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Mechanism of H_2S formation from the metabolism of anetholedithiolethione and anetholedithiolone by rat liver microsomes

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Abbreviations

ADT, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione; ADTSO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione sulfoxide; ADO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-one; ADOSO, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-one sulfoxide; Bz-ImH, *N*-benzyl-imidazole; CYP, cytochrome P450; dmADT, 5-(p-hydroxyphenyl)-3H-1,2-dithiole-3-thione; DT, 5-(p-methoxyphenyl)-1,2-dithiolium hydrogensulfate; HLM, human liver microsomes, HPLC-

HRMS, high performance liquid chromatography-high resolution mass spectrometry, β-NF, β-naphthoflavone; PB, Phenobarbital; pMA, para-methoxy-acetophenone; pHA, para-hydroxy-acetophenone; RLM, rat liver microsomes; UT-RLM: liver microsomes from untreated rats.

Abstract

The drug anetholedithiolethione, ADT, and its analogs have been extensively used as H_2S donors. However, the mechanism of H_2S formation from ADT under biological conditions remains almost completely unknown. This article shows that only small amounts of H_2S are formed during incubation of ADT and of its metabolite anetholedithiolone, ADO, with rat liver cytosol or with rat liver microsomes (RLM) in the absence of NADPH, indicating that H_2S formation under these conditions is only to a minor extent of hydrolytic origin. By contrast, much greater amounts of H_2S are formed upon incubation of ADT and ADO with RLM in the presence of NADPH and dioxygen with a concomitant formation of H_2S and para-methoxy-acetophenone, pMA. Moreover, H_2S and pMA formation under those conditions are greatly inhibited in the presence of *N*-benzyl-imidazole indicating the involvement of cytochrome P450-dependent monooxygenases. Mechanistic studies show the intermediate formation of the ADT-derived 1,2-dithiolium cation and of the ADO sulfoxide during microsomal metabolism of ADT and ADO, respectively. This article proposes first detailed mechanisms for the formation of H_2S from microsomal metabolism of ADT and ADO in agreement with those data and with previously published data on the metabolism of compounds involving a C=S bond. Finally, this article shows for the first time that ADO is a better H_2S donor than ADT under those conditions.

Significance statement: Incubation of anetholedithiolethione, ADT, or its metabolite anetholedithiolone, ADO, in the presence of rat liver microsomes, NADPH and O_2, leads to H_2S. This article shows for the first time that this H_2S formation involves several steps catalyzed by microsomal monooxygenases and that ADO is a better H_2S donor than ADT. We propose first detailed mechanisms for the formation of H_2S from the microsomal metabolism of ADT and ADO.

Introduction

Anetholedithiolethione (ADT, formula in Figure 1) is a drug that has been used for many years for its choleretic and sialogogic properties (Christen, 1995; Hamada et al., 1999; Nagano et al., 2001). ADT and several dithiolethiones are also tested as cancer chemoprevention agents whose mechanisms of action involve activation of Nrf2 signaling and induction of Phase 2 enzymes (Zhang and Munday, 2008; Ansari et al. 2018). ADT and its O-demethylated derivative (dmADT) were also extensively used as H_2S donors, and the coupling of dmADT with numerous anti-inflammatory drugs have led to a variety of compounds described for their H_2S-donor properties and their therapeutic effects (li et al., 2007; Chen et al., 2010; Lee et al., 2010; Sparatore et al., 2011; Kashfi and Olson, 2013; Couto et al., 2015; Szabo and Papapetropoulos, 2017; Powell et al., 2018; Ansari et al., 2018). However, the mechanism of H_2S formation from ADT, dmADT and their derivatives under biological conditions remains almost completely unknown (Szabo and Papapetropoulos, 2017; Powell et al., 2018; Ansari et al., 2018).

We have recently reported that the oxidative metabolism of ADT by liver microsomes not only leads to dmADT but also to several products deriving from an S-oxidation of ADT, such as ADTSO, ADO and pMA (Figure 1) (Dulac et al., 2018). Under these conditions, the corresponding products deriving from dmADT, such as dmADTSO, dmADO, and para-hydroxy-acetophenone (pHA) are also formed (Figure 1) (Dulac et al., 2018). Metabolism of ADO by the same microsomes leads to pMA, a reaction in which the two sulfur atoms and one carbon atom of ADO are lost. Is there formation of H_2S during the cleavage of the heterocycle of ADT and ADO? In the study described in this article, we have followed the formation of H_2S during the metabolism of ADT and ADO by rat liver microsomes (RLM), and found that ADO is a much better H_2S donor than ADT under these conditions. H_2S formation mainly

occurs in the presence of liver microsomes and NADPH, the necessary cofactor of microsomal monooxygenases. Possible mechanisms for H_2S and pMA formation from ADT and ADO are proposed on the basis of these results.

