2011a). In control incubations NQO1 or its cofactor (NADPH) was omitted from the mixtures. After extraction with ethyl acetate, DNA was isolated from the residual water phase as described above.

DNA adduct analysis by 32P-postlabelling
DNA adduct formation was analysed using the nuclelease P1 enrichment version of the thin-layer chromatography (TLC) 32P-postlabelling method (Schmeiser et al. 2013). DNA digestion, adduct enrichment and labelling were performed as described (Stiborova et al. 2003; 2011a). Chromatographic conditions for TLC on polyethyleneimine-cellulose plates (10×20 cm; Macherey-Nagel, Düren, Germany) were: D1, 1.0 M sodium phosphate, pH 6.8; D2, 3.5 M lithium-formate, 8.5 M urea, pH 4; D3, 3.5 M lithium chloride, 0.5 M Tris-HCl, 8.5 M urea, pH 9; D5, 1.7 M sodium phosphate, pH 6. After chromatography TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labelling) were calculated as described (Stiborova et al. 2003; 2011a). Results were expressed as DNA adducts/108 normal nucleotides.

NQO1 mRNA contents in rat liver, kidney and lung
NQO1 mRNA expression in rat liver, kidney and lung was determined as described previously (Stiborova et al. 2006; 2008a). Total RNA was isolated from frozen
livers, kidneys and lungs of untreated and dicoumarol-treated rats (n=3; each group) and mRNA was quantified by qRT-PCR exactly as described (Stiborova et al. 2008a). Briefly, the qPCR data were analyzed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (Ct) method for relative quantification of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then \( \Delta \Delta Ct \) was evaluated according to following equations: \( \Delta Ct = Ct (\text{target}) - Ct (\text{internal standard}) \), \( \Delta \Delta Ct = \Delta Ct \text{treated} - \Delta Ct \text{control} \) where \( \Delta Ct \) for treated rats and \( \Delta Ct \) control is \( \Delta Ct \) for untreated rats. The induction of mRNA expression of studied target gene in pretreated animals was evaluated as \( 2^{-\Delta \Delta Ct} \).

**RESULTS**

**Dicoumarol inhibits bioactivation of AAI by rat and human NQO1 to AAI-DNA adducts in vitro**

As described in our former studies, rat and human NQO1 are capable of activating AAI to form AA-DNA adducts in vitro (Stiborova et al. 2002; 2003; 2008b; 2008c; 2011a; 2011b; 2014b; Martinek 2011). In the presence of NADPH, the cofactor of NQO1, AAI was reductively activated by NQO1 to species generating the same pattern of DNA adducts as that found in patients suffering from AAN and BEN (Schmeiser et al. 1996; 2009; 2014; Nortier et al. 2000; Nortier et al. 2000; Arlt et al. 2002a; 2002b). The adduct pattern formed consisted of two major adducts, 7-(deoxyadenosin-N\(^6\)-yl)aristolactam I (dA-AAI) and 7-(deoxyguanosin-N2-yl)aristolactam I (dG-AAI), and one minor adduct, 7-(deoxyadenosin-N\(^6\)-yl)aristolactam II (dA-AAII) (see insert Figure 1). If dicoumarol was added to the incubation mixture AAI-DNA adduct formation catalyzed by rat and human NQO1 in vitro, was inhibited by 96 and 98%, respectively (Table 1). These results confirmed not only the efficiency of NQO1 to activate AAI to AAI-DNA adducts, but also showed the potency of dicoumarol to inhibit the activity of this reductase in vitro.

<table>
<thead>
<tr>
<th>Enzymatic system</th>
<th>RAL(^a) (mean ± SEM/10(^8) nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human NQO1</td>
<td></td>
</tr>
<tr>
<td>without cofactor</td>
<td>n.d.</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>32.6±3.7</td>
</tr>
<tr>
<td>+ NADPH + dicoumarol(^b)</td>
<td>0.5±0.05***</td>
</tr>
<tr>
<td>(2%)(^c)</td>
<td></td>
</tr>
<tr>
<td>Rat NQO1 without cofactor</td>
<td>n.d.</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>4.2±0.4</td>
</tr>
<tr>
<td>+ NADPH + dicoumarol</td>
<td>0.2±0.02***</td>
</tr>
<tr>
<td>(3.9%)</td>
<td></td>
</tr>
</tbody>
</table>

