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SHORT COMMUNICATION

Involvement of microsomal NADPH-cytochrome P450 reductase in metabolic reduction of drug ketones

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ABSTRACT: Recently, it was found that the carbonyl group of 1-[3-(4-phenoxyphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**5**), an inhibitor of the pro-inflammatory enzyme cytosolic phospholipase $A_2\alpha$, is easily reduced by rat liver S9 fractions *in vitro*. Determination of the inhibitory potency of certain putative inhibitors of carbonyl reducing enzymes on the transformation of the ketone derivative **5** to its alcohol **6** by recombinant microsomal NADPH-cytochrome P450 reductase and by recombinant cytosolic carbonyl reductase-1 now reveals that these compounds show a lack of specificity for these two enzymes in part. Thus, an assignment of the roles of different carbonyl reductases in metabolic keto reduction by the use of inhibitors is problematic. In addition, the ability of NADPH-cytochrome P450 reductase and carbonyl reductase-1 to reduce the ketone groups of the drugs haloperidol and daunorubicin was examined. Under the conditions applied, a pronounced reductive metabolism was only observed for daunorubicin in the presence of microsomal NADPH-cytochrome P450 reductase. Similarly, in rat liver S9 fractions a marked reduction of daunorubicin was seen, while haloperidol was only slightly metabolized to its alcohol. After separation of the S9 homogenate into a microsomal and a cytosolic fraction, it became evident that the ketone groups of daunorubicin, haloperidol and compound **5** were mainly reduced by cytosolic enzymes. However, since microsomes also catalysed these carbonyl reductions to some extent, it can be concluded that microsomal NADPH-cytochrome P450 reductase can contribute to metabolic keto reductions in xenobiotics. Copyright © 2015 John Wiley & Sons, Ltd.

Key words: NADPH-cytochrome P450 reductase; carbonyl reductase-1; haloperidol; daunorubicin; keto-reduction

Introduction

The most important metabolic pathway of xenobiotics is the oxidative biotransformation by cytochrome P450 (CYP) enzymes. However, reduction processes also play a significant role in phase I metabolism. For example, a variety of drugs bearing a carbonyl group such as haloperidol (**1**) and daunorubicin (**2**) are reduced to alcohols (**3** and **4**, respectively) (Figure 1) [1,2]. The reduction of aldehyde and ketone functions of xenobiotic compounds is thought to be catalysed mainly by two classes of enzymes: the short chain dehydrogenase/reductase (SDR) and the aldo-keto reductase (AKR) [3–5]. In addition, NADPH-cytochrome P-450 reductase was reported to be capable of reducing ketones to alcohols [6].

In past years, 1-heteroarylpropan-2-one derivatives were developed as inhibitors of cytosolic phospholipase $A_2\alpha$ and fatty acid amide hydrolase, two enzymes involved in inflammatory processes [7,8]. Because the carbonyl group of such

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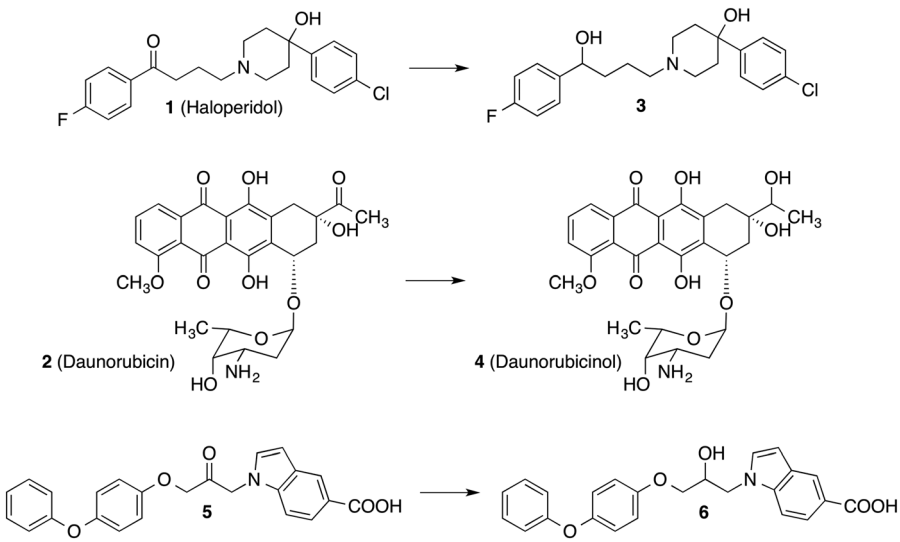