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Materials and Methods

General reagents and synthesis of authentic samples

The commercial origins of ADT, pHA, pMA, NADPH, N-benzyl-imidazole (Bz-ImH) and the solvents used were indicated previously (Dulac et al., 2018). All authentic metabolites of ADT were prepared as described previously (Dulac et al., 2018) and displayed ^{1}H and ^{13}C NMR, UV-visible and mass spectra in accordance with their structures. Nuclear magnetic resonance spectra were recorded on a Bruker 500 AV2 spectrometer. The mass spectra were recorded on an Exactive HRMS-instrument (Thermo, Les Ulis, France).

Synthesis of Dithiolium hydrogensulfate (DT)

DT was prepared from ADT following a previously described protocol (Klingsberg, 1961). Five mmoles ADT (1.2 g) were dissolved in 50 mL acetone and 2.8 g of 40% peracetic acid were added at 0°C. After 10 min, the precipitate was filtered, washed with acetone and dried (1.4 g, 92%). ^{1}H NMR (D_2O): 10.04 (d, 1H, J = 5.0), 8.71 (d, 1H, J = 5.0), 8.06 (d, 2H, J = 9.0), 7.17 (d, 2H, J = 9.0), 3.97 (s, 3H). ^{13}C NMR (D_2O): 191.3, 171.2, 165.5, 134.8, 131.9, 123.4, 115.9, 56.0. HRMS: m/z value for $C_{10}H_9OS_2$: calc.: 209.0089, found: 209.0084. UV (MeOH) λ_{max}: 413 and 260 nm; UV (0.1 M HCl) λ_{max}: 404 and 290 nm.

Synthesis of ADOSO (formula in Figure 3)

ADOSO was prepared from ADO by oxidation with dimethyldioxirane following modifications of a previously described protocol (Tardif and Harp, 2000). A solution of ADO (11.2 mg, 0.05 mmole) in 1 mL acetone was cooled to – 78°C and 1.4 mL of a solution of 0.04M dimethyldioxirane in acetone (prepared as indicated in Adam et al. 1987) was added. The temperature was allowed to rise to room temperature in 1 h and the solvent was evaporated

under vacuum. The crude product was found to be pure ADOSO. ^{1}H NMR ($CDCl_3$): 7.71 (d, 2H, J = 9.0), 7.02 (d, 2H, J= 9.0), 6.81 (s, 1H), 3.88 (s, 3H). ^{13}C NMR ($CDCl_3$): 190.2, 171.6, 163.6, 130.7, 121.8, 121.6, 115.0, 55.9. HRMS: m/z value for $C_{10}H_8O_3S_2$: calc.: 239.9915, found: 239.9908. UV (MeOH) λ_{max}: 306 nm.

Origin of rat liver cytosols and microsomes, and of human liver microsomes

Rat liver microsomes and cytosols were prepared as previously reported (Kremers et al., 1981) from male Sprague-Dawley rats (Charles River, L'Arbresle, France) untreated (UT-RLM) or treated either with phenobarbital (PB) (50 mg.kg^{-1}, in 0.9% NaCl, i.p. for 4 days), or β-naphthoflavone (β-NF) (50 mg.kg^{-1}, in corn oil, i.p. for 4 days), two inducers of cytochromes P450 (CYP) (Williams et al., 2005). Human liver microsomes (HLM) were from Corning Inc (Corning, Amsterdam, The Netherlands). Protein concentrations and CYP contents were determined as described previously (Bradford, 1976, and Omura and Sato, 1964, respectively).

