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Comparison of various aryl-dithiolethiones and aryl-dithiolones as hydrogen sulfide, H_2S, donors in the presence of rat liver microsomes

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Running title: Dithiolethiones and dithiolones as H_2S donors

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Introduction: 336 words

Results and Discussion: 1788 words

Abbreviations

ADT, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione; ADO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-one; ADOSO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-one-1-sulfoxide; ADTSO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione-sulfoxide; β-ME, β-mercapto-ethanol; Bz-ImH,

N-benzyl-imidazole; CYP, cytochrome P450; Cys, L-cysteine; dmADT, 5-(p-hydroxyphenyl)-3H-1,2-dithiole-3-thione; DPD, *N,N*-dimethylamino-*para*-phenylene-diamine; DO, dithiolone; DT, dithiolethione; DTT, dithio-threitol; GSH, glutathione; NAC, *N*-acetyl-L-cysteine; PB, Phenobarbital; pMA, para-methoxy-acetophenone.

Abstract

It has been reported that microsomal metabolism of ADT (anetholedithiolethione, Sulfarlem) and ADO (anetholedithiolone) led to formation of H_2S mainly derived from oxidations catalyzed by cytochrome P450 (CYP)-dependent monooxygenases, and that ADO was a better H_2S-donor than ADT under these conditions. This article compares the H_2S-donor abilities of 18 dithiolethione and dithiolone analogs of ADT and ADO upon incubation with rat liver microsomes. It shows that, for all the studied compounds, maximal H_2S formation was obtained after incubation with microsomes and NADPH, and that this formation greatly decreased in the presence of *N*-benzyl imidazole (Bz-ImH), a known inhibitor of CYP. This indicates that H_2S formation from all the studied compounds requires, as previously observed in the case of ADT and ADO, oxidations catalyzed by CYP-dependent monooxygenases. Under these conditions, the studied dithiolones were almost always better H_2S-donors than the corresponding dithiolethiones. Interestingly, the best H_2S yields (up to 75%) were observed in microsomal oxidation of ADO and its close analogs, pCl-Ph-DO and Ph-DO, in the presence of glutathione (GSH), whereas only small amounts of H_2S were formed in microsomal incubations of those compounds with GSH but in the absence of NADPH. A possible mechanism for this effect of GSH is proposed on the basis of results obtained from reactions of GSH with ADOSO (ADO sulfoxide), the ADO metabolite involved in H_2S formation in microsomal oxidation of ADO.

Significance Statement

A series of 18 dithiolethiones and dithiolones was compared for their ability to form hydrogen sulphide (H_2S) in oxidations catalyzed by microsomal monooxygenases. The studied dithiolones were better H_2S-donors than the corresponding dithiolethiones and the addition of glutathione to the incubations strongly increased H_2S formation. A possible mechanism for this effect of GSH is proposed on the basis of results obtained from reactions of GSH with 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-one-1-sulfoxide, a metabolite of the choleretic and sialologic drug Sulfarlem.

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Introduction

Anetholedithiolethione (ADT, Sulfarlem, Figure 1) is a well known choleretic and sialologic drug (Christen. 1995; Nagano et al., 2001). Its O-demethylated derivative dmADT, and many compounds resulting from the coupling of dmADT with various anti-inflammatory drugs have been extensively described for their H_2S-donor properties and therapeutic effects (Chen et al., 2010; Couto et al., 2015; Sparatore et al., 2009; Sparatore et al., 2011; Kashfi and Olson, 2013; Ansari et al., 2018; Powell et al., 2018). Only very small amounts of H_2S are formed upon incubation of ADT with rat or human liver microsomes in the absence of NADPH, whereas much greater amounts of H_2S are formed upon incubation with liver microsomes containing NADPH and O_2, the two cofactors required for microsomal monooxygenases (Dulac et al., 2019). Microsomal oxidation of ADT leads to the concomitant formation of H_2S and *para*-methoxy-acetophenone (pMA) (Dulac et al., 2019), and to other metabolites such as dmADT and products resulting from an ADT S-oxidation such as anetholedithiolone, ADO and ADTSO (Fig. 1) (Dulac et al., 2018). Interestingly, ADO was found as a much better H_2S donor than ADT under these conditions of microsomal oxidative metabolism (Dulac et al., 2019). Moreover, a detailed mechanism for H_2S formation upon microsomal oxidation of ADT and ADO, implying the intermediate formation of ADOSO (Fig. 1) that results from an S-oxidation of ADO, was proposed (Dulac et al., 2019).

In this article, one compares the abilities of several dithiolethione (DT) and dithiolone (DO) analogs of ADT and ADO to act as H_2S donors in the presence of NADPH-containing liver microsomes, and shows that, in a general manner, dithiolones are better H_2S donors than dithiolethiones under these conditions. The greatest amounts of H_2S were obtained upon microsomal oxidation of close analogs of ADO, and the presence of glutathione (GSH) led to a great increase of H_2S formation (yields based on starting dithiolone up to 75%). A possible

mechanism for this GSH effects is proposed on the basis of experiments performed between ADOSO, the S-oxide of ADO, with GSH.

