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Gambogic Acid Deactivates Cytosolic and Mitochondrial Thioredoxins by Covalent Binding to the Functional Domain

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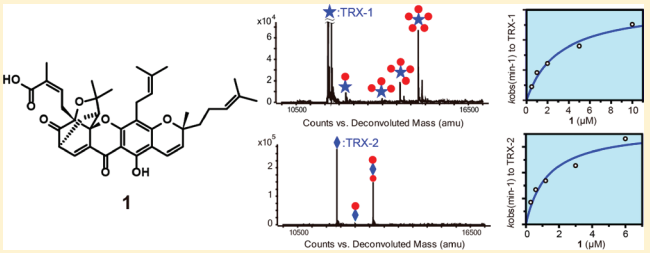
Supporting Information

ABSTRACT: Gambogic acid (**1**) is a cytotoxic caged xanthone derived from the resin of *Garcinia hanburyi*. Compound **1** selectively induces apoptosis in cancer cells, at least partially, by targeting the stress response to reactive oxygen species (ROS). However, the molecular mechanism of ROS toxicity stimulated by **1** remains poorly understood. In this study, mass spectrometric and biochemical pharmacological approaches were used that resulted in the identification of both cytosolic thioredoxin (TRX-1) and mitochondrial thioredoxin (TRX-2) as the molecular targets of **1**. The results obtained showed that **1** deactivates TRX-1/2 proteins by covalent binding to the active cysteine residues in the functional domain via Michael addition reactions. Since both TRX-1 and TRX-2 play key roles in regulating the redox signaling of cancer cells, the present findings may shed light on the relationship between protein binding and cellular ROS accumulation induced by **1**. This provides support for the current clinical trials of gambogic acid (**1**) being conducted alone or in combination with other agents that appear to increase ROS generation in order to selectively kill cancer cells.



Gambogic acid (**1**, Figure 1A) is a caged xanthone derived from the resin of *Garcinia hanburyi* Hook.f. (Clusiaceae).[1−3] The anticancer activity of **1** has been demonstrated previously, and a variety of mechanisms have been proposed by which **1** interferes with tumor growth and progression.[1] Our group has reported that apoptosis induced by **1** may act, at least partially, through a reactive oxygen species (ROS)-related mitochondrial pathway in human hepatoma cells.[4] Ortiz-Sanchez et al.[5] have shown that **1** increases the generation of ROS in a panel of hematopoietic malignant cell lines, which may play an important role in the cytotoxicity induced by this compound. However, the molecular basis of ROS toxicity stimulated by **1** remains poorly understood.

In order to protect against oxidative stresses, living cells have had to develop some "regulators" to maintain redox balance by scavenging ROS. Unlike normal cells, one common biochemical change in cancer cells is the increase in ROS generation.[6] For protection against stress-dysregulated redox signaling, cancer cells that exist in an oxidative stress environment have to depend more on these "regulators" than normal cells.[7,8] Thioredoxin proteins are the key regulators of cellular redox homeostasis, especially in cancer cells. There are two main thioredoxins, the cytosolic form (TRX-1) and the mitochondrial form (TRX-2). Both TRX-1 and TRX-2 play important roles in cellular redox signaling through sulfhydryl reactions via reduction of cysteine residues of, as well as interaction with, various components of signal transduction pathways for the redox response against oxidative stress.[9] Thus, TRXs, the key antioxidant defensive molecules in cancer cells, may serve as attractive molecular targets for new cancer therapeutic agent discovery.[10]

Notably, all TRX proteins have a canonical CGPC catalytic motif located on a highly conserved fold. The cysteine residues of the CGPC motif are the key players used by TRX proteins to break disulfide bonds in oxidized substrate proteins and to scavenge intracellular ROS directly.[11] Therefore, it is conceivable that the α,β-unsaturated ketone moiety of **1** will readily react with these cysteines via Michael addition reaction. In fact, **1** can react rapidly with free thiol groups on reduced glutathione (GSH) by Michael addition.[12] In addition, Palempalli et al.[13] showed that **1** modifies covalently the cysteines of the IκK-β subunit. On the other hand, structure−activity relationship studies have also demonstrated that the α,β-unsaturated ketone moiety of **1** is important for its

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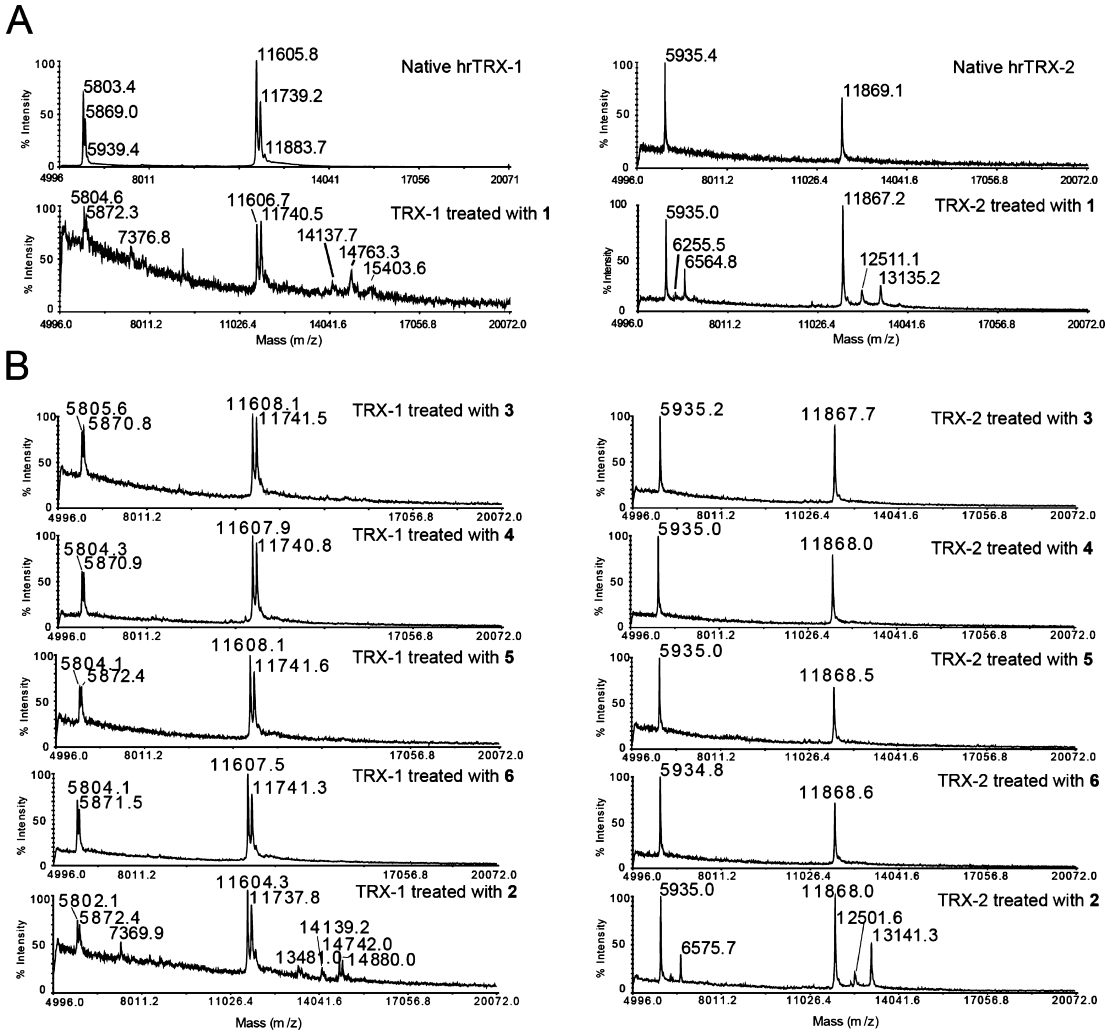