Typical microsomal incubation procedures with measurement of H_2S formation

Hydrogen sulfide formation was measured using the methylene blue method (Fogo and Popowsky, 1949; Li et al. 2007; Giustarini et al., 2014). In this assay, hydrogen sulfide is trapped by $ZnSO_4$ forming ZnS. Under acidic conditions, H_2S redissolves and reacts with *N,N*-dimethyl-para-phenylenediamine in an oxidative coupling reaction catalyzed by ferric chloride forming methylene blue detected spectrophotometrically (λ_{max}: 670 nm). The usual assay mixture (final volume 300 μL) contained cytosolic (2.5 mg/mL) or microsomal (1.5 mg/mL, about 2.5 μM CYP) proteins, 0.1 mM substrate, and 1 mM $ZnSO_4$ in 50 mM phosphate buffer pH 7.4. The mixtures were pre-incubated for 2 min at 37°C and the reactions were started by the addition of 1 mM NADPH. Usual incubations were carried out for 0-90 min at 37°C. The reactions were quenched by the addition of 100 μl of 20 mM *N,N*-dimethyl-para-

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phenylenediamine sulfate (in 7.2 M HCl) and 100 µl of 30 mM $FeCl_3$ (in 1.2 M HCl). After 10 min at room temperature, the proteins were precipitated by centrifugation (13000 rpm, 10 min) and absorbance at 690 nm of aliquots (200 µl) was determined using a 96-well microplate reader (PowerWaveXS, BioTek Instrument, Colmar, France). The H_2S concentrations were calculated against a calibration curve of Na_2S treated under identical conditions as the incubation mixtures. Incubations performed in the absence of O_2 were conducted as above, except that all components were prepared in potassium phosphate buffer previously bubbled with Argon for 30 min. Data are means $\pm$ S.D. from 3 to 6 experiments. Square deviation errors were determined by using the Microsoft Office Excel 2007 (STDEVPA function) software.

Analyses of metabolites

Incubations (final volumes 200 µL) were performed under identical conditions than above, except that $ZnSO_4$ was omitted and reactions were quenched by the addition of 100 µl of cold methanol containing 50 µM 5-(p-chlorophenyl)-3H-1,2-dithiole-3-thione (internal standard). Treatment of the mixtures and their study by HPLC-MS were done as described previously (Dulac et al., 2018).

Results and Discussion

Formation of H_2S upon microsomal metabolism of ADT and ADO.

The formation of H_2S was followed by using the methylene blue method (Fogo and Popowsky, 1949; Giustarini et al., 2014) during the metabolism of ADT and ADO by liver cytosol or microsomes from PB-pretreated rats (see Materials and Methods). Table 1 shows that H_2S was formed upon incubation of 100 µM ADT with liver microsomes from PB-treated rats in the presence of NADPH in a yield of about 13% after 1h. Interestingly, metabolism of ADO led to a much better H_2S yield (about 40%) under identical conditions. It is noteworthy that only low amounts of H_2S were formed under the same conditions but in the absence of NADPH. Moreover, incubation of ADT or ADO with rat liver cytosol under the same conditions in the presence or in the absence of NADPH led to very low amounts of H_2S (Table 1). H_2S formation from metabolism of ADT or ADO with liver microsomes in the presence of NADPH greatly decreased in incubations performed under anaerobic conditions (Table1). These data indicated that microsomal formation of H_2S from ADT or ADO mainly comes from an oxidation of these substrates catalyzed by microsomal monooxygenases, The inhibition of H_2S formation in aerobic microsomal incubations performed in the presence of Bz-ImH, an usual inhibitor of CYP (Testa and Jenner, 1981; Correira and Ortiz de Montellano, 2005) (Table 1), showed that the microsomal oxidations of ADT and ADO responsible for H_2S formation are mainly catalyzed by CYP-dependent monooxygenases.

Similar results were obtained when using either liver microsomes from untreated or β-NF-pretreated rats (UT-RLM or β-NF RLM) or human liver microsomes (HLM), in the presence of NADPH, that led to yields of about 6 and 22% (UT-RLM), 12 and 42% (β-NF-RLM) or 9 and 17% (HLM) H_2S formation from ADT and ADO, respectively (data not shown).

When one simultaneously followed the formation of H_2S and the organic products derived from ADT or ADO in the presence of NADPH-supplemented microsomes, one always observed a concomitant formation of H_2S and pMA as a function of time. This is illustrated in Figure 2 that shows the formation of H_2S and pMA during the metabolism of ADO by liver microsomes from PB-pretreated rats (35% yield based on starting ADO, corresponding to about 14 total CYP turnovers). This is also illustrated in Table 1 that shows that similar amounts of H_2S and pMA are formed in all the conditions used to metabolize ADT and ADO. Altogether these data indicate that H_2S is formed during the metabolism of ADT and ADO to pMA. This is in agreement with the much lower yield of H_2S observed upon microsomal metabolism of ADT that was found to lead to dmADT as a major product (about 65% yield) and only to lower amounts of pMA under similar conditions (Dulac et al., 2018). The concomitant formation of H_2S and pMA from microsomal oxidation of ADT and ADO is in agreement with the fact that both H_2S (this work) and pMA (Dulac et al., 2018) are mainly derived from reactions catalyzed by microsomal monooxygenases.