Figure 1. Metabolism of haloperidol, daunorubicin and 1-[3-(4-phenoxyphenoxy)-2- oxopropyl]indole-5-carboxylic acid (**5**) by carbonyl reducing enzymes

compounds is readily reduced to an alcohol in rat liver homogenate *in vitro* as well as in mice *in vivo* resulting in a loss of their inhibitory potency [7], our interest was focused on this metabolic reaction. Recently, it was found that in a mixture of human recombinant CYP enzymes (CYP1A2, 2C8, 2C9, 2C19, 2D6, 3A4) and NADPH-cytochrome P450 reductase, the latter-enzyme was responsible for the observed pronounced reduction of 1-[3-(4-phenoxyphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**5**) to 1-[2-hydroxy-3-(4-phenoxyphenoxy)propyl]indole-5-carboxylic acid (**6**) (Figure 1) [9]. In rat liver S9 fractions, compound **5** was even completely metabolized to the alcohol **6**. Here, cytosolic SDRs (cSDRs) are held mainly to be responsible for this reaction, because the keto-reduction could be inhibited by certain compounds, which were reported in the literature to be inhibitors of cSDR enzymes [10,11].

In the present study, first the specificity of these inhibitors was re-evaluated by measuring their effect on the transformation of compound **5** to compound **6** by NADPH-cytochrome P450 reductase and by one of the most important cSDRs, the carbonyl reductase-1. Furthermore, the study explored whether these two enzymes are able to reduce the carbonyl-bearing drugs haloperidol and daunorubicin to the corresponding alcohols.

Materials and Methods

Materials

Inhibitors and other reagents and solvents used were purchased from standard suppliers; microsomal NADPH-cytochrome P450 reductase was obtained from Corning Life Sciences (Amsterdam, The Netherlands; product no. 456244) and consisted of a microsomal preparation of insect cell-expressed human P450 reductase and human cytochrome b_5 in 0.5 ml 100 mM potassium phosphate buffer, pH 7.4; the protein content was 2.5 mg/0.5 ml, NADPH-cytochrome P450 reductase activity present in the preparation was 400 nmol/min/mg protein and cytochrome b_5 content was 750 pmol/mg protein. The preparation was used as delivered. Recombinant human carbonyl reductase-1 produced in *E. coli* (20 μg/20 μl) by ProSpec was purchased from Hoelzel Diagnostika (Cologne, Germany; product no. enz-415); the preparation (20 μl) was diluted with 480 μl of a 9:1 mixture of Tris-HCl buffer (20 mM, pH 8.5 at 20 °C) and glycerol prior to usage. The amount of NADPH-cytochrome P450 reductase and carbonyl reductase-1, respectively, used in the incubation procedures led to about a 60–70% conversion of compound **5** to its alcohol metabolite **6**.

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*Inhibition of NADPH-cytochrome P450 reductase- and carbonyl reductase-1-catalysed reduction of compound **5** by carbonyl reductase inhibitors, and metabolism of haloperidol and daunorubicin by these enzymes*

*Inhibition of NADPH-cytochrome P450 reductase-catalysed formation of the alcohol derivative **6** from the ketone derivative **5**.* To a 1.57 mM DMSO solution of compound **5** (2 μl) was added DMSO (0.5 μl) in the case of the controls or a DMSO solution of the appropriate inhibitor (see Table 1) (0.5 μl). After treatment with a 19.5:0.5 (v/v) mixture of phosphate buffered saline and 0.1 M aqueous $MgCl_2$ solution (92.5 μl), the NADPH-cytochrome P450 reductase preparation (5 μl) was added. Then the enzyme reactions were started by the addition of a 15 mM solution of NADPH in a 19.5:0.5 (v/v) mixture of phosphate buffered saline and 0.1 M aqueous $MgCl_2$ solution (25 μl). The final volume was 125 μl, the final concentration of compound **5** was 25 μM, and the final concentration of NADPH was 3 mM. Incubations were carried out at 37 °C for 1 h. Then the reactions were stopped by the addition of acetonitrile (250 μl). The samples were allowed to stand at room temperature for 10 min and then centrifuged at 2000 × *g* and room temperature for 5 min. For calculation of the relative amount of compound **5** metabolized, a mixture of a 1.57 mM DMSO solution of compound **5** (2 μl), DMSO (0.5 μl), phosphate buffered saline (117.5 μl) and the NADPH-cytochrome P450 reductase preparation (5 μl) was incubated in the same manner. The supernatants were analysed directly for metabolite formation by HPLC/UV-MS (for details see Supplementary Material).