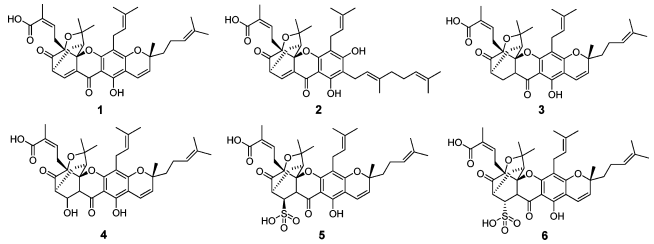
Figure 1. The α,β-unsaturated ketone moiety in gambogic acid (**1**) is required for covalent binding to TRX-1 and TRX-2. (A) MALDITOFMS analysis of hrTRX-1/2 modification by **1**. (B) MALDITOFMS analyses of TRX-1 and TRX-2 modification by analogues of **1**.

antitumor activity,[14,15] suggesting that the potent activities exhibited by **1** can be attributed to its Michael addition reactivity. Taken together, it was hypothesized that **1** may deactivate TRX-1/2 by covalent binding to nucleophilic cysteine residues in their functional domains, break the redox homeostasis in cancer cells, and ultimately trigger the death program of these cells. Of interest, many cellular responses triggered by **1** are regulated by TRX-1 or TRX-2,[15−18] which further enhances the possibility that **1** mediates its effects by modulating cytosolic or mitochondrial thioredoxin signaling.

The purpose of the present study was to verify the aforementioned hypothesis and to reveal the possible molecular basis of ROS insults and for the downstream electrophilic cellular responses stimulated by **1** in human hepatoma cells. The implications in the clinical therapeutic regimen design of this anticancer drug candidate are also discussed.

■ RESULTS AND DISCUSSION

Covalent Binding of Gambogic Acid (1) to TRX-1 and TRX-2. It has been shown that **1** can react with small nucleophilic molecules, such as GSH and a cysteine-containing peptide, via a Michael addition reaction.[12,13] However, whether **1** can covalently modify intact proteins with a certain spatial configuration has never been investigated so far. Thus, the



native and **1**-treated TRX-1/2 proteins were analyzed using MALDITOFMS. When the TRX-1 protein was incubated with **1**, some new peaks appeared in the spectrum with $[M + H]^+$ ions at m/z 14137.7, 14763.3, and 15403.6 (Figure 1), which are consistent with native TRX-1 plus four, five, and six bound molecules of **1**, respectively. Likewise, the spectrum obtained from the **1**-treated TRX-2 protein also showed two new peaks with $[M + H]^+$ ions at m/z 12511.1 and 13135.2 (Figure 1), respectively, which are close to the molecular mass of TRX-2 plus one and two bound molecules of **1**. Furthermore, to elucidate whether the β-carbon (C-10) in the α,β-unsaturated carbonyl moiety of **1** is involved in the reactions with TRX-1/2, selected analogues of **1** with or without an unsaturated ketone moiety were incubated with these proteins and analyzed by MALDITOFMS. As expected, reactions with 9,10-dihydrogam-

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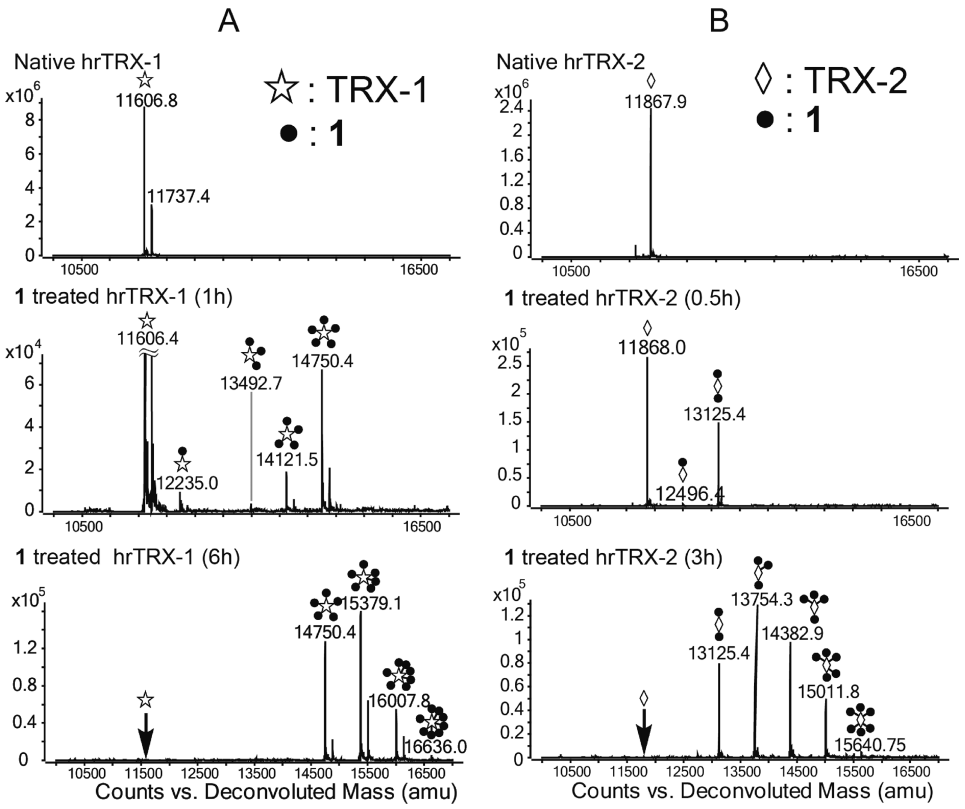

Figure 2. Covalent binding of gambogic acid (**1**) to TRX-1 and TRX-2 in a time-dependent manner. (A) LC-ESIQTOFMS analysis of native hrTRX-1 and hrTRX-1 treated with **1**. (B) LC-ESIQTOFMS analysis of native hrTRX-2 and hrTRX-2 treated with **1**.