**The effect of dicoumarol on NQO1 enzyme activity and AAI-DNA adduct formation in liver, kidney and lung cytosols of rats treated with this compound**

In further experiments, we investigated the effects of dicoumarol on NQO1 activity in vitro, namely, the influence of treatment of rats with dicoumarol on NQO1 activity in vivo, namely, the influence of treatment of rats with dicoumarol on NQO1 activity in liver, kidney and lung of these rats. Because NQO1 is a cytosolic enzyme, NQO1 activity (measured with its two substrates, menadione and AAI) was analyzed in cytosols isolated from these rat organs. Hepatic, renal and pulmonary cytosols isolated from control and dicoumarol-treated rats were analyzed for their efficiencies to reduce these substrates.

The activity of NQO1 with menadione as substrate decreased down to 50% in liver cytosol when rats were treated with dicoumarol (Figure 2A). These results correspond to the strong inhibitory effect of dicoumarol on NQO1 enzyme activity in vitro (Hosoda et al. 1974; Asher et al. 2006). However, in contrast to these results, unexpectedly a 1.8- and 1.7-fold increase in the activity of this enzyme was found in kidney and lung cytosols isolated from rats treated with the higher dicoumarol dose (Figure 2A).

Since AAI is also a substrate of NQO1, its reductive bio-activation to species forming AAI-DNA adducts was analyzed. Cytosols were incubated with AAI, DNA and NADPH, and analyzed for formation of AA-DNA adducts by \(^3\)P-postlabelling. AAI was reductively activated by all rat cytosols isolated from untreated (control) animals, as evidenced by AAI-DNA adduct formation (Figure 3). The DNA adduct patterns generated were the same as those analyzed in patients suffering from AAN and BEN (Schmeiser et al. 1996; 2009; 2014; Nortier et al. 2000; Arlt et al. 2002a; 2002b). Analogy to NQO1 activity with menadione as substrate, a 2-fold decrease in AAI-DNA adduct levels was found in incubations of liver cytosolic fractions isolated from dicoumarol-treated rats. However, a 2.6- and 1.9-fold increase in levels of AAI-DNA adducts was detected in DNA incubations with AAI and kidney or lung cytosols, respectively, from rats exposed to 120 mg dicoumarol compared to controls (Figure 3). Changes in AAI-DNA adduct formation in cytosols from each of these organs correlated with the NQO1 activities determined with menadione in these cytosols. The absolute
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Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N2-yl)aristolactam I; NR, nitro-reduction; UGT, UDP glucuronosyltransferase; SULT, sulfotransferase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from control rats and DNA using the nuclease P1 enrichment version of the 32P-postlabeling assay.

Fig. 2. NQO1 enzyme activity (A) and NQO1 protein expression (B) in cytosols isolated from liver, kidney and lung of untreated (control) rats or rats treated with total doses of 60 or 120 mg/kg bw dicoumarol (see Materials and Methods for details). NQO1 enzymatic activity in cytosols (A) was determined as described in chapter 2.4. NQO1 protein expression in cytosols (B) was determined by Western blotting (see insert). Human recombinant NQO1 (Sigma) was used to identify the rat NQO1 band in rat cytosols (data not shown). Glyceraldehyde phosphate dehydrogenase was used as loading control. All values are given as means ± SD (n = 3). Values significantly different from control rats: **p<0.01, ***p<0.001 (Student’s t-test). Numbers above columns (“F”) indicate fold changes in protein level or enzyme activity compared to control. DC – dicoumarol.
NQO1 activities in the two extrahepatic organs were, however, not consistent with AAI-DNA adduct formation. Although NQO1 activity was higher in lung than kidney cytosols, the levels of AAI-DNA adducts formed by lung cytosols were almost one order of magnitude lower than by cytosols of liver or kidney (compare Figures 2 and 3).

**The effect of dicoumarol on expression of NQO1 protein in liver, kidney and lung of rats treated with this compound**

The activities of a variety of enzymes including NQO1 are usually determined by their expression levels, but other factors influencing their activities cannot be excluded. We, therefore, evaluated whether expression of NQO1 proteins is the cause of changes in NQO1 activities found in tested organs of rats treated with dicoumarol. A method of Western blotting, suitable to evaluate expression of proteins, was used for such investigations. Using this technique, we explained the unexpected increase in NQO1 activity in kidney and lung of rats treated with dicoumarol relative to controls.