Inhibition of carbonyl reductase-1-catalysed formation of compound **6** from compound **5** and metabolism of haloperidol (**1**) and daunorubicin (**2**) by NADPH-cytochrome P450 reductase and carbonyl reductase-1 was measured similarly (for details see Supplementary Material).

*Inhibition of the reduction of compound **5** in rat liver S9 fractions by carbonyl reductase inhibitors, and metabolism of haloperidol and daunorubicin by rat liver S9 fractions*

Inhibition of the reduction of compound **5** to compound **6** in rat liver S9 fractions by ethacrynic acid and diphenyleneiodonium chloride and metabolism of haloperidol and daunorubicin by rat liver S9 fractions was measured according to a published procedure [9] (for details see Supplementary Material).

*Metabolism of compound **5**, haloperidol and daunorubicin by rat liver microsomes and rat liver cytosol*.

Rat liver S9 fractions were separated into microsomes and cytosol by centrifugation at 100 000 × *g* for 60 min. Metabolism of compound **5**, haloperidol and daunorubicin by microsomes and by cytosol was measured in a similar way as for the experiments with S9 fractions [9] (for details see Supplementary Material).

Results and Discussion

Recently, it was shown that compound **5** can be reduced to compound **6** by a mixture of human

Table 1. Effect of different carbonyl reductase inhibitors on reduction of compound **5** to compound **6** by rat liver S9 fractions, human recombinant NADPH-cytochrome P450 reductase and human recombinant carbonyl reductase-1

		Inhibition of the formation of **6** from **5** (%)[a]		
Inhibitor	Conc. (μM)	Rat liver S9 fractions	NADPH-CYP450 reductase	Carbonyl reductase-1
Quercetin	200	39 ± 10	48 ± 3.2	87 ± 1.5
Menadione	200	41 ± 2.6	85 ± 1.2	n.s.[b]
Ethacrynic acid	1000	28 ± 7.5	64 ± 3.1	n.s.[b]
Alpha-lipoic acid	5000	7 ± 1.2	17 ± 8.2	75 ± 6.4
Diphenyleneiodonium chloride	20	n.s.[b]	n.s.[b]	n.s.[b]
Phenolphthaleine	20	n.s.[b]	13 ± 7.0	n.s.[b]
Flufenamic acid	20	n.s.[b]	n.s.[b]	n.s.[b]
18β-Glycyrrhetinic acid	2	n.s.[b]	n.s.[b]	n.s.[b]

[a]Values are means ± standard deviations of independent determinations (n = 3).
[b]n.s.: not significant.

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recombinant CYP enzymes as well as by rat liver S9 fractions [9]. In the microsomal CYP preparation the accessory protein NADPH-cytochrome P450 reductase, which catalyses electron transfer from the co-factor NADPH to the CPYs, was responsible for the carbonyl reduction. This was shown, among other things, by the observation that an excessive conversion of ketone **5** to the alcohol **6** took place in a preparation solely containing NADPH-cytochrom P450 reductase.

The enzymes contributing to the reductive transformation of compound **5** in rat liver S9 fractions were evaluated with the aid of enzyme inhibitors [9]. For the reduction of ketone functions of xenobiotics in the organism, mainly two classes of enzymes are thought to be responsible, namely the AKRs and the SDRs [3–5]. Therefore, the effect of published inhibitors of these enzymes [10,11] on the reduction of compound **5** was determined in this former study [9]. Since the AKR inhibitors flufenamic acid and phenolphthalein did not affect the formation of the alcohol **6** from ketone **5** (Table 1), a contribution of AKRs in this metabolic reaction was ruled out. Also, 18β-glycyrrhetinic acid, an inhibitor of microsomal SDRs (mSDRs) showed no inhibition at a concentration reported in the literature to be effective. In contrast, menadione and quercetin, which have been assigned as inhibitors of cytosolic forms of SDR (cSDR), significantly inhibited the reduction of the ketone group of compound **5**. Therefore, it was assumed that cSDRs play an important role in the reductive metabolism of compound **5** in rat liver S9 fractions. Although quercetin was reported to be an inhibitor of NADPH-cytochrome P450 reductase too [12], the primary role of this enzyme in the metabolism of compound **5** was excluded, because the NADPH-cytochrome P450 reductase inhibitor alpha-lipoic acid [13,14] only slightly inhibited the formation of the alcohol **6** in S9 fractions.