Table 1. Gambogic Acid (1)-Modified Peptide Fragments of TRX-1 and TRX-2 Detected by LC-ESIQTOFMS in the Tryptic Digests[a]

sample	position	charge	*m/z*	calcd mass (Da)	theor mass (Da)	match diff (ppm)	peptide sequence
native TRX-1	22−36	2	812.8969	1623.7734	1623.7789	−3.39	LVVVDFSATWCGPCK
	49−72	2	1360.6077	2718.1934	2718.2041	−3.94	YSNVIFLEVDVDDCQDVASECEVK
	73−81	1	1148.5286	1147.5140	1147.5195	−4.72	CMPTFQFFK
TRX-1 treated with **1**	22−36	2	1441.6994	2880.3840	2880.3862	−0.74	LVVVDFSATWC[b]GPC[b]K
	49−72	3	1325.2780	3972.7901	3972.7956	−1.38	YSNVIFLEVDVDDC[b]QDVASEC[b]EVK
	73−81	2	888.9120	1775.8100	1775.8230	−7.37	C[b]MPTFQFFK
native TRX-2	15−35	2	1158.1096	2314.0853	2314.0875	−0.95	VVNSETPVVVDFHAQWCGPCK
TRX-2 treated with **1**	15−35	3	1191.9068	3570.6890	3570.6947	−1.59	VVNSETPVVVDFHAQWC[b]GPC[b]K

[a]Some modified peptide fragments by **1** derived from being incompletely digested were excluded. [b]Covalent modification with **1**.

bogic acid (**3**), 10-hydroxygambogic acid (**4**), gambogic acid 10α-sulfonic acid (**5**), or gambogic acid 10β-sulfonic acid (**6**) did not cause any modification (Figure 1C). However, gambogenic acid (**2**) modified TRX-1/2 in the same manner as **1** (Figure 1C). These findings demonstrate that the reactive carbon in **1** is indeed C-10.

To confirm the formation of the gambogic acid−protein covalent adducts and to improve the accuracy and sensitivity of detection, a LC-ESIQTOFMS system was employed to reanalyze the native and **1**-treated TRX proteins, and similar results were obtained. Moreover, it was found that **1** modified covalently TRX-1 and TRX-2 in a time-dependent manner. As shown in Figure 2, some modified TRX-1/2 subunits were observed when they were treated with **1**, respectively, corresponding to the addition of one, three, four, and five molecules of **1** to TRX-1 and to the addition of one and two molecules of **1** to TRX-2. It is well recognized that Michael addition reactivity is most efficient for cysteine, among all amino acids, in reacting with α,β-unsaturated ketones.[19] Thus, these findings might suggest that five cysteines in TRX-1 and two cysteines in TRX-2 were modified randomly by **1**. Further incubations resulted in a complete disappearance of the native proteins and a more complicated binding profile of **1** to these proteins (Figure 2). It is understandable that once all the free thiols over the surface of TRX-1/2 are covalently modified by gambogic acid (**1**), other types of nucleophilic amino acid residues on these proteins will begin to react with excess **1** but at much slower rates. However, this scenario may not occur in living cells, because α,β-unsaturated ketones generally display selective reactivity with cysteine residues within complex proteomes in intact cells, although they may display substantial reactivity with all nucleophilic amino acids in solution.[19]

Identification of the Gambogic Acid (1) Modification Sites in TRX-1 and TRX-2. The catalytic activity of TRX-1 or TRX-2 resides in its conserved active site where the two redox-active cysteine residues (C32 and C35 for TRX-1; C31 and

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C34 for TRX-2) undergo reversible oxidation/reduction. The covalent modification of these active-site cysteines may directly associate with the abolition of its redox regulatory functions. Except for the conserved cysteine residues in the catalytic site, three additional structural cysteine residues (C62, C69, and C73) are present on TRX-1. They can be subjected to *S*-nitrosocysteine (a form of oxidative post-translational modification) and/or interchain reaction, which endows them with the ability to scavenge ROS and to preserve the redox regulatory activity of the proteins. Hence, chemical modification on these "noncatalytic" cysteines on TRX-1 may also result in an increase in ROS accumulation. Furthermore, it is important to stress that these "noncatalytic" cysteines locate on a binding area of TRX-1 to thioredoxin reductase (TR).[20,21] Conceivably, once one of these cysteines is modified covalently, the TRX-1/TR redox signaling may also be perturbed to some extent for steric reasons. To further elucidate whether the aforementioned functional cysteines on TRX-1/2 are exactly the binding sites of **1**, a LC-MS/MS-based shotgun protein modification analysis was performed.

For the digested sample of native TRX-1, there are three expected peptides containing cysteine residues (Table 1), which incorporate the functional region of the protein. It was speculated that all the cysteines on TRX-1 are modified by **1**, and the resulting mass spectra of the digested gambogic acid-modified TRX-1 sample indeed identified several complete digested cysteine-containing sequences with adducts of this compound (Table 1). Next, MS/MS analysis was used to further identify the exact sites of adduction. Figure 3A shows the MS/MS spectrum of the $[M + 2H]^{2+}$ ion of the **1**-modified peptide LVVVDFSATWCGPCK at m/z 1441.6994. The product ions at m/z 629.3, corresponding to the $[M + H]^+$ ion of **1**, as well as m/z 601.3, 573.3, and 487.3, relative to the diagnostic fragment ions of **1**,[22,23] confirmed the inclusion of the moiety of **1** in this peptide fragment. Moreover, the singly charged b-ion series from b2 to b10 had no alteration compared with those of the unmodified peptide, indicating that the modification may take place from residues 32 to 36. The singly charged y-ion series (y2−y4) and b-ion (b11) were observed to increase by 628 Da, suggesting that one of the modification sites by **1** is in the sequence on C35. The singly charged y-ion series from y5 to y11 were observed to increase 1256 Da, suggesting that another modification site of **1** is C32. The ions of two other **1**-modified peptides (YSNVIFLEVDVDDCQDVASECEVK and CMPTFQFFK) were also subjected to MS/MS analysis, and the results indicated that all the cysteines (C62, C69, and C73) on these peptides are modified covalently by **1** as well (data not shown).

For the digested sample of native TRX-2, only one cysteine-containing peptide fragment was expected, which incorporates the redox active site region of the protein (Table 1). In the digested **1**-modified TRX-2 sample, the peptide ions of 3570.6890 mass units were detected, as $[M + 3H]^{3+}$ at m/z 1191.9068, corresponding to the residues 15−35 plus two molecules of **1** (Table 1). These ions were then subjected to MS/MS analysis, and the results are shown in Figure 3B. Product ions at m/z 629.3, 601.3, 573.3, and 487.3 relative to moiety **1** were also detected. In addition, both C31 and C34 as the **1**-adduct sites on TRX-2 were assigned unequivocally according to the observed b-ion and y-ion series.

