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Glutathione Reductase Is Inhibited by Acetaminophen-glutathione Conjugate In Vitro

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Summary

The aim of the present work was to investigate a new mechanism likely contributing to the toxic action of acetaminophen, especially to explore the possible inhibition of glutathione reductase through an acetaminophen-glutathione conjugate (APAP-SG). APAP-SG conjugate was synthesized by organic synthesis and purified by column chromatography. The inhibitory effect of the conjugate on two types of glutathione reductase (from yeasts and rat hepatocytes) was tested spectrophotometrically. We found that the enzyme activity was reduced similarly after the treatment with 2.96 mM acetaminophen-glutathione conjugate in both yeast and hepatocyte glutathione reductases (GR); the enzyme activity was inhibited to 52.7±1.5 % (2.4±0.3 mU/ml) in yeast GR (control activity was 5.6±0.3 mU/ml) and to 48.1±8.8 % (2.2±0.2 mU/ml) in rat hepatocytes lysate GR (control activity was 5.2±0.2 mU/ml). In addition, the enzyme activity (from hepatocytes lysate) was decreased to 79±7 %, 67±2 % and 39±7 %, in 0.37, 1.48 and 3.7 mM concentration of the conjugate, respectively. We found that glutathione reductase, the essential enzyme of the antioxidant system, was dose-dependently inhibited by the product of acetaminophen metabolism – the conjugate of acetaminophen and glutathione.

Key words

Acetaminophen toxicity • Glutathione reductase • Glutathione • Hepatotoxicity

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Introduction

Acetaminophen (APAP) is at present one of the mostly used analgesics and antipyretics. It is considered to be a safe drug when used at therapeutic doses. On the other hand, the acetaminophen overdosing is the most frequent cause of acute liver failure in men (Lee 2004). Hence, the mechanisms of acetaminophen toxicity have been studied very intensively recently.

At therapeutic doses, acetaminophen is detoxified by three major pathways in the liver. The most of APAP dose is conjugated with glucuronate and sulfate (about 80 % and 10 %, respectively). Remaining part of APAP is oxidized by cytochrome P450 to a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). This compound is detoxified by either spontaneous or enzyme-catalyzed reaction with glutathione (GSH) resulting in a conjugate APAP-SG, 3-(glutathion-S-yl) acetaminophen. In APAP overdose, the glucuronidation and sulfation pathways are saturated, acetaminophen is being oxidized to NAPQI in much higher extent and GSH stores become depleted. Consequently, NAPQI binds to

various proteins, the APAP-protein adducts are produced, and due to GSH depletion, there is an increase of oxidative stress in the cell (Jollow *et al.* 1973). These actions result in hepatocellular death seen as centrilobular necrosis in the liver (Mitchell *et al.* 1973, Jaeschke and Bajt 2006).

So far, numerous mechanisms contributing to the hepatocyte injury have been found. Except of GSH depletion and reactive oxygen species (ROS) production, the lipoperoxidation, mitochondrial permeability transition pore opening (Kon *et al.* 2004) and impairment of mitochondrial respiration have also been mentioned. Despite the processes cited above, the crucial causative mechanism of the toxicity remains unknown (Kaplowitz 2004, Jaeschke and Bajt 2006). Two possible theories have been postulated so far – the oxidative and the metabolic one. The oxidative theory proposes the explanation of the damage by an increase of oxidative stress, the latter one by binding of NAPQI to SH-groups of proteins supposing their function to be impaired (James *et al.* 2003). Unfortunately, neither the oxidative nor the metabolic theory explain the entire toxicity found in acetaminophen-treated liver cells at all points.

As we have recently described (Roušar *et al.* 2009), acetaminophen toxicity is linked to reduced activity of glutathione reductase (GR) *in vitro*. It is a crucial enzyme in glutathione metabolism because it reduces glutathione disulphide (GSSG) back to the reduced form, GSH. Thus, this enzyme is essentially important during oxidative stress, where the level of GSSG increases and the inhibition of glutathione reductase could be a principal mechanism in acetaminophen toxicity. Since the cause of the enzyme inhibition remains unknown, the aim of our work was focused on an attempt to find and describe the reason of decreased activity of glutathione reductase (using two different types of glutathione reductases, i.e. from yeast and from rat hepatocytes). In addition, we wanted to prove the hypothesis that APAP-SG conjugate may play an important role in the mechanism of APAP toxicity. The experiments were carried out *in vitro.* The outcomes were aimed to serve as the preliminary data for following testing in cells and *in vivo*.