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Materials and Methods

General reagents, authentic samples and proteins

The commercial origins of ADT, pMA, NADPH, Bz-ImH and the solvents used were indicated previously (Dulac et al., 2018). Naproxene, Aspirine (sodium salts), *N.N*-dimethyl-para-phenylenediamine sulfate (DPD) and Oltipraz were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Authentic metabolites of ADT, ADO, dmADT and dmADO, were prepared as described previously (Dulac et al., 2018). ADOSO was prepared from ADO following modifications of a previously reported protocol (Tardif and Harp, 2000; Dulac et al., 2019). NMR data were obtained from a Bruker 500 AV2 spectrometer. Mass spectra were recorded on an Exactive HRMS-instrument (Thermo, Les Ulis, France). Preparation of liver microsomes from phenobarbital (PB)-pretreated rats, measurements of protein concentrations and CYP contents were made as reported previously (Dulac et al., 2019).

Chemistry

Dithiolethiones were obtained by treatment of the corresponding β-keto-esters with Lawesson's reagent and elemental sulfur in anhydrous toluene following a previously described method (Pedersen and Lawesson, 1979; Biard et al., 1992).

5-(Phenyl)-3H-1,2-dithiole-3-thione (**Ph-DT**). ^{1}H NMR (d_6-acetone) 7.90 (d, 2H, J = 8.0), 7.65 (t, 1H, J = 8.0), 7.61 (s, 1H), 7.58 (m, 2H); HRMS (ESI$^+$): *m/z* value for $C_9H_6S_3 + H^+$ 210.9711, found 210.9704.

5-(4-Chlorophenyl)-3H-1,2-dithiole-3-thione (**p-Cl-Ph-DT**). ^{1}H NMR (d_6-acetone) 7.93 (d, 2H, *J* = 8.0), 7.62 (d, 1H, *J* = 8.0), 7.61 (s, 1H); HRMS (ESI$^+$): *m/z* value for $C_9H_5ClS_3 + H^+$ 244.9321, found 244.9662.

5-(2-Chlorophenyl)-3H-1,2-dithiole-3-thione (**o-Cl-Ph-DT**). ^{1}H NMR (d_6-acetone) 7.77 (dd, 1H, *J* = 8.0 and 1.5), 7.67 (d, 1H, *J* = 8.0), 7.61 (td, 1H, *J* = 7.5 and 1.5), 7.54 (t, 1H, *J* = 8.0), 7.37 (s, 1H); HRMS (ESI$^+$): *m/z* value for $C_9H_5ClS_3 + H^+$ 244.9321, found 244.9458.

5-(3-Hydroxy-4-methoxy-phenyl)-3H-1,2-dithiole-3-thione (**o-OH-ADT**). ^{1}H NMR (d_6-acetone) 7.56 (s, 1H), 7.47 (d, 1H, *J* = 2.0), 7.42 (d, 1H, *J* = 8.0), 6.98 (d, 1H, *J* = 8.0), 3.90 (s, 3H); HRMS (ESI$^+$): *m/z* value for $C_{10}H_8O_2S_3 + H^+$ 256.9765, found 256.9755.

Naproxene-dmADT (ATB345) and aspirine-dmADT (ACS 14) were prepared as previously described (Wallace et al., 2007) by reaction of naproxene or acetylsalicylic acid with dmADT in the presence of hydroxybenzotriazole and di-*cyclo*-hexyl-carbodiimide in dry *N,N*-dimethylformamide.

4-(5-Thioxo-5*H*-[1,2]dithiol-3-yl)phenyl-2-(2-methoxynaphthaleny-6-yl)propanoate (**Naproxene-dmADT**). ^{1}H NMR (d_6-acetone) 7.88 (d, 2H, *J* = 8.5), 7.85-7.82 (m, 3H), 7.55 (s, 1H), 7.53 (d, 1H, *J* = 1.5), 7.31 (d, 1H, *J* = 3.0), 7.24 (d, 2H, *J* = 8.5), 7.17 (dd, 1H, *J* = 9.0 and 2.5), 4.24 (q, 1H, *J* = 7.0), 3.91 (s, 3H), 1.96 (d, 3H, *J* = 7.0); HRMS (ESI$^+$): *m/z* value for $C_{23}H_{18}O_3S_3 + H^+$ 439.0497, found 439.0681.

4-(5-Thioxo-5H-1,2-dithiol-3-yl)phenyl-2-acetoxybenzoate (**Aspirine-dmADT**). ^{1}H NMR (d_6-acetone) 8.24 (dd, 1H, *J* = 7.5 and 2.0), (8.03 d, 2H, *J* = 9.0), 7.78 (t, 1H, *J* = 7.5), 7.64 (s, 1H), 7.49 (m, 3H), 7.32 (d, 1H, J = 8.0), 2.32 (s, 3H); HRMS (ESI$^-$): *m/z* value for $C_{18}H_{12}O_4S_3 - H^+$ 386.9819, found 386.9819.

