Dicoumarol inhibits rat NAD(P)H:quinone oxidoreductase *in vitro* and induces its expression *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 ^{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 *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 *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 *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 genotoxicty of AAI. It acts both as an NQO1 inhibitor mainly *in vitro* and as an NQO1 inducer if administered to rats.

ORIGINAL ARTICLE

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-N^6-yl)aristolactam I
dA-AAII	- 7-(deoxyadenosin-N^6-yl)aristolactam II
dG-AAI	- 7-(deoxyguanosin-N^2-yl) aristolactam I
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

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; Jelakovic *et al.* 2012; Yun *et al.* 2012). The most abundant DNA adduct detected in AAN patients is 7-(deoxyadenosin-N^6-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), but also *in vivo* (Stiborova *et al.* 2013b; 2014a; 2014b; 2014c). A role of NQO1 in renal AAI nitroreduction *in vivo* was 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 AAI and DNA.

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 sacrified 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). Immunoquantification 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).

Cytosolic 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 ^{32}P-postlabelling

DNA adduct formation was analysed using the nuclease P1 enrichment version of the thin-layer chromatography (TLC) ^{32}P-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 polyethylenimine-cellulose plates (10×20 cm; Macherey-Nagel, Düren, Germany) were: D1, 1.0 M sodium phosphate, pH 6.8; D3: 3.5 M lithium-formate, 8.5 M urea, pH 4; D4, 0.8 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/10^8 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 (c_T) 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 $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard), $\Delta\Delta c_T = \Delta c_{T\ treated} - \Delta c_{T\ control}$, where $\Delta c_{T\ treated}$ is Δc_T for treated rats and $\Delta c_{T\ control}$ is Δc_T for untreated rats. The induction of mRNA expression of studied target gene in pretreated animals was evaluated as $2^{-\Delta\Delta c_T}$.

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 *et al.* 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; 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-N^2-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*.

Tab. 1. The effect of dicoumarol on the AAI-DNA adduct formation from AAI (0.1 mM) by NQO1 *in vitro*.

	RAL[a] (mean ± SEM/10^8 nucleotides)			
Enzymatic system	**dG-AAI**	**dA-AAI**	**dA-AAII**	**Total**
Human NQO1 without cofactor	n.d.			
+ NADPH	32.6±3.7	50.2±5.6	8.2±0.9	91.0±9.5
+ NADPH + dicoumarol[b]	0.5±0.05***	1.1±0.1***	0.2±0.02***	1.8±0.2***
				(**2%**)[c]
Rat NQO1 without cofactor	n.d.			
+ NADPH	4.2±0.4	15.4±1.6	1.0±0.1	20.6±2.1
+ NADPH + dicoumarol	0.2±0.02***	0.6±0.07***	n.d.	0.80±0.1***
				(**3.9%**)

Numbers are averages ± SEM (n=4) of duplicate *in vitro* incubations, each DNA sample was determined by two postlabeled analyzes.
[a] Relative adduct labeling; [b] 10 μM dicoumarol; [c] % of control
Significantly different from levels of AAI-DNA adducts without dicoumarol: ***p<0.001 (Student's t-test).

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 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 AAI-DNA adducts by ^{32}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; 2012; 2014; Nortier *et al.* 2000; Arlt *et al.* 2002a; 2002b) or *in vitro* in incubations with rat and human NQO1 (Stiborova *et al.* 2002; 2003; 2011a; Martinek 2011) (see insert Figure 1). No adducts were observed in control incubations carried out in parallel (data not shown). Analogously 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