Although many TRX inhibitors exhibit some selectivity on the functional cysteines in TRXs, the present results showed clearly a nearly random distribution of **1**-cysteine residue

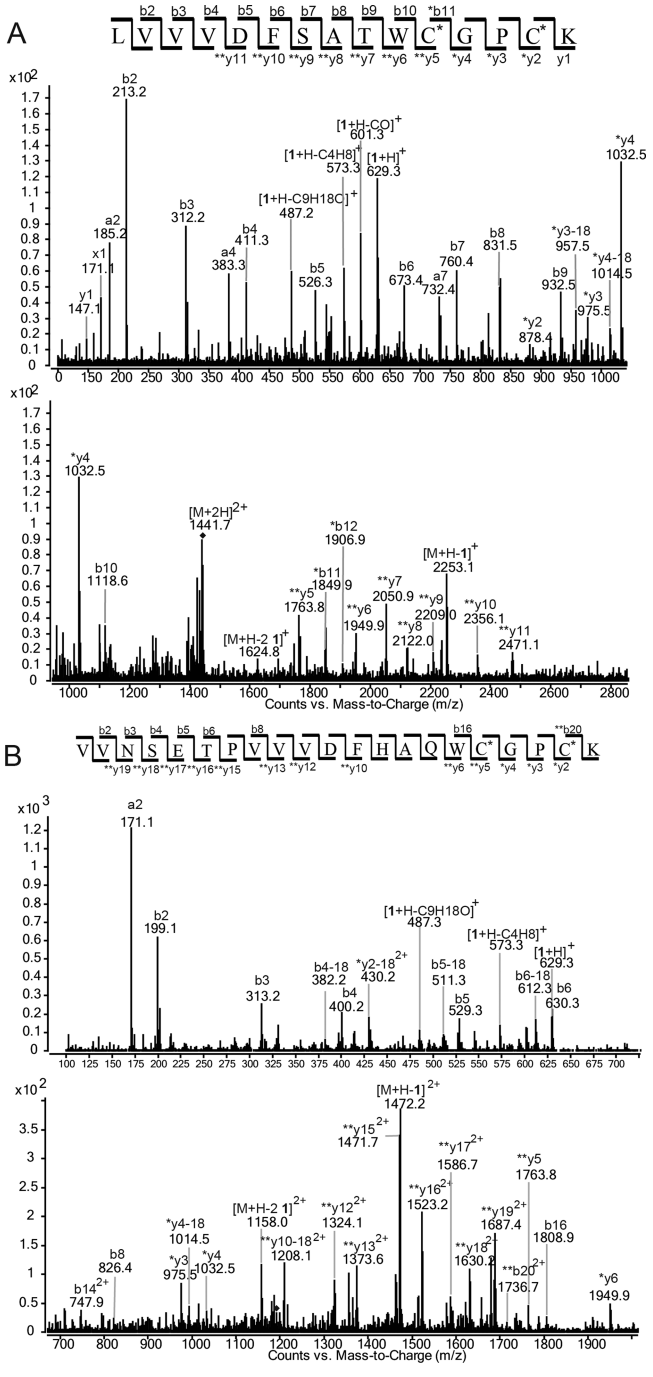

Figure 3. Identification of the gambogic acid (**1**) modification sites in TRX-1 and TRX-2 using LC-ESIQTOFMS. (A) Product-ion spectrum of the $[M + 2H]^{2+}$ at m/z 1442.2. (B) Product-ion spectrum of the $[M + 3H]^{3+}$ at m/z 1191.9. The most common cleavage sites are at the CO−NH bonds that gave rise to the b- and/or the y-ions, and all those marked with an asterisk on the spectrum are ions modified by **1**.

adducts over the surface of both TRX-1 and TRX-2. It is probable that **1**, unlike most known TRX inhibitors,[24−26] contains a large conjugated π-electron system, thereby possessing an extremely strong total electrophility.[27] Generally, the reactive functionality present in highly electrophilic compounds exhibits an extremely high level of chemical reactivity, and these electrophiles typically modify those nucleophilic residues on cellular proteins that are most accessible from the medium.[28] In addition, a common feature

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of catalytic cysteines, and of "noncatalytic" active-site cysteines, as well as those that undergo various forms of oxidative modification in proteomes is hyperactivity.[29,30] The lack of selectivity between **1** and the cysteines on TRX-1 or TRX-2 may therefore also contribute to the intrinsic hyperactivity of the cysteines on these proteins.

Covalent Binding of Gambogic Acid (1) to Endogenous TRX-1 and TRX-2 in Human Hepatoma SMMC-7721 Cells. Considering that **1** readily reacts with reductive GSH[12] and that cancer cells generally have high levels of GSH, it was examined initially whether the interactions between TRX-1/2 and **1** are modulated by this physiological cellular thiol. It was found that the binding interactions between **1** and TRX-1/2 were able to withstand the reducing conditions to some extent when a large amount of GSH was evident during the reaction (data not shown). Thus, it seems that GSH did not completely inhibit the formation of **1**-TRX-1/TRX-2 adducts within cancer cells. Knowing that it has already been demonstrated that **1** causes dysregulation of cellular redox homeostasis in SMMC-7721 cells,[4] cell-based experiments using SMMC-7721 cells were therefore performed to evaluate whether the present in vitro observations are compatible with the in vivo effects caused by **1**. Cells were treated with 3.0 μM biotinylated gambogic acid (**7**, Figure 4A), which retains the α,β-unsaturated ketone moiety and the activity of **1**.[18] Then, the distribution of **7** as well as endogenous TRX-1/2 was examined after a 2 h treatment. In control groups, no biotin staining (blue) was detected, and the TRX-1 or TRX-2 proteins (red) were distributed evenly inside the cells (Figure 4B). However, in treated cells, co-localization of **7** and TRX-1 or TRX-2 was observed (purple) (Figure 4B). Thus, it is very likely that chemical modification on TRX-1/2 proteins by gambogic acid (**1**) occurs in the cellular context.

Moreover, the **1**/TRX-1 and **1**/TRX-2 adducts were also detected in SMMC-7721 cells exposed to **7** using affinity capture (Figure 4C). Thus, these findings supported the ability of gambogic acid (**1**) to react with endogenous TRX-1 or TRX-2 in intact SMMC-7721 cells with a high level of GSH or L-cysteine to an appreciable extent. Of interest, the presence of high concentrations of GSH also could not inhibit completely the reaction with the corresponding target proteins of other natural Michael reaction acceptor molecules, such as leptomycin B,[31] curcumin,[32] and (−)-epigallocatechin-3-gallate.[24] This may be attributed to the high pK_a or hydrophilicity of small reductive molecules such as GSH and L-cysteine, which prevents them from efficiently accessing these electrophilic compounds.[25,30] It is therefore possible that the relatively low pK_a values of the cysteine residues on the functional regions of TRX proteins, as well as the distribution of the hydrophobic amino acid side chains in these regions,[33] play major roles in allowing these proteins to react efficiently with **1**. On the other hand, the high hydrophobicity and unique chemical skeleton of **1** may also facilitate its preferential affinity to the functional domain of TRX proteins.