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Dithiolones were obtained by treatment for 24 h at room temperature of the corresponding dithiolethiones with mercuric acetate in a dichloromethane and acetic acid mixture following a previously described method (Klingsberg, 1972).

5-Phenyl-3H-1,2-dithiole-3-one (**Ph-DO**): ^{1}H NMR (*d*$_6$-acetone): 7.83 (d, 2H, *J* = 7.0), 7.61 (m, 1H), 7.56 (m, 2H), 7.10 (s, 1H); HRMS (ESI$^+$): *m/z* value for $C_9H_6OS_2 + H^+$ 194.9939, found 194.9964.

5-(4-Chlorophenyl)-3H-1,2-dithiole-3-one (**p-Cl-Ph-DO**): ^{1}H NMR (*d*$_6$-acetone) 7.85 (d, 2H, *J* = 8.5), 7.61 (d, 2H, *J* = 8.5), 7.13 (s, 1H); HRMS (ESI$^+$): *m/z* value for $C_9H_6ClOS_2$ 228.9549, found 228.9581.

5-(2-Chlorophenyl)-3H-1,2-dithiole-3-one (**o-Cl-Ph-DO**): ^{1}H NMR ($CDCl_3$) 7.51 (d, 2H, *J* = 8.5), 7.43 (t, 1H, *J* = 8.5), 7.36 (t, 1H, *J* = 8.0), 6.77 (s, 1H); HRMS (ESI$^+$): *m/z* value for $C_9H_5ClOS_2 + H^+$ 228.9549, found 228.9580.

5-(3-Hydroxy-4-methoxy-phenyl)-3H-1,2-dithiole-3-one (**o-OH-ADO**): ^{1}H NMR ($CDCl_3$) 7.18 (dd, 1H, *J* = 8.5 and 2.0), 7.05 (d, 1H, *J* = 2.0), 6.97 (d, 1H, *J* = 8.5), 6.73 (s, 1H), 6.00 (s, 1H), 3.94 (s, 3H); HRMS (ESI$^-$): *m/z* value for $C_{10}H_8O_3S_2 - H^+$ 238.9836, found 238.9834.

4-Methyl-5-pyrazinyl-3H-1,2-dithiole-3-one (**Oltipraz-DO**) (O'Dwyer et al., 1997; Ko et al., 2006): ^{1}H NMR ($CDCl_3$) 8.98 (s, 1H), 8.69 (d, 2H, *J* = 7.5), 2.32 (s, 3H); HRMS (ESI$^+$): *m/z* value for $C_8H_7ON_2S_2$ 211.0001, found 210.9991.

Naproxene-dmADO and Aspirine-dmADO were obtained by reaction of naproxene or acetylsalicylic acid with dmADO in the presence of hydroxybenzotriazole and di-*cyclo*-hexyl-

carbodiimide in dry *N,N*-dimethylformamide following a described protocol (Wallace et al., 2007).

4-(5-Oxo-5*H*-[1,2]dithiol-3-yl)phenyl-2-(2-methoxynaphthaleny-6-yl)propanoate (**Naproxene-dmADO**). ^{1}H NMR (d_6-acetone); 7.85-7.82 (m, 3H), 7.68 (d, 2H, *J* = 8.5), 7.54 (dd, 1H, *J* = 8.5 and 2.0), 7.30 (d, 1H, *J* = 2.5), 7.22 (d, 2H, *J* = 8.5), 7.17 (dd, 1H, *J* = 9.0 and 2.5), 7.04 (s, 1H), 4.23 (q, 1H, *J* = 7.0), 3.90 (s, 3H), 1.66 (d, 3H, *J* = 7.0); HRMS (ESI$^+$): *m/z* value for $C_{23}H_{18}O_4S_2 + H^+$ 423.0726, found 423.0786.

4-(5-Oxo-*5H*-1,2-dithiol-3-yl)phenyl-2-acetoxybenzoate (**Aspirine-dmADO**) ^{1}H NMR (d_6-acetone) 8.22 (dd, 1H, *J* = 7.5 and 1.5), 7.93 (d, 2H, *J* = 8.0), 7.78 (t, 1H, *J* = 7.5), 7.49 (m, 1H), 7.46 (d, 2H, *J* = 8.0), 7.30 (d, 1H, *J* = 7.5), 7.12 (s, 1H), 2.26 (s, 3H); HRMS (ESI$^-$): *m/z* value for $C_{18}H_{12}O_5S_2 - H^+$ 371.0047, found 371.0045.

Typical microsomal incubation procedures with measurement of H_2S formation

Hydrogen sulfide formation was measured using the methylene blue method (Fogo and Popowsky, 1949; Giustarini et al., 2014) using microsomal incubation procedures previously described (Dulac et al. 2019). HPLC-HRMS analyses of metabolites from incubations of ADO and ADOSO were performed as previously described (Dulac et al., 2018).