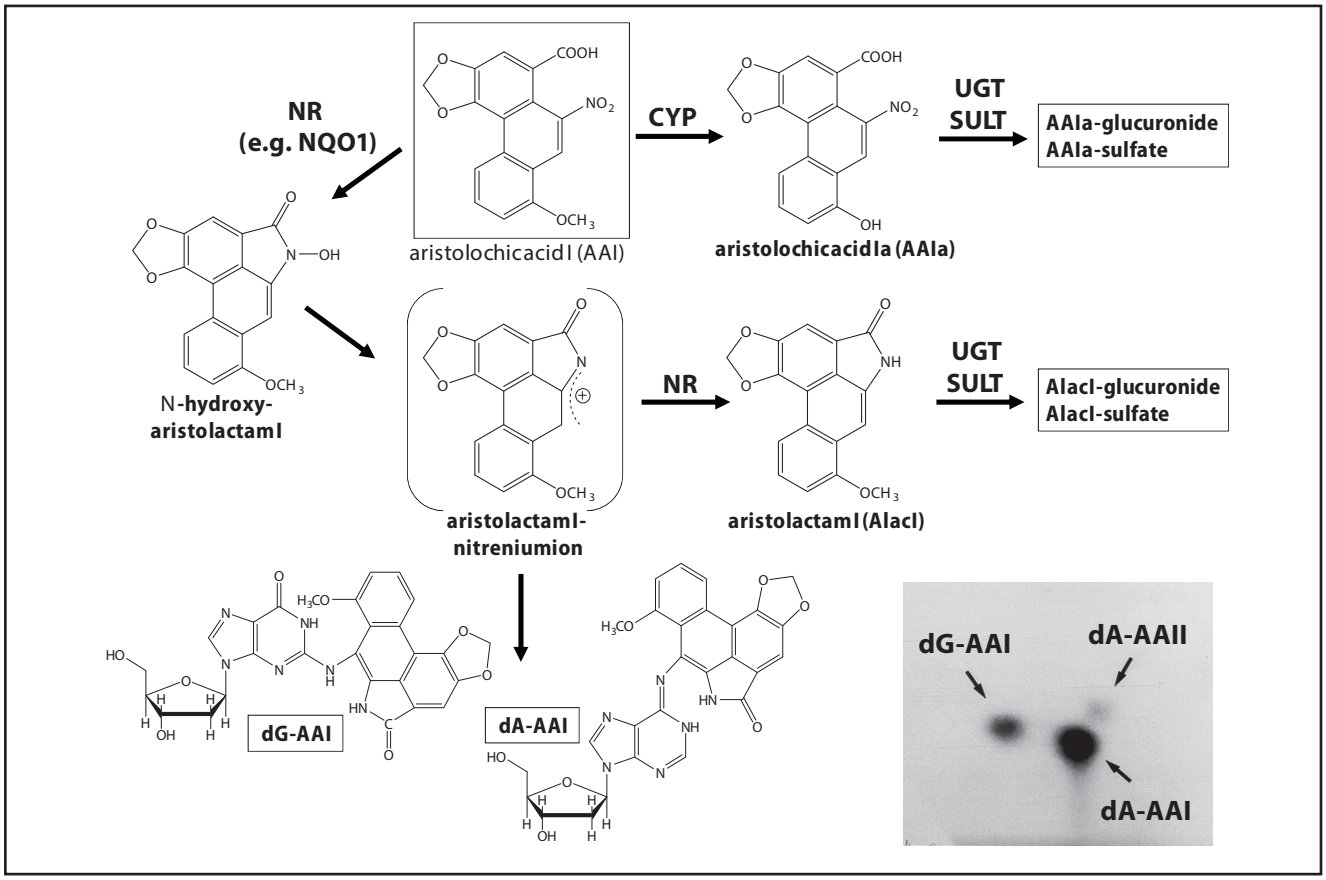

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitro-reduction; UGT, UDP glucuronosyltransferase; SULT, sulfotransferase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from control rats and DNA using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay.