In the present study two more carbonyl reductase inhibitors were first tested for their effect on the metabolism of compound **5** in S9 fractions. The cSDR inhibitor ethacrynic acid [15] significantly blocked the reductive reaction, while the NADPH-cytochrome P450 reductase inhibitor diphenyleneiodonium chloride [16,17] did not show any inhibitory potency at a concentration reported to be sufficient to inhibit this enzyme effectively (Table 1). Thus, these results confirmed our previous findings.

Nevertheless, we wanted to repeat the inhibition experiments with pure carbonyl reductase-1, which is one of the main cSDRs in the organism [18] and NADPH-cytochrome P450 reductase. From both enzymes human recombinant forms are commercially available. First, it was verified that carbonyl reductase-1 is also able to reduce compound **5** to compound **6**. In the following inhibition experiments, the activities of the two enzymes were chosen in a way that about 60–70% of substrate **5** was converted to product **6**. The concentrations of the inhibitors were selected on the basis of inhibition data published in the literature [10,11]. The results obtained (Table 1) were very surprising. Quercetin, menadione and ethacrynic acid, which have been reported to be inhibitors of cSDRs, showed a pronounced inhibition of NADPH-cytochrome P450 reductase, while the cSDR carbonyl reductase-1 only was inhibited by quercetin and not by menadione and ethacrynic acid. Vice versa, the NADPH-cytochrome P450 reductase inhibitor alpha-lipoic acid inhibited carbonyl reductase-1 with a much higher potency than NADPH-cytochrome P450 reductase itself. For diphenyleneiodonium chloride, published to be an inhibitor of NADPH-cytochrome P450 reductase, neither an effect on this enzyme nor on carbonyl reductase-1 could be measured. The AKR inhibitor phenolphthaleine slightly affected NADPH-cytochrome P450 reductase, while the AKR inhibitor flufenamic acid showed no inhibitory effect on the formation of compound **6**. The mSDR inhibitor 18β-glycyrrhetinic acid also was ineffective. Taken together, these results indicate that it is difficult to assign the roles of carbonyl reducing enzymes with the aid of the published inhibitors, because these inhibitors lack specificity in part or do not have an effect on these enzymes at all.

Like compound **5**, haloperidol and daunorubicin are drugs with metabolically sensitive carbonyl groups. Enzymes that have actually been described to reduce daunorubicin at the C13 position to yield the hydroxy metabolite daunorubicinol (**4**) (Figure 1) are carbonyl reductase-1 [19–23], carbonyl reductase-3 [23] and certain AKRs [19,22,24–27]. Haloperidol has been reported to be reduced by AKRs with low affinity and low

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efficiency and by an unspecified carbonyl reductase with low affinity but high efficiency [19,28]. With purified NADPH-cytochrome P450 reductase and carbonyl reductase-1 to hand, we were also interested to what extent daunorubicin and haloperidol are reduced by these two enzymes.

The results are shown in Table 2. Under the conditions applied, about 60–70% of the propan-2-one **5** was converted to the alcohol **6** by both enzymes. NADPH-cytochrome P450 reductase only reduced a small amount of haloperidol (**1**) to its alcohol **3** as determined by HPLC/UV-MS studies, while it extensively metabolized daunorubicin (**2**) to the alcohol metabolite daunorubicinol (**4**) (for HPLC chromatograms see Supplementary Material). To our knowledge, a reduction of the keto group of daunorubicin by NADPH-cytochrome P450 reductase has not been reported before. After incubation of haloperidol with carbonyl reductase-1 under these conditions no carbonyl reduction was seen, and only a slight transformation of daunorubicin to its alcohol form occurred in the presence of this enzyme.

Finally, the rate of metabolism of haloperidol and daunorubicin in rat liver S9 fractions was studied. Only little metabolic transformation of haloperidol appeared in this environment. One minor metabolite found could be identified as the alcohol derivative **3**. In contrast, compound **5** and daunorubicin were extensively metabolized giving rise to one main metabolite in each case, which were identified by LC/MS as daunorubicinol (**4**) and the alcohol **6**, respectively (for HPLC-chromatograms see Supplementary Material). Similar results have been obtained in the experiments with recombinant microsomal NADPH-cytochrome P450 reductase described above. To clarify, whether the latter enzyme is the key player in the reduction of daunorubicin, haloperidol and compound **5** in rat liver homogenate, S9 fraction was separated into microsomes and cytosol by centrifugation at 100 000 × *g* and the substances were incubated with these preparations. While the protein content in the experiments with S9 fractions had been 2.0 mg/ml measured with the Bradford method, in the incubation solutions of the isolated microsomes and the cytosol it was 0.16 mg/ml and 1.7 mg/ml, respectively.