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Results and Discussion

Formation of H_2S upon incubation of various dithiolethione analogs of ADT

The formulae of the compounds used in this study are shown in Figure 2. This includes Oltipraz, a drug available for treatment of schistosomiasis and also tested as a cancer chemopreventive agent (Bueding et al., 1982; Rao et al., 1993; O'Dwyer et al., 1997; Ko et al., 2006), close analogs of ADT, such as Ph-DT, p-Cl-Ph-DT, o-Cl-Ph-DT, dmADT, o-OH-ADT, and compounds resulting from acylation of the OH group of dmADT by anti-inflammatory drugs, Naproxene-dmADT, and Aspirine-dmADT. The H_2S-donor properties of these compounds were compared to those of the corresponding dithiolone derivatives in which the C=S function is replaced with a C=O function. They are indicated in Table 2 by abbreviations similar to those used for the corresponding dithiolethiones (Table 1 and Figure 2) in which DT was replaced with DO. The origin or synthesis of all these compounds is described in Materials and Methods.

Formation of H_2S was measured by using the methylene blue method (Giustarini et al., 2014) during incubation of the DT and DO compounds with liver microsomes from PB-pretreated rats. As previously reported (Dulac et al., 2019), incubation of 100 µM ADT with those liver microsomes in the presence of 1mM NADPH led to H_2S in a yield of about 12% after 1h (Table 1). Without NADPH or in the presence of 1mM Bz-ImH, a known inhibitor of CYP-dependent monooxygenases (Testa and Jenner, 1981; Correia and Ortiz de Montellano, 2005), only low amounts of H_2S were formed (Table 1, Dulac et al., 2019). Table 1 also shows that the eight ADT analogs used in this study are generally less efficient H_2S-donors than ADT after microsomal metabolism, if one excepts o-OH-ADT that gave similar H_2S yields. Moreover, for most of these compounds one observed, as in the case of ADT, a decrease of H_2S formation

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after incubation without NADPH or in the presence of Bz-ImH. These results indicate that microsomal H_2S formation from the studied dithiolethiones would mainly derive, as in the case of ADT (Dulac et al., 2019), from an oxidation catalyzed by CYP-dependent monooxygenases.

When incubation of ADT with NADPH supplemented microsomes was performed under identical conditions but in the presence of 1 mM glutathione, one observed a decrease of H_2S yield (Table 1). Microsomal incubations in the presence of GSH of the other DT indicated in Table 1 led to H_2S yields either slightly higher or lower than that found in the case of ADT (Table 1).

The above data show that the eight ADT analogs used in this study are generally less efficient H_2S-donors than ADT after microsomal metabolism, if one excepts o-OH-ADT that gave similar H_2S yields (Table 1). The first step of the microsomal metabolism of ADT leading to H_2S formation seems to be the S-oxidation of the ADT C=S group (Dulac et al., 2018). This S-oxidation is in competition with the CYP-dependent oxidative demethylation of ADT leading to dmADT (Dulac et al., 2018). The lower yields of H_2S formation observed with most other DT could be due either to competitive microsomal oxidations more efficient than the ADT oxidative demethylation, or to less efficient C=S S-oxidation compared to ADT. In that context, we have performed preliminary HPLC-MS studies of microsomal oxidations of two other DTs mentioned in Table 1: dmADT, and o-OH-ADT. In the case of dmADT, a metabolite characterized by a molecular ion at *m/z* = 242.9596, that should result from the hydroxylation of the phenyl ring of dmADT, was detected (*m/z* calculated for $C_9H_7O_2S_3$ (dmADT + O) + H^+) = 242.9609). Finally, in microsomal oxidation of o-OH-ADT, a metabolite characterized by a molecular ion at *m/z* 242.9595 was found and should result from an oxidative demethylation of o-OH-ADT (*m/z* calculated for $C_9H_7O_2S_3$ (o-OH-ADT - CH_2) + H^+ = 242.9609). Other kinds of reactions are occurring in competition with the S-oxidation of the C=S function of Aspirine-

ADT, Naproxene-ADT and Oltipraz. In the particular case of Aspirine-ADT and Naproxene-ADT, we found that incubation of these compounds with liver microsomes led to dmADT that should result from the hydrolysis of their ester function by microsomal esterases. In the case of Oltipraz, literature data reported that several metabolites not resulting from the S-oxidation of its C=S group are formed in high amounts (Bieder et al., 1983).