In contrast to the rat liver, where non-significant changes in the levels of NQO1 protein were detected, this NQO1 inhibitor acted as an inducer of NQO1 protein expression in rat kidney and lung; up to 2.1- and 1.5-fold higher levels of NQO1 protein were found in kidney and lung cytosols of dicoumarol-treated rats relative to those of control animals, respectively (Fig. 2B). The levels of NQO1 protein in kidney and lung correlated with the enzyme activity in cytosols of these organs. Expression levels of NQO1 and its enzyme activities measured with menadione as a substrate in hepatic and renal cytosols essentially corresponded also to the bioactivation of AAI to species forming DNA adducts. However, such a correlation was not found in lung cytosols; low levels of AAI-DNA adducts did not correspond to the high expression of NQO1 and enzyme activity in cytosols of this organ (compare Figures 2 and 3). The reason responsible for this phenomenon is not known and remains to be explored.

**NQO1 mRNA levels in rat liver, kidney and lung**

Changes in NQO1 mRNA levels were determined by qRT-PCR analysis. As shown in Table 2, treatment of rats with both doses of dicoumarol induced significant increases in NQO1 mRNA levels in the liver; a 1.7- and 2.5-fold increase in levels of NQO1 mRNA was found after exposure of rats to 60 and 120 mg/kg bw of dicoumarol, respectively (Table 3). The dicoumarol effect on NQO1 mRNA in the other two organs was either none (lung) or not significant (kidney) (Table 2).

**DISCUSSION**

Our results show that dicoumarol, a strong inhibitor of NQO1 (Hosoda et al. 1974; Asher et al. 2006), plays a dual role on this enzyme in Wistar rats *in vivo*. We show that *in vitro* dicoumarol inhibits the reductive bioactivation of AAI catalyzed by rat and human NQO1; this reaction was inhibited up to 98% by dicoumarol. The effects of dicoumarol *in vivo* were, however, not so clear

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Tab. 2. Expression of NQO1 mRNA in rats treated with dicoumarol NQO1.

<table>
<thead>
<tr>
<th>Rats treated with 60 mg/kg dicoumarol</th>
<th>ΔcT</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6.40±0.21</td>
<td>1.7*</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.82±0.47</td>
<td>1.4</td>
</tr>
<tr>
<td>Lung</td>
<td>7.35±0.11</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rats treated with 120 mg/kg dicoumarol</th>
<th>ΔcT</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>5.09±0.15</td>
<td>2.5***</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.83±0.73</td>
<td>1.0</td>
</tr>
<tr>
<td>Lung</td>
<td>7.37±0.19</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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*Results shown are mean ± SD (n = 3) of untreated (control) rats or rats treated with 60 or 120 mg/kg bw dicoumarol.

*The Fold Change refers to fold increase in NQO1 mRNA expression in treated animals over control group evaluated by 2-(ΔΔcT) method (see Material and Methods for details).

Significantly different from controls: *p<0.05; ***p<0.001 (Student’s t-test).
cut. In liver cytosols from dicoumarol-treated rats isolated 24 h after the last administration NQO1 activity is either inhibited or unchanged (see Figure 2). It is possible that dicoumarol is still binding to liver NQO1 because it has strong protein binding properties (Aشر et al. 2006). It is expected that due to its long elimination half-life of 5–25 h in rats (Lai et al. 1976) residual dicoumarol may remain in tissues, particularly in liver as its main site of metabolism. The decreased NQO1 enzyme activity resulted in lower DNA adduct formation in ex-vivo incubations with AAI using hepatic cytosols (see Figure 3). The patterns of protein levels, enzyme activities and AAI-DNA adduct formation also show inhibition of NQO1 at 60 mg, but a recovery at 120 mg for reasons unknown. However, the situation in the kidney and lung was more complex. In kidney and lung, dicoumarol induced NQO1 protein levels and also enzyme activity in a dose dependent manner (see Figures 2 and 3). An up to a 2-fold induction in NQO1 protein expression was observed in kidney after exposure of rats to dicoumarol. The NQO1 protein levels correlated with enzyme activity. However, reduction of AAI by rat cytosols to a cyclic acylnitrenium ions forming DNA adducts (Schmeiser et al. 2009; Stiborova et al. 2014a; 2014b; 2014c) correlated well only in liver and kidney with NQO1 expression and enzyme activity. The situation in rat lung was completely different from that in the other two organs investigated. Basal and dicoumarol-induced NQO1 protein levels in rat lung were up to 3-times higher than in liver and kidney. Likewise, the efficiency of rat lung cytosols to reduce menadione was up to 5-fold higher than those of liver and kidney cytosols. However, the activity of lung cytosols to reduce AAI to species forming DNA adducts was low. Several reasons for this observation are possible: some inhibitors that compete with AAI to binding to the NQO1 active centre might be present in rat lung cytosol or the lung NQO1 protein has undergone allosteric effects in AAI reduction. Which of these reasons might be most important in our experiments is hard to say and remains to be explained.