Inhibitory Effect of Gambogic Acid (1) on the Activities of TRX-1 and TRX-2. An insulin reduction assay was carried out to assess the ability of **1** to inhibit the reductive function of TRX-1/2 and the kinetics of inactivation. As shown in Figure 5A, **1** inhibited insulin reduction both dose- and time-dependently, yielding k_i (or IC_{50}) values of 3.11 ± 0.72 and 1.31 ± 0.44 μM for TRX-1 and TRX-2, respectively, as well as a k_{inact} (inactivation rate constant) of 0.063 ± 0.006 and 0.037 ± 0.0046 min^{-1}, in turn, for TRX-1 and TRX-2. Of note, the inhibitory effect of **1** on TRX-1 is comparable with two high-potency antitumor quinols targeting TRX-1, namely, 4-(benzothiazol-2-yl)-4-hydroxycyclohexa-2,5-dienone and 4-(1-

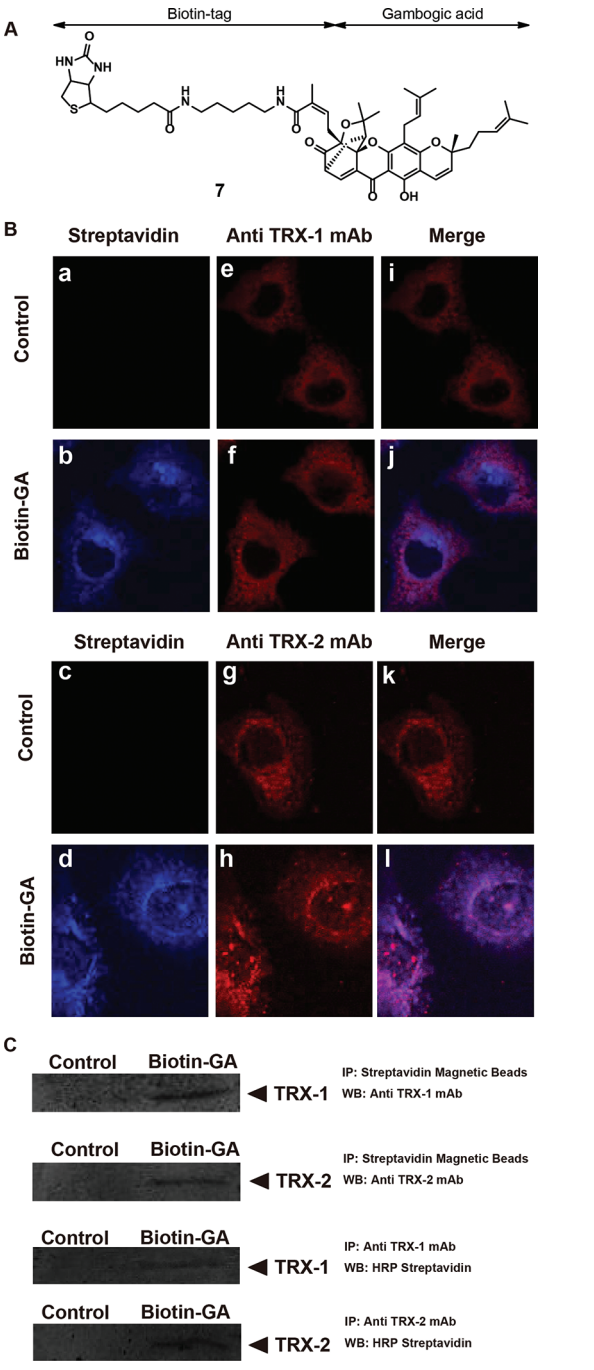

Figure 4. Covalent binding of gambogic acid (**1**) to endogenous TRX-1 and TRX-2 in human hepatoma SMMC-7721 cells. (A) Chemical structure of biotinylated gambogic acid (**7**). (B) Immunofluorescence detection of the cellular accumulation of **7** and the distribution profile of TRX-1/2 in SMMC-7721 cells. DyLight 405 fluorescence (**7**, blue) is shown in the left column of panels a–d; the distribution of TRX-1 and TRX-2 (red) is shown in the center column of panels e, f and g, h, respectively; and the corresponding merged (superimposed) images are shown in the right column of panels i–l (purple represents co-localization). The images were obtained at 1000× magnification. (C) The adducts modified by **1** of TRX1/2 were captured and detected by immunological approaches, as indicated. All experiments were repeated at least three times with the same results.

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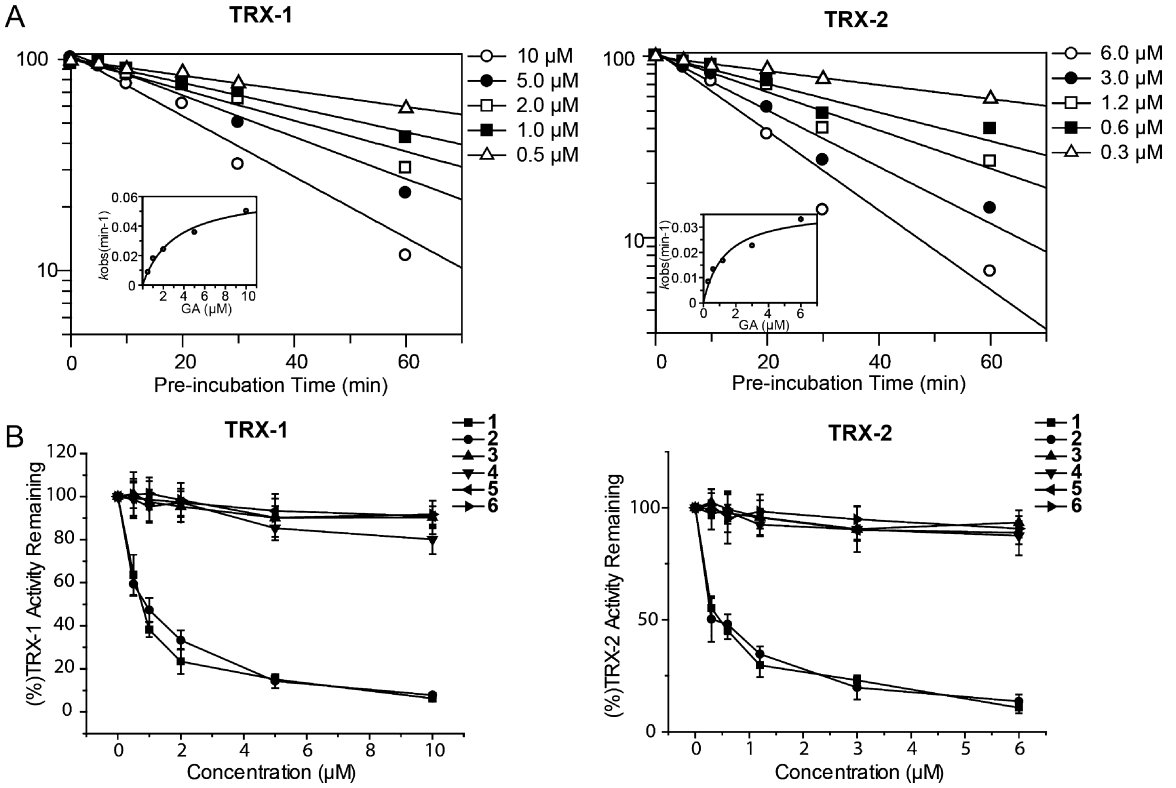