Formation of H_2S upon incubation of various dithiolone analogs of ADO

As reported previously, incubation of ADO with NADPH-supplemented liver microsomes under the above described conditions led to H_2S yields higher than those observed with ADT (Table 2 and Dulac et al., 2019). Without NADPH or in the presence of 1mM Bz-ImH, the H_2S yields were very low which confirmed that H_2S formation is mainly catalyzed by CYP-dependent monooxygenases (Table 2 and Dulac et al., 2019). Similar or even higher H_2S yields (22 to 36%) were observed after microsomal metabolism of Ph-DO, p-Cl-Ph-DO and o-Cl-Ph-DO (Table 2). On the contrary, o-OH-ADO, Oltipraz-DO, Naproxene-dmADO and Aspirine-dmADO led to lower H_2S yields (about 10%). Moreover, all studied DO (except o-OH-ADO) led to higher H_2S yields than the corresponding DT (compare Tables 1 and 2). The H_2S yields are 2.5 to 4.8 fold increased in the case of ADO, Ph-DO, p-Cl-Ph-DO and o-Cl-Ph-DO, but only 1.5 to 2.0 fold increased in the case of Oltipraz-DO, Naproxene-dmADO and Aspirine-dmADO). In a general manner, in incubations performed without NADPH or in the presence of Bz-ImH, all studied DO led to low H_2S yields. This indicates that H_2S formation from these DO mainly depends on CYP-dependent monooxygenases. This would be in agreement with the mechanism previously proposed for H_2S formation from microsomal oxidation of ADO that would involve an S-oxidation leading to ADOSO (Figure 1) as the first step (Dulac et al., 2019).

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Table 2 also shows that the presence of 1 mM GSH in microsomal incubation of ADO and the other DO led, in many cases, to an increase in the H_2S yields. This increase is particularly important in the case of ADO, p-Cl-Ph-DO, o-Cl-Ph-DO and Ph-DO, as incubation of Ph-DO with NADPH- and GSH-supplemented microsomes led to H_2S in a 75% yield. It is noteworthy that such high H_2S yields were not observed upon incubation of the DO with GSH alone or with GSH and liver microsomes without NADPH (yields observed with ADO under those conditions were 2.0 ± 0.8 and 3.0 ± 1.0 %. respectively).

The mechanism previously proposed for H_2S formation upon microsomal oxidation of ADO (Dulac et al., 2019) involves an S-oxidation of ADO with formation of ADOSO, as a first step (Figure 3). The next steps would be the opening of ADOSO by H_2O leading to intermediate **1**, the hydrolysis of the HSC=O function of **1** leading to H_2S and **2**, and the decarboxylation of **2** leading to the disulfoxide of *para*-methoxy-thioacetophenone **3**. Finally, monooxygenations of **3** would lead to pMA and HSO_4^-, as previously found in microsomal oxidation of compounds involving a C=S bond (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vannelli et al., 2002; Testa and Kramer, 2007). The same mechanism could be at the origin of H_2S formation upon microsomal metabolism of the other studied DO. In the presence of GSH, a nucleophilic attack of GSH on the S=O group of ADOSO with opening of the SO-S bond leading to intermediate **4** could occur. Hydrolysis of the HSC=O function of **4** should lead to H_2S (Figure 4). One could expect that the opening of the O=S-S bond of ADOSO by GSH would be faster than the opening of this bond by H_2O, a much weaker nucleophile.

H_2S formation in reaction of ADOSO with GSH

In order to better understand the effects of GSH on H_2S formation upon ADO microsomal oxidation, we have studied the reaction of ADOSO with GSH and found that incubation of 100

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μM ADOSO with 250 μM GSH in phosphate buffer pH 7.4, 1h at 37°C, led to H_2S in a yield of about 95% based on starting ADOSO (Table 3), whereas incubations of ADOSO alone in the same buffer or incubations of ADO with GSH under identical conditions only led to H_2S yields lower than 3% (Table 3). Figure 5 shows that the H_2S yields observed after reaction of ADOSO with increasing concentrations of GSH (from 100 μM to 1 mM) increased from 30 to about 200%. Other thiols (all at 250 μM), such as β-mercapto-ethanol (β-ME), dithiothreitol (DTT), L-cysteine (Cys), or N-acetyl-L-cysteine (NAC) reacted with ADOSO with formation of H_2S in yields between 70 and 130% (Table 3). Those very high H_2S yields, often higher than 100%, obtained upon reaction of ADOSO with thiols indicated that more than one sulfur atom of ADOSO was converted to H_2S. A possible explanation for those results is shown in Figure 4 in which intermediate **5**, resulting from the opening of ADOSO by GSH and hydrolysis of the HSC=O function of **4**, is reduced by GSH with formation of **6** and intermediate **7**. Decarboxylation of **7** should lead to *para*-methoxy-thioacetophenone **8**. Finally, reaction of GSH with **8** would lead to H_2S and pMA (Figure 4). In agreement with this hypothesis, an HPLC-MS study of the reaction of 100 μM ADOSO with 250 μM GSH in phosphate buffer pH 7.4 (1 h at 37°C) showed the formation of pMA in a 84% yield (data not shown).