Materials and Methods

Chemicals

Glutathione reductase (from *Saccharomyces cerevisiae*; 160 U/mg prot.), GSH, glutathione disulphide, sodium phosphate buffer, potassium phosphate buffer, hydrochloric acid, acetaminophen and NADPH were purchased from Sigma-Aldrich (USA). The reagents used to the synthesis and purification of APAP-SG conjugate were purchased from Lachema (Czech Republic).

Preparation of acetaminophen-glutathione conjugate

The APAP-SG conjugate was synthesized according to the method of Thatcher and Murray (2001), the separation of the conjugate was performed as a modification of the method described by Allameh and Alikhani (2002). Briefly, sodium hydroxide solution (8 g in 250 ml of distilled water) was added to the solution of silver nitrate (4.224 g in 65 ml of distilled water) and the precipitated silver oxide was filtered off using glass sinter and washed.

Acetaminophen (0.428 g) was suspended in 100 ml of dry chloroform and just prepared silver oxide (2.2 g) was added. The suspension was stirred, filtered and the NAPQI solution was obtained. Glutathione (0.857 g) was dissolved in 250 ml of 0.1 M sodium phosphate buffer pH 7.4 and freshly prepared NAPQI solution was added drop wise. Reaction mixture was separated and the water was evaporated at 40 °C. Residue of the mixture was stirred in methanol for 3 h and filtered. Methanol was evaporated using vacuum evaporator.

APAP-SG conjugate was separated using column chromatography on Silicagel 60 (Merck, Germany) where the separation of the reaction residue containing APAP, APAP-SG and GSSG was performed; mobile phase consisted of methanol/water (9:1). The fractions (10 ml) were collected after separation and analyzed by thin layer chromatography (TLC) according to Allameh and Alikhani (2002). The detection was carried out using TLC on Silicagel 60 F254 (Merck, Germany) with methanol/water (9:1) as a mobile phase; bands were visualized by $\lambda = 254$ nm (APAP, APAP-SG) or after reaction with 0.2 % ninhydrin (APAP-SG, GSSG). The Rf values for APAP, APAP-SG and GSSG were approximatelly 0.9, 0.7 and 0.3, respectively. The Rf values of GSSG and APAP were determined after comparison with standard values. APAP-SG conjugate was obtained as a solid by desiccation of the fractions with proved APAP-SG only.

Preparation of hepatocyte lysates

Hepatocytes were isolated from male albino Wistar rats (250-280 g; Biotest, Czech Republic) by

collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes was more than 90 % as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in Williams' E medium and diluted to final density of 10^6 cells per ml. The cells were sonicated (Bandelin Sonopuls sonicator, Germany) and the lysates were centrifuged (4 °C, 10 min, 10000 g). The inhibition of glutathione reductase was tested in supernatant which was diluted in distilled water to gain the final GR activity similar to samples containing yeast glutathione reductase. The specific activity of rat hepatocytes lysate GR was 22 mU/mg prot.

All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

GR activity assay

The principle of the method is the reduction of oxidized glutathione by glutathione reductase in the presence of NADPH (Carlberg and Mannervik 1975). Activity of both yeast (from *Saccharomyces cerevisiae*; 160 U/mg prot.) and hepatocytes lysate GR was determined at 25 °C in 0.2 M potassium phosphate buffer (pH 7.5) by monitoring of NADPH absorbance decline (λ=340 nm) using well-plate spectrophotometer INFINITE M200 (Tecan, Austria). The volumes of solutions were 50 µl GR, 25 µl GSSG (3.7 mM) and the assay was started by addition of 50 µl NADPH (0.7 mM); the values in the brackets mean the final concentrations of a compound in a well. The decline of absorbance was monitored during 20 min and the results were presented as a dependence of absorbance on time. One Unit was defined as an amount of the enzyme which will reduce 1 µmole of oxidized glutathione per minute at pH 7.6 at 25 °C, using a molar extinction coefficient of 6.22 x 10^3 for NADPH.