Possible mechanism of H_2S formation during microsomal metabolism of ADO

The above described results indicate that H_2S formation from microsomal metabolism of ADO is catalyzed by monooxygenases. A possible mechanism for this reaction is shown in Figure 3. It would involve an S-oxidation of ADO followed by the opening of the S-S bond of the intermediate (ADOSO) by H_2O to give intermediate **1**. The hydrolysis of the HSC=O function of **1** should lead to H_2S and compound **2**. Decarboxylation of **2** would lead to **3**, the disulfoxide of para-methoxy-thioacetophenone. Further monoxygenation of **3** should lead to pMA, as previously found in microsomal oxidation of compounds involving a C=S bond (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007).

The sulfoxide intermediate proposed in Figure 3, ADOSO, was synthesized following a previously described method (Tardif and Harp, 2000) (see Materials and Methods) and found to be stable in the buffer used for microsomal incubations (Table 2). In the presence of RLM without NADPH, it led to H_2S formation in a 33% yield after 1h incubation. This result is in agreement with the mechanism shown in Figure 3, in which a simple hydrolysis of ADOSO leads to the formation of H_2S. However, hydrolysis of the O=S-S bond of ADOSO appears to be catalyzed by liver microsomal proteins as it does not occur in buffer alone (Table 2). In the presence of liver microsomes and NADPH, the H_2S yield increased up to about 61% (Table 2). This could be due to an S-oxidation of the S=O group of ADOSO catalyzed by microsomal monooxygenases followed by the hydrolytic opening of the resulting O_2S-S bond and hydrolysis of the HSC=O bond.

Possible mechanism of H_2S formation during microsomal metabolism of ADT

The possible formation of intermediates in the metabolism of ADT to H_2S and pMA was evaluated from an HPLC-HRMS study of incubates of ADT with liver microsomes from PB-pretreated rats in the presence of NADPH. During the metabolism of ADT under these conditions, one could detect, in addition to the previously reported metabolites dmADT, ADTSO, ADO, dmADO, and pMA (Dulac et al., 2018), small amounts of the 1,2-dithiolium cation, DT (Figure 4), derived from a loss of the sulfur atom of the ADT C=S group. DT was characterized by its HRMS molecular peak at m/z = 209.0084 (theoretical m/z value for $C_{10}H_9OS_2$ = 209.0089). The formation of the hydrogensulfate of such a 1,2-dithiolium ion has been reported in the oxidation of 4-phenyl-1,2-dithiol-3-thione, an ADT analog, by 3 equivalents of peracetic acid (Klingsberg, 1961). Oxidation of ADT by peracetic acid under such conditions led us to DT hydrogensulfate, the cation of which was characterized by ^1H NMR and HRMS (see Materials and Methods). The HRMS spectrum of this authentic

compound was identical to that of DT detected during ADT microsomal oxidation. Incubation of 100 µM DT hydrogensulfate with liver microsomes from PB-pretreated rats in the presence of NADPH led to H_2S (38% yield, Table 2) and pMA (30% yield, data not shown), respectively, after 1h.

Thus, ADO and DT are formed during microsomal metabolism of ADT and lead to H_2S and pMA when incubated with RLM in the presence of NADPH. Possible mechanisms for their formation upon microsomal oxidation of ADT are shown in Figure 4. They would involve a common intermediate previously found in microsomal S-oxidation of compounds involving a C=S function (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007), that results from two successive S-oxidations of the C=S function (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007), intermediate **4** in the case of ADT. Monooxygenation of **4** leading to **5** could be followed by an attack of H_2O either on the carbon atom of **5** leading to **6** (Figure 4, Pathway A) or on the highly oxidized sulfur atom of **5** leading to **8** (Figure 4, Pathway B). In pathway A, further S-oxidation of intermediate **6** would lead to intermediate **7**. The loss of a hydrogensulfate moiety from **7** would then lead to ADO (Figure 4). Possible microsomal mechanisms for the formation of H_2S from ADO have been discussed in the previous paragraph (Figure 3). In pathway B, the loss of a hydrogensulfate moiety of intermediate **8** would lead to the DT cation. According to this mechanism, oxidation of ADT to DT should require three successive monooxygenations catalyzed by microsomal monooxygenases. This is in agreement with the oxidation of 1,2-dithiolthiones to dithiolium hydrogensulfates by peracetic acid that was reported to consume 3 moles of peracetic acid (Klingsberg, 1961).