Those reactions between ADOSO and GSH could occur in microsomal incubations of ADO in the presence of NADPH and GSH, and this could explain the increase of the H_2S yields observed when GSH is added to microsomal incubations. However, in such microsomal incubations, the formation of ADOSO by monooxygenation of ADO is in competition with other oxidations of ADO such as its oxidative O-demethylation (Dulac et al., 2018). Moreover, in such incubations of ADO with NADPH-supplemented microsomes in the presence of GSH, reactions of intermediates **5**, **6** and **8** with GSH leading to H_2S (Figure 4) should be in competition with monooxygenation of those intermediates that would eventually lead to HSO_4^-

as in the case of **3** (Figure 3). The occurrence of those competitive reactions in microsomal incubations of ADO should explain the much lower H_2S yields observed (Table 2) when compared to those found in reactions of ADOSO with GSH in the absence of microsomes (Table 3 and Figure 5).

The mechanisms proposed for H_2S formation from ADO in Figures 3 and 4 should be operative for H_2S formation from the other studied DO. Accordingly, the results concerning Ph-DO, o-Cl-Ph-DO and p-Cl-Ph-DO were very similar to those observed with ADO (Table 2). The much lower H_2S yields obtained in microsomal oxidation of dmADO, o-OH-ADO, Oltipraz-DO, Naproxene-dmADO, and Aspirine-dmADO (Table 2) could be related to monooxygenations of those molecules occurring at positions different from the S atom involved in the formation of ADOSO from ADO.

Conclusion

This article compares the H_2S-donor abilities of a series of 18 dithiolethione or dithiolone analogs of ADT or ADO incubated with NADPH-supplemented rat liver microsomes. It shows that only minor amounts of H_2S are formed in incubations performed without NADPH or with NADPH and Bz-ImH, which indicates that H_2S formation requires oxidations catalyzed by CYP-dependent monooxygenases. With NADPH-supplemented microsomes, the best DT H_2S-donors were ADT itself and o-OH-ADT, and the studied DO were almost always better H_2S-donors than the corresponding DT. The presence of GSH in microsomal incubations had only minor effects on the H_2S yields observed with DT. However, it led to a great increase of the H_2S yields in microsomal incubations of ADO and its close analogs, o-Cl-Ph-DO, p-Cl-Ph-DO and Ph-DO, the best H_2S yield (75%) being obtained in the case of Ph-DO. A possible mechanism for this effect of GSH is proposed on the basis of a study of reactions of GSH with

ADOSO, the ADO metabolite involved in H_2S formation in microsomal oxidation of ADO. Since GSH is present in most cells, one should expect that DO related to ADO act as particularly good H_2S-donors in *in vivo* situations.

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Authorship contributions

Participated in research design: Dansette, Boucher, Mansuy.

Conducted experiments: Dali, Boucher.

Contributed new reagents or analysis tools: Dali, Boucher, Dansette.

Performed data analysis: Dali, Boucher, Dansette, Mansuy.

Wrote or contributing to the writing of the manuscript: Mansuy, Boucher, Dansette.

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Figure Legends

Figure 1: Formulae of ADT and some of its metabolites formed upon microsomal oxidation.

Figure 2: Formulae of the dithiolethiones used in this study.

Figure 3: Mechanism previously proposed for H_2S formation upon metabolism of ADO by NADPH-supplemented liver microsomes (Dulac et al., 2019). Ar = p-methoxyphenyl. +"O" on some arrows indicates that the corresponding step involves a monooxygenation of the substrate.

Figure 4: Possible mechanism for the formation of H_2S upon reaction of ADOSO with GSH.

Figure 5: Representative curve showing the H_2S yields obtained after the reaction of 100 µM ADOSO with increasing GSH concentrations in 50 mM phosphate buffer pH 7.4. ADOSO (100 µM) in 50 mM phosphate buffer pH 7.4 was incubated for 1 h at 37°C in the presence of increasing concentrations of GSH and H_2S formation was measured as indicated in Materials and Methods. Data are means $\pm$ S.D. from 3 experiments.

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Table 1: H_2S formation upon metabolism of various dithiolethione analogs of ADT by liver microsomes from PB-treated rats.

Dithiolthiones	C.S. [a]	C.S. -NADPH [b]	C.S. + Bz-ImH [c]	C.S. + GSH [d]
Ph-DT	7.5 ± 2.7	2.0 ± 0.2	3.6 ± 1.1	8.5 ± 1.0
ADT	12.0 ± 3.5	3.1 ± 1.3	2.9 ± 1.0	6.4 ± 1.6
dmADT	2.5 ± 1.0	2.9 ± 1.7	2.5 ± 1.9	5.5 ± 1.8
p-Cl-Ph-DT	7.0 ± 1.1	2.4 ± 2.3	2.0 ± 0.8	3.3 ± 1.3
o-Cl-Ph-DT	6.0 ± 0.3	1.2 ± 0.3	1.5 ± 1.0	1.8 ± 0.8
o-OH-ADT	12.4 ± 2.5	3.5 ± 1.5	3.0 ± 0.8	9.3 ± 1.0
Oltipraz	5.7 ± 3.0	1.5 ± 0.5	1.9 ± 1.4	1.5 ± 1.7
Naproxene-dmADT	6.3 ± 0.7	2.3 ± 1.8	4.5 ± 1.0	4.7 ± 0.7
Aspirine-dmADT	5.1 ± 1.0	4.5 ± 0.7	2.2 ± 1.1	7.4 ± 3.1

[a] C.S.: Complete system. H_2S yields (%) were measured after 1h incubation of 100 µM dithiolethione in phosphate buffer, pH 7.4 in the presence of liver microsomes (~2.5 µM CYP) and 1 mM NADPH, as described in Materials and Methods. Data are yields based on starting compound and are means ± S.D. from 3 to 8 experiments. [b] Identical conditions but in the absence of NADPH. [c] Identical conditions but 1 mM Bz-ImH was added in the solution. [d] Identical conditions but in the presence of 1 mM GSH.