Figure 5. Inhibitory effect of gambogic acid (**1**) on the reductive activities of TRX-1 and TRX-2. (A) Time- and concentration-dependent inhibitory effects of **1** on the insulin reduction activities of TRX-1 and TRX-2, respectively. The slopes of the lines represent the observed first-order rate constants (k_{obs}) of the inactivation reaction at a given concentration of **1**. The insets show the nonlinear regression plot for TRX-1/2 inactivation. (B) Effects of **1** and its analogues on TRX-1/2. Different concentrations of **1** and its analogues (with or without an α,β-unsaturated ketone unit) were preincubated with TRX-1/2 for 60 min at 37 °C and then subjected to the assay systems to determine insulin reduction activity. Points represent mean of triplicate experiments; bars, SD.

benzenesulfonyl-1*H*-indol-2-yl)-4-hydroxycyclohexa-2,5-dienone.[34] The effective inhibitory concentration levels of **1** on TRX proteins would be attainable easily in plasma or tissues in either animals or humans after an iv dose of gambogic acid (**1**),[27,35,36] suggesting that significant inhibition by **1** on TRX proteins could occur in vivo. In addition, in vivo experiments using SMMC-7721 cells were carried out to determine whether **1** can inhibit cellular TRX activity. When SMMC-7721 cells were exposed to different concentrations of **1** for 2 h, respectively, the cellular TRX activity decreased dose-dependently (Figure S1, Supporting Information), indicating that inhibition of TRX may be a significant contributor to the mechanism of action of **1**.

On the other hand, if the irreversible inhibitory effects of **1** on TRXs are mediated by its Michael-reaction activity, an α,β-unsaturated ketone motif should be present in the molecule. Different concentrations of **1** and its analogues were preincubated with TRX-1 or TRX-2 for 60 min at 37 °C and then subjected to bioassay to determine the insulin reduction activity. Indeed, **1** and **2** both gave similar potent inhibitory effects for TRX-1 and TRX-2, whereas the other analogues of **1** showed no effects on these proteins (Figure 5B), confirming that the α,β-unsaturated ketone moiety is required for the inhibitory effect of **1** on TRX proteins.

The data described above demonstrate firmly that **1** can deactivate TRX proteins by covalent binding to the active nucleophilic residues in their functional domain. Considering the key roles of both TRX-1 and TRX-2 in maintaining the cellular ROS homeostasis,[9,10] the inhibitory effects of **1** on TRXs may, at least partially, be involved in the accumulation of ROS induced by this compound in cancer cells. In addition, previously reported cellular responses modulated by **1** are regulated by TRX-1 or TRX-2, such as transcriptional activation of p38-MAPK,[4] p53,[16] and other apoptotic pathways[18] as well as inhibition of Bcl-2 gene expression[15] or the NF-κB pathway.[17] It is possible therefore that the irreversible inhibitory effects of **1** on TRX-1 and -2 may function upstream of these cellular responses.

Of note, because most functional cysteine residues in proteomes are hyper-reactive,[29,30] gambogic acid (**1**) will conceivably interact with a broad range of proteins, especially those in cellular antioxidant systems that bear critical thiol or selenol groups, such as thioredoxin reductase, glutaredoxins, and glutathione S-transferase, although some functional cysteines may be reactive inherently, but inaccessible to **1** for steric reasons. On the other hand, the possibility cannot be ruled out of other protein targets involved in shared cellular responses, such as the aforementioned transcriptional changes. Indeed, some of these gene expression changes can also be accounted for partially by the **1**-induced inhibition of the transferrin receptor[18] or Hsp90.[37] Therefore, future studies will be needed to clarify a more integrated targeting profile by **1** and the network relating protein modification and gene expression changes induced by this compound using systems biology strategies.[38,39]

Collectively, the present results provide at least a plausible molecular basis of ROS toxicity and for the downstream electrophilic cellular responses stimulated by gambogic acid (**1**)

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in cancer cells. Mounting evidence indicates that cancer cells are generally more vulnerable to further oxidative insults induced by compounds that abrogate the antioxidant systems in cells[40] and that a potential synergetic effect for inactivating cancer cells can be achieved by combining different chemical agents that may induce ROS production or alter redox conditions.[41−44] One therapeutic implication is that **1**, as an irreversible inhibitor of two key redox regulators (TRX-1 and TRX-2 proteins), may synergize with other agents that appear to increase ROS generation for selectively killing cancer cells, although this will require more experimental evidence.

EXPERIMENTAL SECTION

Compounds and Reagents. Gambogic acid (**1**) and gambogenic acid (**2**) were provided by Kanion (Jiangsu, People's Republic of China), while 9,10-dihydrogambogic acid (**3**), 10-hydroxygambogic acid (**4**), gambogic acid 10α-sulfonic acid (**5**), gambogic acid 10β-sulfonic acid (**6**), and biotinylated gambogic acid (**7**) were synthesized in our laboratory according to previously described procedures.[12−14,45,46] All chemical structures were confirmed from their UV, NMR, and MS data and by comparison of the spectroscopic data with those published in the literatures. The purities of these compounds were all greater than 96% based on HPLC analysis by an internal normalization method.

Human recombinant TRX-1 and TRX-2 were purchased from ProSpec (Rehovot, Israel). Bovine serum albumin (BSA), paraformaldehyde, Tris, NaCl, EDTA, NP-40, PMSF, NaF, SDS, and DTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal antibodies to both TRX-1 and TRX-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pierce Classic IP kit, DyLight 405 conjugated streptavidin, HRP-conjugated streptavidin, streptavidin magnetic beads, and SuperSignal West Femto maximum sensitivity substrate were obtained from Thermo Pierce (Rockford, IL, USA). HPLC grade acetonitrile and formic acid were obtained from Fisher Scientific (Shanghai, People's Republic of China). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA).

Cell Culture. The human hepatoma SMMC-7721 cell line was purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, People's Republic of China) and passaged in the laboratory for fewer than six months after receipt. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsland, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Beyotime, People's Republic of China). The cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

Mass Spectrometry (MALDITOFMS). hrTRX-1 (0.1 mg/mL, 8.6 μM) or hrTRX-2 proteins (0.1 mg/mL, 5.4 μM) were preincubated in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 1 mM TCEP for 30 min at 37 °C in a Bioyong MK10/20 incubator. Then, gambogic acid (**1**) and its analogues (**2**−**6**) with the same final concentration (0.1 mM for hrTRX-1 and 0.05 mM for hrTRX-2, 5% DMSO) or vehicle alone was added, respectively. The protein samples were mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature. The analyses were performed using an ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) with a nitrogen laser (337 nm). All analyses were in the positive-ion mode, and the instrument was calibrated immediately prior to each series of studies. Analytical data were acquired using Data Explorer software (Applied Biosystems).

Mass Spectrometry (LC-ESIQTOFMS). hrTRX-1 and hrTRX-2 proteins were preincubated as described above. Gambogic acid (**1**) (50 or 20 μM final concentration, 5% DMSO) or vehicle alone was added. After incubation for different time points at 37 °C, 1 μL aliquots were partially injected onto an Agilent 6510 LC-ESI-Q/TOF-MS system (Agilent Technologies, Santa Clara, CA, USA) to detect changes in the protein mass between the intact native TRXs and those treated by **1**. These samples were all separated on a Poroshell 300 SB-C_3 column (2.1 × 75 mm, 5 μm; Agilent Technologies) using a linear gradient of water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) (time: 0−1 min, 95% A; 5−10 min, 5% A; 12−15 min, 95% A). The flow rate was 0.5 mL/min. The QTOF mass spectrometer equipped with an ESI source was set with the drying gas (N_2) flow of 11 L/min, a nebulizer pressure of 45 psig, a drying gas temperature of 300 °C, a capillary voltage of 3500 V, and a cycle time of 0.5 s, with the positive-ion mode used. The fragmentor voltage was set at 150 V. The mass spectra were acquired with a full MS scan (m/z 500−3000). Analytical data were acquired using MassHunter software (Agilent Technologies). The resultant ion spectra were then deconvoluted using a BioConfirm unit (Agilent Technologies).