Dicoumarol as an NQO1 inhibitor has been employed in a mouse model (male C57BL/6 mice) by Chen and collaborators (Chen et al. 2011) to evaluate the role of NQO1 in AAI nitroreduction in kidney in vivo. In their study, modulation of the AAI metabolism by dicoumarol and phenindione, another NQO1 inhibitor (Chen et al. 1999), was investigated (Chen et al. 2011). Whereas inhibition of NQO1 activity decreased the amount of the reductive metabolite aristolactam I in kidney in dicoumarol-pretreated mice 30 min after a single i.p. injection of AAI, the amounts of AAI and its detoxication metabolite AA1a increased in mouse serum. Furthermore, pretreatment of mice with dicoumarol decreased AAI-induced nephrotoxicity as well as the survival rate of these mice (Chen et al. 2011). These results suggested that dicoumarol acts as an inhibitor of AAI nitroreduction in this mouse model. However, the effect of dicoumarol was only investigated a short period (30 min) after pretreatment of mice with it.

In our previous study using Wistar rats as the experimental model, these were treated with dicoumarol 22 h prior to AAI administration (Stiborova et al. 2014c). As a measure of genotoxicity we found higher levels of AAI-DNA adducts in liver and kidney. The induction of NQO1 protein and enzyme activity by dicoumarol found in the present study provides an explanation for our previous results. We demonstrated that a long exposure time to dicoumarol in Wistar rats of 24 h resulted in an increase in NQO1 protein expression and its activity in rat kidney and lung. Such an induction increased the potency of kidney cytosols to reductively activate AAI and led to elevated AAI-DNA adduct levels in ex-vivo incubations of AAI with renal cytosolic fractions and DNA. However, because AAI is also reductively activated to form AAI-DNA adducts or oxidatively detoxified to AA1a by CYP enzymes such as CYP1A1 and/or 2C (Stiborova et al. 2001a; 2012; 2014c; Arlt et al. 2011; Levova et al. 2011), their influencing by dicoumarol might also be important to modulate levels of AAI-DNA adducts formed in vivo. We have previously found that higher protein levels and activities of CYP1A1 in kidney and CYP1A2 in liver were found in rats treated with dicoumarol. But, this increase was less important for an increase in AAI-DNA adduct levels in rat liver and kidney treated with dicoumarol prior to administration to AAI in vivo than induction of NQO1 (Stiborova et al. 2014c). On the contrary, inhibition of CYP2C6 and 2C11 enzymatic activities in rats treated with dicoumarol was found and led to a decrease in AAI oxidative detoxification to AA1a. This finding indicates that higher adduct levels found in the liver of rats pretreated with dicoumarol prior to AAI administration might be, beside induction of NQO1, also caused by decreased AAI detoxification to AA1a due to the dicoumarol-mediated CYP2C inhibition (Stiborova et al. 2014c). Because dicoumarol is used as an anticoagulant drug that functions as a vitamin K antagonist also in human medicine (for a review, see Wallin et al. 2008; Rajan & Moliterno 2012), an induction of NQO1 and inhibition of CYP activities might increase AAI-DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans.