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Table 2: H_2S formation upon metabolism of various dithiolone analogs of ADO by liver microsomes from PB-treated rats. Abbreviations used for the dithiolones are similar to those used for the corresponding dithiolethiones (Table 1 and Figure 2) in which DT was replaced with DO.

Dithiolones	C.S. [a]	C.S. – NADPH [b]	C.S. + Bz-ImH [c]	C.S. + GSH [d]
Ph-DO	36.5 ± 1.0	1.5 ± 0.1	3.2 ± 2.0	74.9 ± 0.4
ADO	31.1 ± 4.6	3.1 ± 2.1	4.1 ± 1.7	44.2 ± 5.1
dmADO	11.4 ± 1.2	3.3 ± 0.5	4.6 ± 2.0	21.4 ± 1.6
p-Cl-Ph-DO	29.8 ± 3.0	1.4 ± 0.3	3.7 ± 0.4	45.5 ± 3.3
o-Cl-Ph-DO	21.7 ± 5.9	1.2 ± 1.7	2.9 ± 2.1	41.2 ± 2.8
o-OH-ADO	8.9 ± 0.1	3.9 ± 1.6	1.4 ± 0.2	4.7 ± 1.8
Oltipraz-DO	10.9 ± 3.3	0.9 ± 1.1	2.9 ± 3.2	9.8 ± 1.1
Naproxene-dmADO	9.9 ± 1.3	2.6 ± 0.2	3.6 ± 0.6	11.8 ± 0.5
Aspirine-dmADO	9.7 ± 0.1	2.6 ± 0.8	4.7 ± 0.4	14.7 ± 0.6

[a] C.S.: Complete system. H_2S yields (%) were measured after 1 h incubation of 100 µM dithiolone in phosphate buffer, pH 7.4, in the presence of liver microsomes (~2.5 µM CYP) and 1 mM NADPH, as described in Materials and Methods. Data are yields based on starting compound and are means ± S.D. from 3 to 8 experiments. [b] Identical conditions but in the absence of NADPH. [c] Identical conditions but 1mM Bz-ImH was added in the solution. [d] Identical conditions but in the presence of 1 mM GSH.

Table 3. Formation of H_2S upon reaction of ADOSO with GSH or other thiols.

Conditions [a]	**H_2S yield (%)**
ADOSO + buffer alone	2.6 ± 0.8
ADOSO + GSH	93.6 ± 10.1
ADOSO + β-ME	114.2 ± 15.8
ADOSO + DTT	128.2 ± 12.8
ADOSO + Cys	97.6 ± 12.8
ADOSO + NAC	73.8 ± 11.0

[a] ADOSO (100 µM) was incubated for 1 h at 37°C in 50 mM phosphate buffer pH 7.4, alone or in the presence of 250 µM of the indicated thiol and H_2S yields (%) were measured as indicated in Materials and Methods. Data are means ± S.D. from 4 to 8 experiments.

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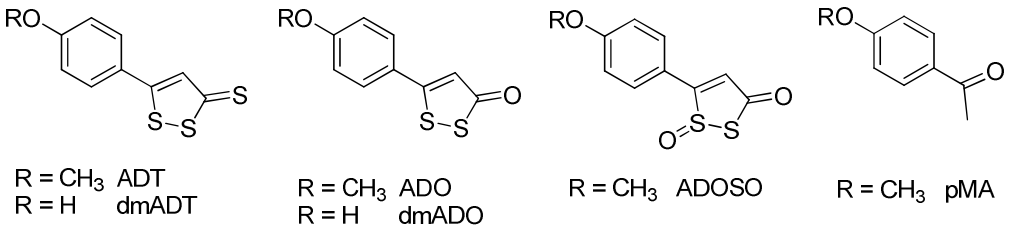