H_2S formation from microsomal metabolism of DT could then involve an addition of H_2O to DT leading to **9**, followed by a S-S bond cleavage resulting in the formation of intermediate **10**. Hydrolysis of the HOC=S function of **10** should lead to H_2S and **11**, the decarboxylation of which should give para-methoxy-thioacetophenone **12** (Figure 4). This mechanism proposed in Figure 4 for the formation of H_2S by simple hydrolysis of DT is in agreement with the results of Table 2 showing that incubation of DT in buffer alone or with microsomes without NADPH did lead to H_2S (with yields of 6 and 26%, respectively). The higher yield observed in the presence of microsomes suggest that the hydrolytic opening of DT would be catalyzed by microsomal proteins. The further increase of the H_2S formation yield (38%, Table 2) in the presence of microsomes and NADPH could be due to an oxidation of DT to ADO, followed by the oxidation of ADO with formation of H_2S as described in Figure 3.

The final steps of the formation of pMA from microsomal metabolism of DT would involve an oxidation of **12** to pMA that should occur as expected in microsomal oxidation of compounds involving a C=S bond (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007). Such an oxidation of **12** to pMA and HSO_4^- should require four monooxygenations, in agreement with previous data showing that the oxidation by H_2O_2 of compounds involving a C=S bond into corresponding products involving a C=O bond requires the consumption of 4 equivalents of H_2O_2 (Böttcher and Lüttringhaus, 1945). According to the mechanisms proposed in Figure 4, the oxidation of ADT to pMA and hydrogensulfate would require 7 monooxygenation steps. This would be in agreement with a previous article reporting that titration of ADT with H_2O_2 reach a plateau after consumption of 7 H_2O_2 equivalents (Böttcher and Lüttringhaus, 1945). It is ironical that the metabolic activation of ADT to release a single molecule of H_2S requires seven monoxygenation steps.

Even though several steps of the mechanisms proposed in Figures 3 and 4 remain to be established, they are in agreement with the previous mechanisms proposed for the microsomal

oxidation of compounds involving a C=S bond (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007). They also are in agreement with the above described experimental data: (i) the detection of the DT cation during ADT microsomal incubations, (ii) the formation of about 0.4 mole of H_2S from the microsomal oxidation of 1 mole of ADO that corresponds to the formation of about 0.4 mole of pMA resulting from the cleavage of the ADO-dithiolone ring with loss of the S atoms, and (iii) the formation of only about 0.13 mole of H_2S from the microsomal oxidation of one mole of ADT that corresponds to the formation of only about 0.2 mole of pMA.

Conclusion

This article shows that only small amounts of H_2S are formed during incubation of ADT and ADO with rat liver cytosol or with RLM in the absence of NADPH. This indicates that H_2S formation under these conditions is only to a minor extent of hydrolytic origin. By contrast, much greater amounts of H_2S are formed upon incubation of ADT and ADO with RLM only in the presence of NADPH and dioxygen, the two necessary reactants of microsomal monooxygenases. Moreover, H_2S formation under those conditions is greatly inhibited in the presence of Bz-ImH, indicating that this formation is mainly catalyzed by CYP-dependent monooxygenases. This article also shows for the first time that ADO is a much better H_2S donor than ADT under these conditions. It also proposes for the first time detailed mechanisms for the formation of H_2S from the metabolism of ADT and ADO, that are in agreement with the above described data on the metabolism of these compounds (Dulac et al., 2018) and the mechanisms previously proposed for the metabolic oxidation of compounds involving a C=S bond (Cashman and Hanzlik, 1982; Hanzlik and Cashman, 1983; Vanelli et al., 2002; Testa and Krämer, 2007).

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Authorship contributions

Participated in research design: Dansette, Boucher, Mansuy.

Conducted experiments: Dulac, Nagarathinan, Boucher.

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

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

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

References

Adam W, Chan YY, Cremer D, Gauss J, Scheutzow D, and Schindler M (1987) Spectral and chemical properties of dimethyldioxirane as determined by experiment and ab initio calculations. *J Org Chem* **62**: 2800-2803.