Interestingly, in the experiments with the microsomes a reduction of daunorubicin (**2**) to daunorubicinol (**4**) was not detected. Thus, it could be concluded that the activity of microsomal NADPH-cytochrome P450 reductase in this preparation is obviously much lower than that applied in the incubation solutions with the recombinant enzyme, and that the impact of this enzyme on metabolic reduction of daunorubicin could be overestimated when performing reduction studies solely with pure enzymes. In contrast, a high reduction rate of daunorubicin was seen in the cytosolic preparation. As for the S9 fractions, about 70–80% of this compound was transformed to daunorubicinol. Compound **5** was quantitatively metabolized in the cytosol resulting in high concentrations of alcohol **6**. In the microsomal preparation compound **5** was reduced too, but only by 10%. In this context it has to be mentioned that in earlier experiments it was found that this compound was nearly quantitatively transformed to

Table 2. Metabolism of 1-heteroarylpropan-2-one **5**, haloperidol and daunorubicin by rat liver S9 fractions, rat liver microsomes, rat liver cytosol, human recombinant NADPH-cytochrome P450 reductase, and human recombinant carbonyl reductase-1

Compound	Metabolic stability (%)[a]				
	Rat liver			NADPH-CYP450 reductase (microsomal)	Carbonyl reductase-1 (cytosolic)
	S9 fractions	Microsomes	Cytosol		
Compound **5**	0	89 ± 2.6	0	36 ± 2.9	30 ± 4.9
Haloperidol (**1**)	84 ± 6.7	90 ± 2.6	91 ± 6.0	90 ± 8.0	100
Daunorubicin (**2**)	28 ± 4.0	96 ± 4.2	21 ± 1.2	23 ± 4.2	95 ± 3.2

[a]Percentage of parent compound remaining after incubation in the presence of the co-factor NADPH; means ± standard deviations, $n = 3$, in case of the experiments with rat liver S9 fractions $n = 6$.
Main metabolites determined by HPLC/UV-MS: alcohol derivative **6** in case of compound **5**, alcohol derivative **3** in case of haloperidol, daunorubicinol (**4**) in case of daunorubicin.

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its alcohol **6** by rat liver microsomes. This can be explained by the fact that in these experiments [7] the concentration of the microsomes was about 14-fold higher than in the present case. Under the conditions applied, haloperidol was metabolized by S9 fractions, isolated microsomes as well as cytosol only to a minor extent. However, in all preparations small amounts of the reduced form of haloperidol (**3**) could be detected by LC/MS. Similar to the results obtained with compound **5**, the amount of the alcohol **3** was about 10-fold higher in the incubations performed with S9 fractions and cytosol, respectively, than in that with the microsomes.

Taken together it was shown that the metabolic reduction of the ketone group of haloperidol, daunorubicin and compound **5** in liver homogenate is predominantly catalysed by cytosolic enzymes. Nevertheless, since the microsomes also catalyse this reaction in the case of haloperidol and compound **5** to some extent, it can be concluded that microsomal NADPH-cytochrome P450 reductase can also contribute to metabolic keto reduction of xenobiotics in principal.

Conclusions

The results obtained in the inhibition experiments indicate that many of the chemical inhibitors used to ascertain the roles of different carbonyl reductases in metabolic processes lack specificity for these enzymes. Furthermore, the investigations on the metabolic reduction of the ketone groups of haloperidol (**1**), daunorubicin (**3**) and the propan-2-one derivative **5** by recombinant microsomal NADPH-cytochrome P450 reductase, recombinant cytosolic carbonyl reductase-1, rat liver S9 fractions as well as its microsomal and cytosolic fractions indicate that the ketone groups of daunorubicin, haloperidol and compound **5** are mainly reduced by cytosolic enzymes. However, since microsomes also catalysed these carbonyl reductions at least to some extent, it can be concluded that microsomal NADPH-cytochrome P450 reductase can contribute to metabolic keto reductions in xenobiotics. Further studies should be performed to clarify the roles of the different carbonyl reducing enzymes for the reductive metabolism of ketone compounds.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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Supporting information

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