To determine the sites of reaction between **1** and the TRX proteins, the remaining samples were digested with trypsin (Promega, San Luis Obispo, CA, USA) overnight using a 1:6 ratio of trypsin to the TRX proteins. Digestion was stopped by addition of 0.1% formic acid, and then the digested peptide samples were separated on an Eclipse Plus C_{18} column (2.1 × 100 mm, 3.5 μm; Agilent Technologies) using a linear gradient of 0−1 min, 95% A; 15−17 min, 5% A; 17.1−20 min, 95% A. The flow rate was 0.25 mL/min, and the column temperature was controlled at 30 °C. The mass spectrometer was set as above. The mass spectra were acquired with a full MS scan (m/z 100−3000) and analyzed using MassHunter software. The resultant peptide maps of the TRX proteins treated with or without **1** were reconstructed using a molecular feature extractor and then matched automatically with the defined sequence (with or without modification) using BioConfirm software. Modified peptides were selected for fragmentation in the MS/MS mode to identify sites of modification.

Immunofluorescence Detection. SMMC-7721 cells were cultured in coverslips. When cells reached about 60% confluence, they were treated with either vehicle (DMSO) or 3.0 μM biotinylated gambogic acid (**7**). After a 2 h treatment, cells were washed with PBS and were fixed with 2 mL of 4% paraformaldehyde in PBS at 1 h intervals. After blocking for 30 min with 2% BSA, cells were incubated with primary monoclonal antibodies against TRX-1 (or TRX-2) overnight at 4 °C. Cells were washed with PBS and incubated sequentially with DyLight 405 conjugated streptavidin and rhodamine-conjugated secondary antibodies (Invitrogen, Carlsland, CA, USA) for 1 h at room temperature with shaking, respectively. Coverslips were washed and mounted, and images were observed and captured with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Biotinylated Gambogic Acid (7) Labeling of TRXs in SMMC-7721 Cells. Cells were incubated with 3 μM **7** for 2 h. The cells were harvested and lysed with immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (w/v) NP-40, 0.2 mM PMSF, 0.1 mM NaF).

Cell lysates (1000 μg) were incubated batchwise with 50 μL of streptavidin beads for 15 min at 25 °C with constant shaking. The beads were rinsed three times with lysis buffer. The proteins were eluted by boiling the beads in Laemmli sample buffer (SDS-PAGE buffer) for 5 min, analyzed by SDS-PAGE, and immunoblotted with anti-TRX1 (or anti-TRX2) monoclonal antibody. Detection was performed with the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA).

In addition, the cell lysates were incubated with 3 μg of anti-TRX-1 or anti-TRX-2 antibody overnight at 4 °C. The mixture was then treated with 50 μL of Pierce protein A/G plus agarose and incubated for 1 h at 4 °C. The mixture was washed three times with lysis buffer and boiled with Laemmli sample buffer. The incorporation of **7** into TRX-1 or TRX-2 immunoprecipitates was then subjected to SDS-PAGE and detected with HRP-conjugated streptavidin and Super-Signal West Femto maximum sensitivity substrate using the Chemidoc XRS + System (Bio-Rad, Hercules, CA, USA).

Insulin Reduction Assay. TRX activity was determined using the well-established insulin reduction assay.[24,34]

hrTRX-1 (1 μM) or hrTRX-2 (0.6 μM) was incubated in solvent alone or with **1** at varying concentrations at 37 °C (up to 60 min). At indicated times (0, 5, 10, 20, 30, and 60 min), aliquots (20 μL) of each

incubation sample were removed, subjected to ultrafiltration to remove excess **1**, and added to separate vials containing 1 μM thioredoxin reductase, 1 mM NADPH, 100 mM PBS (pH 7.4), and 5 mM EDTA. Insulin and NADPH were added to initiate the reaction, and the reaction was allowed to proceed for an additional 30 min at 37 °C. The reaction was stopped by the addition of 125 μL of 6 M guanidine-HCl, 100 mM PBS (pH 7.4), and 10 mM DTNB, and absorbance was measured at 412 nm to determine the remaining TRX activity. The inhibitory capacity of the analogues on the TRX-1/TR or TRX-2/TR system-mediated insulin reduction was also detected. Plots and kinetic parameters were all determined by a nonlinear regression analysis using the GraFit 7 software (Erithacus Software Limited, Horley, UK).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Inhibitory effect of gambogic acid (**1**) on cellular TRX activity (Figure S1), NMR and ESIMS data of compounds **1**–**7** (Figures S2–20), and complete Western blotting gels from Figure 4C (Figure S21) are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Chantarasriwong, O.; Batova, A.; Chavasiri, W.; Theodorakis, E. A. *Chemistry* **2010**, *16*, 9944–9962.

(2) Han, Q. B.; Xu, H. X. *Curr. Med. Chem.* **2009**, *16*, 3775–3796.

(3) Ren, Y.; Yuan, C.; Chai, H. B.; Ding, Y.; Li, X. C.; Ferreira, D.; Kinghorn, A. D. *J. Nat. Prod.* **2011**, *74*, 460–463.

(4) Nie, F.; Zhang, X.; Qi, Q.; Yang, L.; Yang, Y.; Liu, W.; Lu, N.; Wu, Z.; You, Q.; Guo, Q. *Toxicology* **2009**, *260*, 60–67.

(5) Ortiz-Sanchez, E.; Daniels, T. R.; Helguera, G.; Martinez-Maza, O.; Bonavida, B.; Penichet, M. L. *Leukemia* **2009**, *23*, 59–70.

(6) Trachootham, D.; Alexandre, J.; Huang, P. *Nat. Rev. Drug Discovery* **2009**, *8*, 579–591.

(7) Tonissen, K. F.; Di Trapani, G. *Mol. Nutr. Food Res.* **2009**, *53*, 87–103.

(8) Powis, G.; Kirkpatrick, D. L. *Curr. Opin. Pharmacol.* **2007**, *7*, 392–397.

(9) Kondo, N.; Nakamura, H.; Masutani, H.; Yodoi, J. *Antioxid. Redox. Signaling* **2006**, *8*, 1881–1890.

(10) Powis, G.; Mustacich, D.; Coon, A. *Free Radical Biol. Med.* **2000**, *29*, 312–322.

(11) Cheng, Z.; Zhang, J.; Ballou, D. P.; Williams, C. H. *Chem. Rev.* **2011**, *111*, 5768–5783.