Ansari M, Khan M, Saquib M, Khatoon S, and Hussain M (2018) Dithiolethiones: a privileged pharmacophore for anticancer therapy and chemoprevention. *Future Med Chem* **10**:1241-1260.

Böttcher B and Lüttringhaus A (1945) Über Trithione. *Justus Ann Chem* **557**: 89-107.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Cashman JR, and Hanzlik RP (1982) Oxidation and other reactions of thiobenzamide derivatives of relevance to theit hepatotoxicity. *J Org Chem* **47**: 4645-4650.

Chen P, Luo Y, Hai L, Qian S, and Wu Y (2010) Design, synthesis, and pharmacological evaluation of the aqueous prodrugs of desmethylanetholetrithione with hepatoprotective activity. *Eur J Med Chem* **45**: 3005-3010.

Christen MO (1995) Anetholedithiolethione: Biochemical considerations, *Methods Enzymol* **252**: 316-323.

Correia MA, and Ortiz de Montellano PR (2005) Inhibition of Cytochromes P450 enzymes, in *Cytochrome P450, Structure, Mechanism, and Biochemistry* 3rd Edition (Ortiz de Montellano PR ed) pp 247-322, Kluwer Academic/Plenum Publishers, New York.

Couto M, de Ovalle S, Cabrera M, Cerecetto H, González M (2015) Searching phase II enzymes inducers, from Michael acceptor-[1,2]dithiolethione hybrids, as cancer chemo preventive agents. *Future Med Chem***7**: 857-871.

Dulac M, Sassi A, Nagarathinan C, Christen MO, Dansette P, Mansuy D, Boucher JL (2018) Metabolism of anethole dithiolethione by rat and human liver microsomes: formation of various products deriving from its O-demethylation and S-oxidation. Involvement of cytochromes P450 and flavin monooxygenases in these pathways. *Drug Metab Dispos* **46**: 1390-1395.

Fogo JK, and Popowsky (1949) Spectrophotometric determination of hydrogen sulfide. *Anal Chem* **21**: 732-734.

Giustarini D, Fanti P, Sparatore A, Matteucci E, and Rossi R (2014) Anetholedithiolethione lowers the homocysteine and raises the glutathione levels in solidtissuesand plasma of rats: a novel non-vitamin homocysteine-lowering agent. *Biochem Pharmacol* **89**: 246-254.

Hamada T, Nakane T, Kimura T, Arisawa K, Yoneda K, Yamamoto T, and Osaki T (1999) Treatment of xerostomia with the bile secretion-stimulating drug anethole trithione: a clinical trial. *Am J Med Sci* **318**: 146-151.

Hanzlik RP, and Cashman JR (1983) Microsomal metabolism of thiobenzamide and thiobenzamide S-oxide. *Drug MetabDispos* **11**: 201-205.

Kashfi K, and Olson KR (2013) Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide-releasing chimeras. *Biochem Pharmacol* **85**: 689-703.

Klingsberg E (1961) The 1,2-Dithiolium Cation. A new pseudoaromatic system. I Preparation and properties of 3-phenyl- and 4-phenyl-1,2-dithiolium salts. *J Am Chem Soc* **83**: 2934-2937.

Kremers P, Beaune P, Cresteil T, de Graeve J, Columelli S, Leroux JP, and Gielen JE (1981) Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. *Eur J Biochem* **118**: 599‑606.

Lee M, Tazzari V, Giustarini D, Rossi R, Sparatore A, Del Soldato P, McGeer E, and McGeer PL (2010) Effects of hydrogen sulfide-releasing L-DOPA derivatives on glial activation: potential for treating Parkinson disease. *J Biol Chem* **285**: 17318-17328.

Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, and Moore PK (2007) Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radical Biol Med* **42**: 706-719.

Nagano T, and Takeyama M (2001) Enhancement of salivary secretion and neuropeptide (substance P, α-calcitonin gene-related peptide) levels in saliva by chronic anetholetrithione treatment. *J Pharm Phamacol* **53**: 1697-1702.

Omura T, and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370-2378.

Powell CR, Dillon KM, and Matson JB (2018) A review of hydrogen sulfide (H_2S) donors: Chemistry and potential therapeutic applications. *Biochem Pharmacol* **149**: 110-123.

Sparatore A, Santus G, Giustarini D, Rossi R, and Del Soldato P (2011) Therapeutic potential of new hydrogen sulfide-releasing hybrids. *Expert Rev Clin Pharmacol* **4**: 109-121.