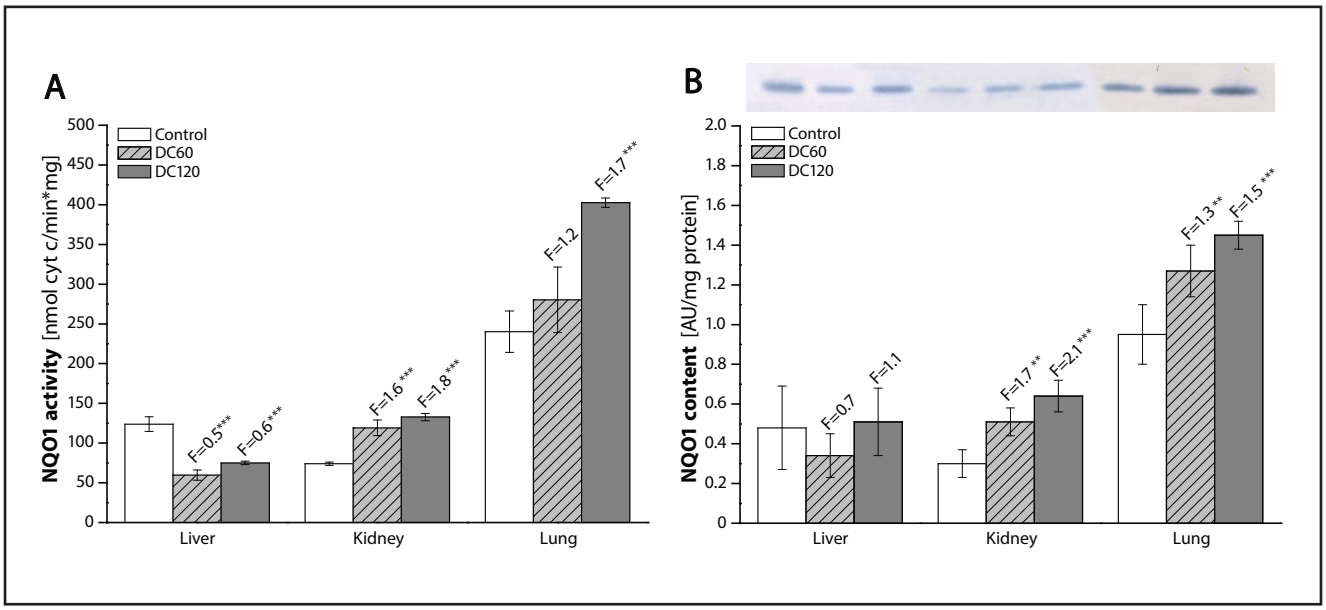

Fig. 2. NQO1 enzyme activity (A) and NQO1 protein expression (B) in cytosols isolated from liver, kidney and lung of untreated (control) rats or rats treated with total doses of 60 or 120 mg/kg bw dicoumarol (see Materials and Methods for details). NQO1 enzymatic activity in cytosols (A) was determined 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

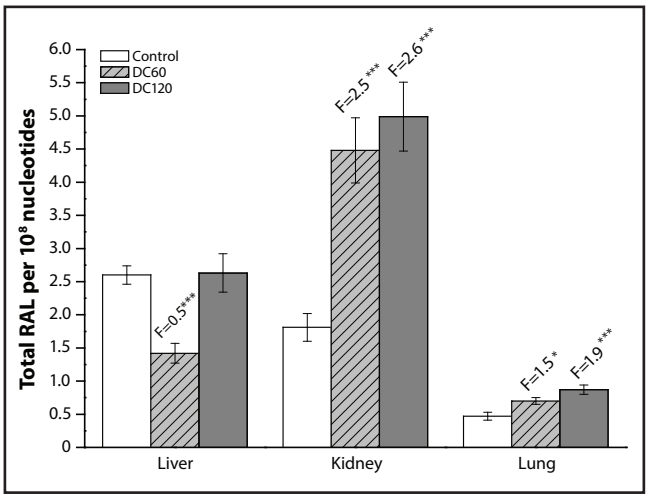

Fig. 3. DNA adduct formation *ex vivo* by AAI in cytosols isolated from untreated (control) rats or rats treated with total doses of 60 and 120 mg/kg bw dicoumarol (see Materials and Methods for details). AAI-DNA adduct formation was determined by ^{32}P-postlabelling in hepatic, renal and pulmonary cytosolic fractions. Values are given as the means SD (n = 3). RAL, relative adduct labeling. Comparison was performed by t-test analysis; $^{*}p < 0.05$, $^{***}p < 0.001$, different from control. Numbers above columns ("F") indicate fold changes in DNA adduct levels compared to control. DC – dicoumarol.

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

Rats	Δc_T[a]	Fold Change[b]
Control rats		
Liver	6.40±0.21	
Kidney	7.82±0.47	
Lung	7.35±0.11	
Rats treated with 60 mg/kg dicoumarol		
Liver	5.61±0.22	1.7*
Kidney	7.29±0.75	1.4
Lung	7.54±0.30	0.9
Rats treated with 120 mg/kg dicoumarol		
Liver	5.09±0.15	2.5***
Kidney	7.83±0.73	1.0
Lung	7.37±0.19	1.0

[a] Results shown are mean ± SD (n = 3) of untreated (control) rats or rats treated with 60 or 120 mg/kg bw dicoumarol.
[b] The Fold Change refers to fold increase in *NQO1* mRNA expression in treated animals over control group evaluated by $2^{-(\Delta\Delta c_T)}$ method (see Material and Methods for detail).
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 (Asher *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 AAIa 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 AAIa by CYP enzymes such as CYP1A 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 AAIa. 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 AAIa 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 sacrified 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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