Estimation of GR inhibition by APAP-SG conjugate

The inhibition of both yeast and rat hepatocytes lysate GR activities by APAP-SG conjugate were assayed in well plates. The stock solution of APAP-SG (100 mM) was prepared. Then, the solutions with various concentrations of APAP-SG (5 mM, 10 mM, 20 mM, 40 mM, 50 mM) were prepared by dilution in distilled water. 10 µl of each solution were added to the mixture of GR (50 µl) and GSSG (25 µl) to assess the inhibitory effect. The measurement was started by the addition of NADPH (50 µl) and monitored by λ = 340 nm spectrophotometrically for 20 min at 25 °C. Control samples were prepared by identical protocol, the distilled water (10 µl) was added instead of APAP-SG conjugate.

Statistical analysis

All experiments were repeated at least two times with negligible differences among results. The results were processed by one-way ANOVA test, followed by Bonferroni *post-hoc* test. The results are expressed as the mean ± S.D. (GraphPad Prism 4.03 for Windows, GraphPad Software, USA). $p<0.05$ was considered as significant.

Results

Purified APAP-SG conjugate was used to estimation of possible inhibitory effect in two types of glutathione reductase from yeast or rat hepatocyte lysate. The results show (Fig. 1) that the activity of both types of GR was decreased similarly in comparison to control in the presence of 2.96 mM APAP-SG. The enzyme activity was inhibited to 52.7±1.5 % (2.4±0.3 mU/ml) in yeast GR (control activity 5.6±0.3 mU/ml) and to 48.1±8.8 % (2.2±0.2 mU/ml) in rat hepatocyte lysate GR (control activity was 5.2±0.2 mU/ml). Based on these results, all other experiments were performed using rat liver GR.

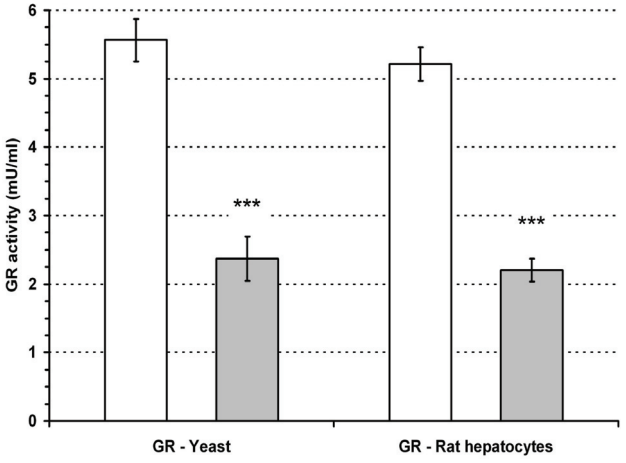

Fig. 1. Comparison of inhibitory effect of acetaminophen-glutathione conjugate, APAP-SG (2.96 mM) on glutathione reductases from yeast and rat hepatocyte lysate. Glutathione reductases from yeast and rat hepatocyte were treated with a solution consisting of 2.96 mM APAP-SG conjugate (gray columns) and the decrease in absorbance (λ = 340 nm) was measured after addition of NADPH for 20 minutes. The results were evaluated and compared to control (white columns) which consisted of the same concentrations of all compounds, excluding APAP-SG conjugate. Results are expressed as mean ± S.D. (n = 4; ***, $p<0.001$, compared to control).