Besides analyses of the effect of dicoumarol on NQO1 enzyme activity and NQO1 protein levels, we also investigated its influence on NQO1 mRNA expression. Although NQO1 protein levels essentially correlated with enzyme activity, no correlation between these parameters was found with regards to NQO1 mRNA expression. A significant increase in NQO1 mRNA expression was found only in the livers of dicoumarol-treated rats, in contrast to protein and activity, but no effect of dicoumarol on mRNA expression of this enzyme was observed in kidney and lung at both dicoumarol doses, despite increases in NQO1 protein levels in these organs (see Table 2 and Figure 2).
Similar discrepancies between induction of mRNAs of NQO1 or several other enzymes and their protein levels were observed previously (Dickins, 2004; Stiborova et al, 2006; 2007a; Aimova et al. 2007). It is known that some inducers might prolong half-lives of mRNAs, while others increase transcription. Therefore, in our experiments, where the rats were sacrificed 24 h after the last dicoumarol administration, mRNA levels may have returned to normal, since half-lives of mRNAs are usually much shorter than those of proteins (for a review, see Dickins, 2004). Furthermore, the NQO1 protein stabilization in kidney and lung might be another explanation for the discrepancy observed. However, the studies investigating the stability of NQO1 protein and the half-life of its mRNA in individual rat organs were not carried out in our laboratory and are also not described in the literature. In addition, detailed analyses of the time dependence of the mRNA expression levels and NQO1 protein levels were not performed in the present study. Therefore, future investigations should address the questions whether the transient induction of NQO1 mRNAs or the different half-lives and/or stabilities for its mRNA and protein are the rational for our observation, and/or how dicoumarol impacts on the stability of mRNA and protein of this enzyme.

Even though we here demonstrated that dicoumarol increased NQO1 protein expression and enzyme activity, the mechanisms of this induction effect are still a matter of debate. NQO1 induction has been widely investigated in a variety of studies (for a review, see Jaiswal 2000; Dinkova-Kostova et al. 2004; Ross 2004; Dinkova-Kostova & Talalay 2010). Protein levels of NQO1 enzyme are influenced by several chemicals. NQO1 expression is regulated by two distinct regulatory elements in the 5' flanking region of the NQO1 gene, the antioxidant response element (ARE) and the xenobiotic response element involving ligand-activated aryl hydrocarbon receptor (Jaiswal 2000; Dinkova-Kostova et al. 2004; Ross 2004; Dinkova-Kostova and Talalay, 2010). In ARE-mediated NQO1 induction, nuclear factor-erythroid 2-related factor 2 (NRF2) and the cytoskeletal-binding protein KEAP1 (Kelch-like ECH-associating protein 1) play an important role (i.e. the NRF2-KEAP1 mechanism of NQO1 induction). ARE-mediated NQO1 gene expression is increased by a variety of antioxidants, tumour promoters and reactive oxygen species (ROS) (Jaiswal, 2000; Dinkova-Kostova et al. 2004; Ross, 2004; Dinkova-Kostova & Talalay, 2010). NQO1 is known to be implicated into the vitamin K metabolism, and was demonstrated to be identical to the so called dicoumarol-inhibited vitamin K reductase (Dinkova-Kostova & Talalay, 2010). Because vitamin K is redox cycled during its metabolism, ROS might be generated in some one-electron redox reactions involved in this metabolism (Gong et al. 2008; Tie et al. 2011). NQO1 competes with enzymes that redox cycle vitamin K and catalyzes two-electron reduction of vitamin K to hydroquinone. This prevents the formation of the semiquinone and ROS. If dicoumarol inhibits NQO1 activity, ROS formation could increase significantly (Gong et al. 2008; Dinkova-Kostova & Talalay, 2010) and might be the cause of ARE activation leading to NQO1 induction. Hence, an increase in dicoumarol-mediated ROS formation caused by NQO1 inhibition of two-electron redox reactions of vitamin K metabolism might be one mechanism by which dicoumarol induces NQO1. However, the question whether ROS formation during these redox cycling reactions is the means by which NQO1 is induced, or by other mechanisms, remains to be explored in future studies.

In conclusion, in this study we demonstrated that dicoumarol, a strong inhibitor of NQO1, increases expression of NQO1 protein and enzyme activity in kidney and lung of rats exposed to this compound and that these effects lead to increased bioactivation of AAI to species binding to DNA mainly in the kidney, the target organ of AAI. Because dicoumarol is a drug used in human medicine, such an NQO1 induction might increase AAI-DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans treated with this drug.

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