Figure 1

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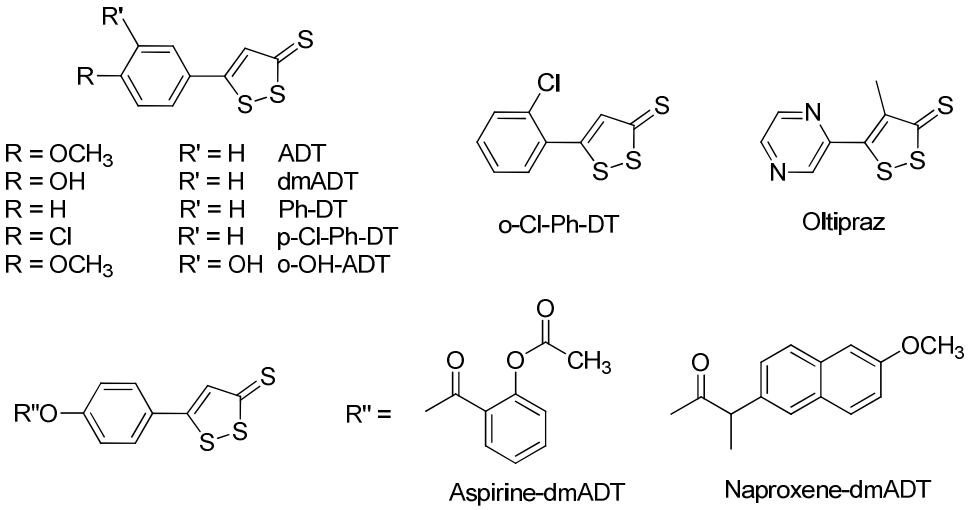

Figure 2

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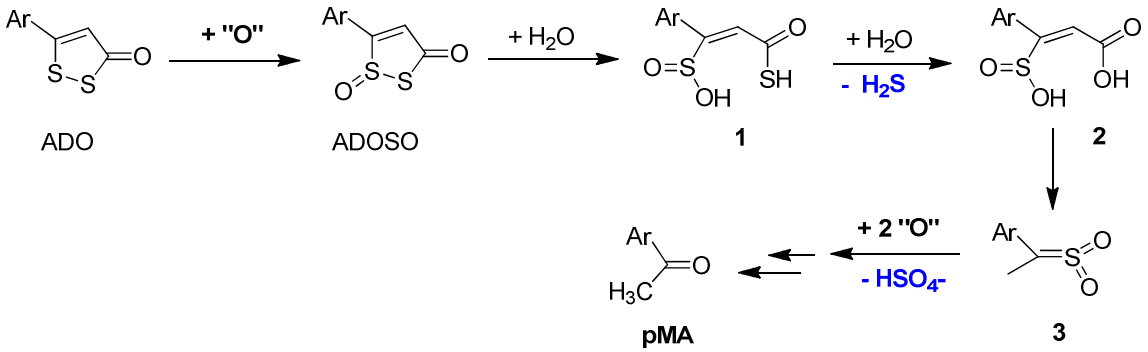

Figure 3

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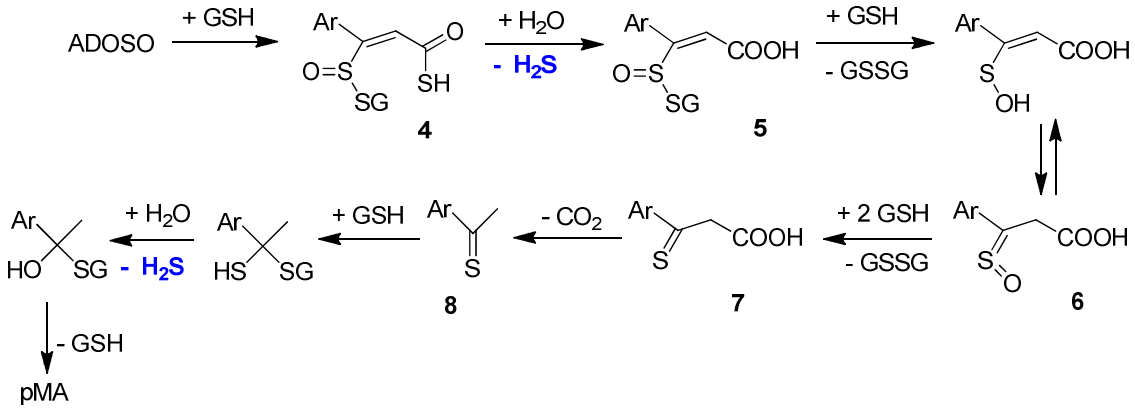

Figure 4

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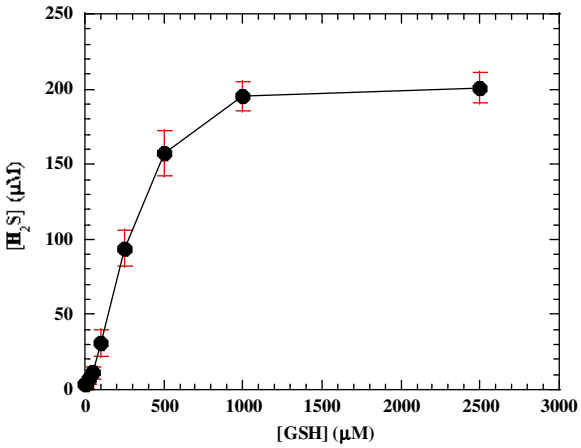

Figure 5