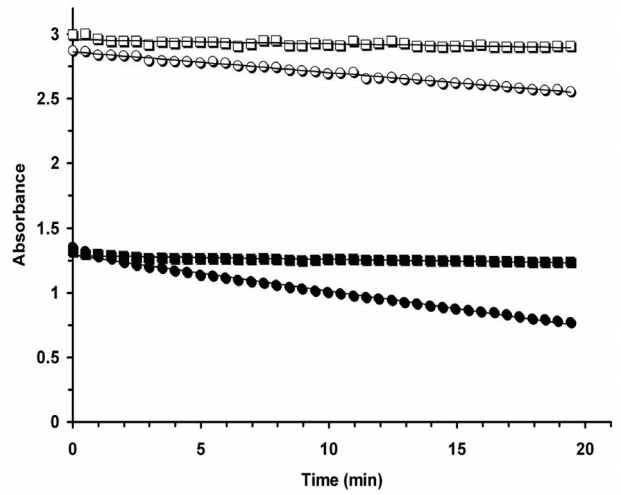

Fig. 2. Time course of the inhibition of glutathione reductase (from rat hepatocytes lysate) by acetaminophen-glutathione conjugate, APAP-SG (2.96 mM). Glutathione reductase (8 mU/ml) was treated with 2.96 mM APAP-SG conjugate (○, open circles) and the decrease in absorbance of NADPH (λ = 340 nm) was measured. After 20 min, the results were evaluated and compared to control (●, closed circles) which consisted of the same concentrations of compounds, excluding APAP-SG conjugate. The blank samples without glutathione disulfide were also assessed - in the presence of the APAP-SG conjugate (□, open squares) and without APAP-SG conjugate (■, closed squares).

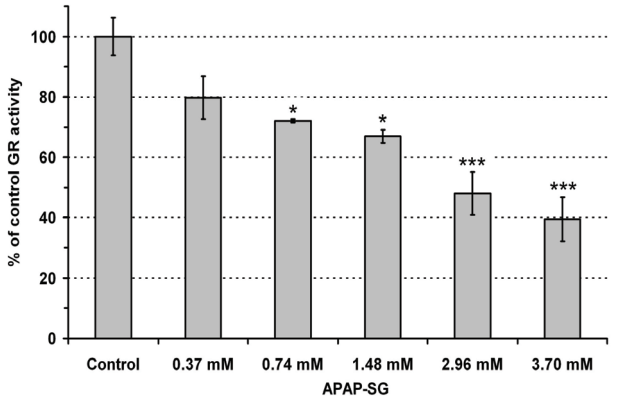

Fig. 3. Inhibition of glutathione reductase (from rat hepatocyte lysate) by acetaminophen-glutathione conjugate (APAP-SG). Ratio of resultant glutathione reductase activities to control signal (8.7±0.5 mU/ml) in samples treated with various concentrations of APAP-SG conjugate (0.37 mM, 0.74 mM, 1.48 mM, 2.96 mM and 3.70 mM). The absorbance decrease of NADPH (λ = 340 nm) was monitored for 20 min. Results are expressed as mean ± S.D. (n = 2-4; *, $p<0.05$, ***, $p<0.001$, compared to control).

We analyzed also blank samples in all experiments. The blank samples did not contain GSSG and the obtained absorbance signals were subtracted in both control and APAP-SG-treated samples. The results show that the blank signal accounts for only 2 % and 4 % of absorbance decrease in control and APAP-SG-treated samples, respectively (Fig. 2). Although we did not purified the hepatocyte GR, the values of blank signals showed, that our conditions of GR assay were rather specific.

The inhibitory effect on hepatocyte glutathione reductase was tested in a number of APAP-SG concentrations. We found that the enzyme activity was decreased proportionally to increasing APAP-SG concentration (Fig. 3). The GR activity was inhibited to 79.8±7.0 %, 72.1±0.5 %, 66.9±2.2 %, 48.0±7.1 % and 39.5±7.4 % in presence of 0.37 mM, 0.74 mM, 1.48 mM, 2.96 mM and 3.7 mM APAP-SG, respectively. GR activity in controls was 8.7±0.5 mU/ml.

Discussion

Acetaminophen toxicity is a complex process where many mechanisms contribute to the hepatocyte impairment. Since the acetaminophen overdosing is one of the mostly found causes of acute liver failure (Lee 2004), the estimation of APAP toxicity has been extensively studied.

Recently, a number of reviews and original papers have been published in which new mechanisms contributing to the liver damage have been described. However, none of the mechanisms explain the cause of the hepatocyte damage completely (James *et al.* 2003, Jaeschke and Bajt 2006).