Szabo C, and Papapetropoulos A (2017) Pharmacological modulation of H_2S levels: H_2S donors and H_2S biosynthesis inhibitors. *Pharmacol Rev* **69**: 497-564.

Testa B, and Jenner P (1981) Inhibitors of Cytochrome P-450s and their mechanism of action. *Drug Metab Rev* **12**: 1-117.

Testa B, and Krämer SD (2007) The Biochemistry of drug metabolism. An Introduction. Part 2. Redox reactions and their enzymes. *Chemistry and Biodiversity* **4**: 257-405.

Tardif S, and Harp D (2000) Chemoselective oxidation of 5-phenyl-1,2-dithiole-3-substituted derivatives to their corresponding 5-phenyl-1,2-dithiole-S-oxides. *Sulfur Lett* **23**: 169-184.

Vannelli TA, Dykman A, and Ortiz de Montellano PR (2002) The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase. *J Biol Chem* **277**: 12824-12829.

Williams SN, Dunham E, and Bradfield CA (2005) Induction of cytochrome P450 enzymes, in *Cytochrome P450, Structure, Mechanism, and Biochemistry* 3rd Edition (Ortiz de Montellano PR ed) pp 323-346, Kluwer Academic/Plenum Publishers, New York.

Zhang Y, and Munday R (2008) Dithiolethiones for cancer chemoprevention: where do we stand? *Mol Cancer Ther* **7**: 3470-3479.

Figure Legends

Figure 1. Formula of the main metabolites formed during microsomal metabolism of ADT (from Dulac et al., 2018).

Figure 2. Representative kinetics of formation of H_2S and pMA upon oxidation of 100 μM ADO by liver microsomes from PB-pretreated rats (2.5 μM P450) in the presence of 1 mM NADPH. Incubations (500 μL) were performed and stopped at the indicated times, as indicated in Materials and Methods. H_2S amounts (●) were measured by the methylene blue assay from 300 μL aliquots whereas the pMA levels (❍) were obtained from 200 μL aliquots analyzed by HPLC with UV-visible detection.

Figure 3. Possible mechanism for the microsomal oxidation of ADO with formation of H_2S and pMA (**Ar** = CH_3O-C_6H_4-). + "O" on some arrows indicates that the corresponding step involves a monooxygenation of the substrate.

Figure 4. Possible mechanisms for the microsomal oxidation of ADT with formation of H_2S and pMA involving DT or ADO as intermediates. Possible mechanism for the microsomal oxidation of ADO with formation of pMA and H_2S are shown in Figure 3. The meaning of Ar and + "O" are given in the legend of Figure 3.

Table 1. Formation of H_2S upon metabolism of ADT and ADO by liver cytosol or microsomes from PB-pretreated rats.

Conditions	H_2S [a]	pMA[a]	Conditions	H_2S [a]	pMA[a]
ADT + NADPH	1.0 ± 0.3	0.1 ± 0.1	ADO + NADPH	1.0 ± 0.5	0.2 ± 0.1
ADT + Cytosol	2.0 ± 1.0	0.1 ± 0.1	ADO + Cytosol	2.0 ± 1.5	3.5 ± 1.5
ADT + Cytosol + NADPH	2.8 ± 1.0	0.1 ± 0.1	ADO + Cytosol + NADPH	3.0 ± 1.8	3.7 ± 1.5
ADT + Microsomes	2.7 ± 0.7	0.8 ± 0.5	ADO + Microsomes	3.0 ± 1.5	0.5 ± 0.2
ADT + Microsomes + NADPH	12.7 ± 2.0	20 .5 ± 5.0	ADO + Microsomes + NADPH	40.0 ± 5.0	41.0 ± 6.0
ADT + Microsomes + NADPH – O_2	3.8 ± 1.5	5.0 ± 2.5	ADO + Microsomes + NADPH – O_2	5.0 ± 2.0	8.2 ± 2.5
ADT + Microsomes + NADPH + Bz-ImH[b]	5.2 ± 1.5	4.5 ± 2.0	ADO + Microsomes + NADPH + Bz-ImH[b]	6.5 ± 1.5	5.5 ± 2.0

[a] H_2S and pMA yields measured after 1h incubation of ADT or ADO (100 μM) in phosphate buffer pH 7.4 in the presence of cytosol (1.5 mg/mL proteins) or microsomes (2.5 μM P450) from PB-pretreated rats, as described in Materials and Methods. Data are % based on starting ADT or ADO and are means ± SD from 3 to 6 experiments. [b] 1 mM *N*-benzyl-imidazole was added in the incubation.