The former assumption explaining a cause of APAP toxicity is a metabolic one. It is linked to increased NAPQI production during metabolic phase in the APAP overdose. After GSH stores are depleted, NAPQI binds to various proteins and the APAP-adducts are produced (Jollow *et al.* 1973, Qiu *et al.* 1998). This was believed to impair the protein function. However, none or only a modest change in their activity was found (Pumford *et al.* 1997).

The oxidative theory proposed that depletion of GSH is followed by enhanced oxidative stress in a short period of time which leads to the hepatocyte injury. Indeed, it was supported by increased lipoperoxidation, ROS production or increased synthesis of nitric oxide (NO) (Hinson *et al.* 1998, Knight *et al.* 2001, Jaeschke *et al.* 2003). However, it is questioned if the oxidative stress is a cause or just a consequence to decreased glutathione levels. It was shown that the increased ROS production follows the GSH depletion (Bajt *et al.* 2004). Another issue was to determine the localization of ROS production. The role of Kupffer cells, increased NO production or mitochondria as a source of ROS were investigated, but the question concerning the role of ROS

as the main cause of cell death has not been answered yet (James *et al.* 2003, Jaeschke and Bajt 2006).

The present work is directed to assess a newly proposed principle contributing to the acetaminophen toxicity. We observed recently that the glutathione reductase activity was reduced in rat hepatocytes treated with acetaminophen (Roušar *et al.* 2009). This inhibition was proved to be dose-dependent. We ascribed the cause of GR inhibition to the impairment of the enzyme by ROS or toxic aldehydes, as described in several other papers (Ochi 1990, Vessey and Lee 1993). Despite this, we have proposed another theory of the cause of GR inhibition. We have hypothesized that the inhibition could be caused by direct effect of APAP-SG, the conjugate of glutathione and acetaminophen (or NAPQI more precisely).

To prove our hypothesis, we decided to prepare APAP-SG conjugate by organic synthesis. We tested two types of glutathione reductase – a commercially available GR from yeast *Saccharomyces cerevisiae* and GR from rat hepatocyte lysate. In accordance to our hypothesis, we repeatedly found that APAP-SG inhibits largely yeast and hepatocyte lysate GR; 2.96 mM APAP-SG was able to inhibit glutathione reductase activity by about 50 %.

The assessed concentrations of APAP-SG (0.37 - 3.7 mM) may be comparable to the APAP-SG levels occurring in hepatocytes. In APAP overdose, the levels of APAP in the cells are in milimolar range (Mitchell *et al.* 1973); the GSH levels in hepatocytes were found in milimolar range as well (Pastore *et al.* 2003). After the glucuronidation and sulfation pathways are saturated, the most of the dose is oxidized to NAPQI. Thus, the described effect of APAP-SG on GR activity is likely to occur in the cells. The possibly substantial role of GR in the acetaminophen toxicity is supported by the work of Armesto *et al.* (1993) who demonstrated that a drug, lobenzarit, enhances GR activity in mice. Since lobenzarit was proved to have hepatoprotective properties in APAP overdose, this effect may be caused just by decreasing of the GR inhibition present in APAP-treated hepatocytes.

We propose that the cause of observed GR inhibition by APAP-SG might be due to similar principle as in the case of inhibition of glutathione reductase by S-nitrosoglutathione (Becker *et al.* 1995). The authors presented that S-nitrosoglutathione was capable of inhibiting the glutathione reductase activity by reversible and/or irreversible mechanism. It follows that GR is able to react with a compound of chemical structure related at least to a half of glutathione disulphide molecule.

The exploration of glutathione reductase inhibition by APAP-SG partially changes the view to acetaminophen toxicity. Indeed, the conjugate of acetaminophen (or more precisely NAPQI) and glutathione always occurs when NAPQI has been formed by cytochrome P450. Moreover, NAPQI production is even catalyzed by glutathione-S-transferase (GST) (Coles *et al.* 1988). The conjugation of APAP and GSH has been considered till now as a protective mechanism which protects the hepatocyte against the binding of NAPQI to various proteins. The conjugate is consequently transported out of the cell to the bile by MRP-2 (multidrug resistance-associated protein-2) localized in canalicular membrane of hepatocyte (Chen *et al.* 2003).

Despite generally accepted mechanisms described above, several original works brought results that were not anticipated before. In 2000, the work of Henderson *et al.* (2000) described the estimation of APAP toxicity in GST-pi knockout mice. GST-pi is an isoenzyme of glutathione-S-transferase which is proved to catalyze the formation of APAP-SG conjugate in the liver (Coles *et al.* 1988, Henderson and Wolf 2005). Remarkably, the results showed that GST-pi knockout mice were much more resistant to APAP toxicity than wild-type mice. The explanation of that phenomenon was attributed to GST-catalyzed redox cycling and enhancement of oxidative stress, or to the role of GST-pi as an inhibitor of the stress inducible Jun N-terminal kinase (Henderson *et al.* 2000). However, the results may be explained convincingly regarding the finding published in this paper.

The hepatocytes from mice lacking GST-pi produce certainly much lower amount of APAP-SG conjugate (Coles *et al.* 1988). Hence, lower concentration of APAP-SG can lead to a slighter inhibition of GR. Indeed, this is supported by different recoveries of glutathione when comparing wild-type and GST-pi knockout mice treated with acetaminophen (Henderson *et al.* 2000). These results show that GSH concentrations were decreased in both strains, although GSH levels remained higher at all time points in knockout mice compared to wild-type ones. In addition, the GSH levels almost recovered to pretreatment levels within 5 h in GST-pi null mice, whereas GSH remained depleted in wild-type mice. The authors tested the protein levels of glutathione biosynthetic enzymes, γ-glutamylcysteine synthetase and glutathione synthetase, which were essentially unchanged. Hence, the GSH recovery cannot be explained in this way and it is likely that the diverse GSH recovery between mice strains could be influenced by different GR activity.

Another paper was concerned in testing of

susceptibility of transport-deficient hyperbilirubinemic, MRP-2 deficient rats to acetaminophen (Silva *et al.* 2005). The treatment of MRP-2 deficient rats with acetaminophen resulted in a finding that mutant rats were more resistant than wild-type rats. The published explanation was that increased expression of certain cytochrome P-450 isoenzymes produced even more NAPQI which caused even larger impairment. On the other hand, the MRP-2 deficiency in rats likely evoked an accumulation of APAP-SG in the hepatocytes leading to the subsequent enhancement of the GR inhibition (Silva *et al.* 2005). Obviously, an attribute of enhanced NAPQI formation is also important, but the increased toxicity may be again due to increased production of APAP-SG.

For further support of our hypothesis, we sought to find another mechanism capable to enhance APAP-SG intracellular levels. Rzucidlo *et al.* (2000) published a paper focused on the estimation of acute APAP toxicity in transgenic mice with elevated hepatic glutathione. They hypothesized that due to overexpression of glutathione synthetase and elevated GSH levels, the transgenic mice should be better protected against APAP toxicity. Surprisingly, the expectations were not fulfilled because transgenic mice showed significantly higher level of hepatotoxicity. The cause of this surprising finding was not fully explained. However, regarding our results presented here, the mechanism of higher hepatotoxicity level in mice with elevated glutathione could be easily explained by increased formation of APAP-SG conjugate and augmented inhibition of glutathione reductase.

Conclusions

The obtained results confirmed our hypothesis that glutathione reductase could be inhibited by APAP-SG conjugate *in vitro* which is supported by published results of the other authors. Generally, depletion of GSH due to reaction with NAPQI in acetaminophen overdose is always related to acetaminophen toxicity. Since glutathione reductase is a crucial enzyme in maintenance of intracellular GSH levels, the decrease of GR activity raises hepatocyte impairment. In addition, it is generally accepted that the GSH exhaustion results in a number of consequent pathological processes (e.g. ROS production, peroxynitrite formation and/or lipoperoxidation) which lead to GSSG production and to the cell death. Hence, our results shed light on a mechanism that could contribute to the acetaminophen toxicity in the liver.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

APAP, acetaminophen; APAP-SG, acetaminophen-glutathione conjugate; GR, glutathione reductase; GSH, glutathione (reduced form); GSSG, glutathione disulphide; GST, glutathione-S-transferase; MRP-2, multidrug resistance-associated protein-2; NAPQI, N-acetyl-p-benzoquinone imine; ROS, reactive oxygen species; TLC, thin layer chromatography.

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