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Table 2: Formation of H_2S upon incubation of ADOSO and DT in the presence of RLM.

Conditions [a]	**H_2S yield (%)**	**Conditions** [a]	**H_2S yield (%)**
ADOSO + buffer alone	0.2 ± 0.1	DT + buffer alone	6 ± 2
ADOSO + RLM	33 ±3	DT + RLM	26 ± 3
ADOSO + RLM + NADPH	61 ± 5	DT + RLM + NADPH	38 ± 5

[a] ADOSO or DT (100 μM) were incubated for 1 h at 37°C in 50 mM phosphate buffer alone, or in the presence of RLM from PB-pretreated rat (2.5 μM P450) in the absence or presence of 1 mM NADPH. Reactions were stopped and H_2S amounts were measured by the methylene blue assay as indicated in Materials and Methods. Data are % based on starting ADOSO or DT and are means ± S.D. from 3 to 4 experiments.

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Figure 1

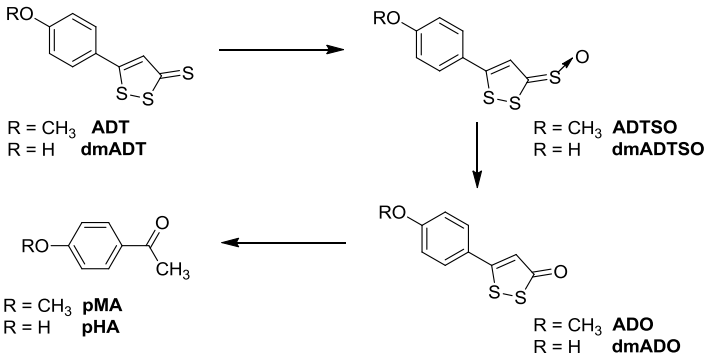

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Figure 2

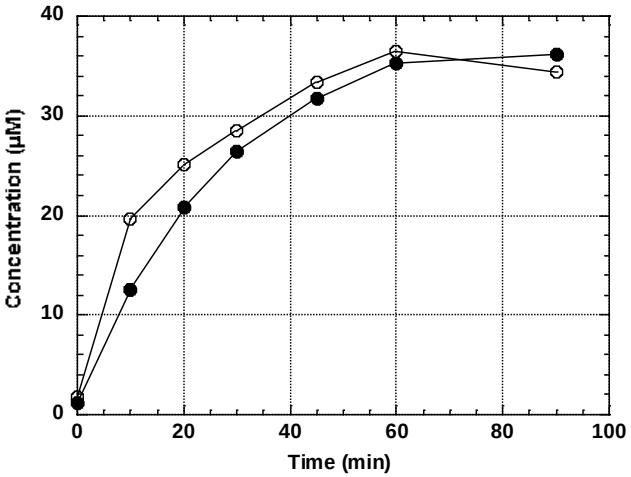

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Figure 3

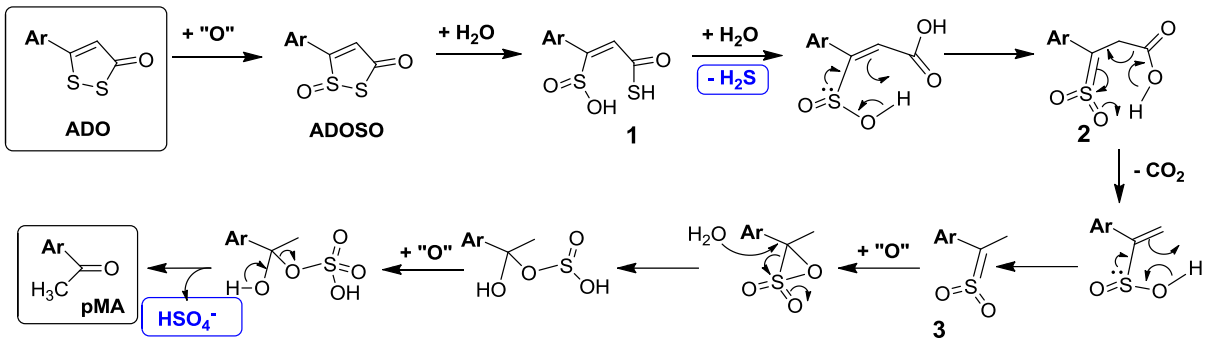

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Figure 4