(12) Yang, J.; Ding, L.; Hu, L.; Qian, W.; Jin, S.; Sun, X.; Wang, Z.; Xiao, W. *Drug Metab. Dispos.* **2011**, *39*, 617–626.

(13) Palempalli, U. D.; Gandhi, U.; Kalantari, P.; Vunta, H.; Arner, R. J.; Narayan, V.; Ravindran, A.; Prabhu, K. S. *Biochem. J.* **2009**, *419*, 401–409.

(14) Zhang, H. Z.; Kasibhatla, S.; Wang, Y.; Herich, J.; Guastella, J.; Tseng, B.; Drewe, J.; Cai, S. X. *Bioorg. Med. Chem.* **2004**, *12*, 309–317.

(15) Zhai, D.; Jin, C.; Shiau, C. W.; Kitada, S.; Satterthwait, A. C.; Reed, J. C. *Mol. Cancer Ther.* **2008**, *7*, 1639–1646.

(16) Gu, H.; Wang, X.; Rao, S.; Wang, J.; Zhao, J.; Ren, F. L.; Mu, R.; Yang, Y.; Qi, Q.; Liu, W.; Lu, N.; Ling, H.; You, Q.; Guo, Q. *Mol. Cancer Ther.* **2008**, *7*, 3298–3305.

(17) Pandey, M. K.; Sung, B.; Ahn, K. S.; Kunnumakkara, A. B.; Chaturvedi, M. M.; Aggarwal, B. B. *Blood* **2007**, *110*, 3517–3525.

(18) Kasibhatla, S.; Jessen, K. A.; Maliartchouk, S.; Wang, J. Y.; English, N. M.; Drewe, J.; Qiu, L.; Archer, S. P.; Ponce, A. E.; Sirisoma, N.; Jiang, S.; Zhang, H. Z.; Gehlsen, K. R.; Cai, S. X.; Green, D. R.; Tseng, B. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 12095–12100.

(19) Weerapana, E.; Simon, G. M.; Cravatt, B. F. *Nat. Chem. Biol.* **2008**, *4*, 405–407.

(20) Sandalova, T.; Zhong, L.; Lindqvist, Y.; Holmgren, A.; Schneider, G. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 9533–9538.

(21) Fritz-Wolf, K.; Kehr, S.; Stumpf, M.; Rahlfs, S.; Becker, K. *Nat. Commun.* **2011**, *2*, 383.

(22) Zhou, Y.; Liu, X.; Yang, J.; Han, Q. B.; Song, J. Z.; Li, S. L.; Qiao, C. F.; Ding, L. S.; Xu, H. X. *Anal. Chim. Acta* **2008**, *629*, 104–118.

(23) Yang, J.; Ding, L.; Hu, L.; Jin, S.; Liu, W.; You, Q.; Guo, Q. *J. Pharm. Biomed. Anal.* **2012**, *60*, 71–79.

(24) Zhang, H.; Cao, D.; Cui, W.; Ji, M.; Qian, X.; Zhong, L. *Free Radical Biol. Med.* **2010**, *49*, 2010–2018.

(25) Shibata, T.; Yamada, T.; Ishii, T.; Kumazawa, S.; Nakamura, H.; Masutani, H.; Yodoi, J.; Uchida, K. *J. Biol. Chem.* **2003**, *278*, 26046–26054.

(26) Callister, M. E.; Pinhu, L.; Catley, M. C.; Westwell, A. D.; Newton, R.; Leaver, S. K.; Quinlan, G. J.; Evans, T. W.; Griffiths, M. J.; Burke-Gaffney, A. *Br. J. Pharmacol.* **2008**, *155*, 661–672.

(27) Yang, J.; Ding, L.; Hu, L.; Jin, S.; Liu, W.; Wang, Z.; Xiao, W.; Yu, Q.; Guo, Q. *Eur. J. Mass Spectrom.* **2010**, *16*, 605–617.

(28) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. *Nat. Rev. Drug Discovery* **2011**, *10*, 307–317.

(29) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. *Nature* **2010**, *468*, 790–795.

(30) Rudolph, T. K.; Freeman, B. A. *Sci. Signaling* **2009**, *2*, re7.

(31) Kudo, N.; Matsumori, N.; Taoka, H.; Fujiwara, D.; Schreiner, E. P.; Wolff, B.; Yoshida, M.; Horinouchi, S. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 9112–9117.

(32) Fang, J.; Lu, J.; Holmgren, A. *J. Biol. Chem.* **2005**, *280*, 25284–25290.

(33) Wakita, C.; Maeshima, T.; Yamazaki, A.; Shibata, T.; Ito, S.; Akagawa, M.; Ojika, M.; Yodoi, J.; Uchida, K. *J. Biol. Chem.* **2009**, *284*, 28810–28822.

(34) Bradshaw, T. D.; Matthews, C. S.; Cookson, J.; Chew, E. H.; Shah, M.; Bailey, K.; Monks, A.; Harris, E.; Westwell, A. D.; Wells, G.; Laughton, C. A.; Stevens, M. F. *Cancer Res.* **2005**, *65*, 3911–3919.

(35) Ding, L.; Huang, D.; Wang, J.; Li, S. *J. Chromatogr. B* **2007**, *846*, 112–118.

(36) Hao, K.; Liu, X. Q.; Wang, G. J.; Zhao, X. P. *Eur. J. Drug Metab. Pharmacokinet.* **2007**, *32*, 63–68.

(37) Davenport, J.; Manjarrez, J. R.; Peterson, L.; Krumm, B.; Blagg, B. S.; Matts, R. L. *J. Nat. Prod.* **2011**, *74*, 1085–1092.

(38) Bottcher, T.; Pitscheider, M.; Sieber, S. A. *Angew. Chem., Int. Ed.* **2010**, *49*, 2680–2698.

(39) Jacobs, A. T.; Marnett, L. J. *Acc. Chem. Res.* **2010**, *43*, 673–683.

(40) Raj, L.; Ide, T.; Gurkar, A. U.; Foley, M.; Schenone, M.; Li, X.; Tolliday, N. J.; Golub, T. R.; Carr, S. A.; Shamji, A. F.; Stern, A. M.; Mandinova, A.; Schreiber, S. L.; Lee, S. W. *Nature* **2011**, *475*, 231–234.

(41) Javvadi, P.; Hertan, L.; Kosoff, R.; Datta, T.; Kolev, J.; Mick, R.; Tuttle, S. W.; Koumenis, C. *Cancer Res.* **2010**, *70*, 1941–1950.

(42) Zhang, X.; Zheng, Y.; Fried, L. E.; Du, Y.; Montano, S. J.; Sohn, A.; Lefkove, B.; Holmgren, L.; Arbiser, J. L.; Holmgren, A.; Lu, J. *Free Radical Biol. Med.* **2011**, *50*, 811–820.

(43) Sun, Y.; St Clair, D. K.; Xu, Y.; Crooks, P. A.; St Clair, W. H. *Cancer Res.* **2010**, *70*, 2880−2890.
(44) Scarbrough, P. M.; Mapuskar, K. A.; Mattsonc, D. M.; Gius, D.; Watson, W. H.; Spitz, D. R. *Free Radical Biol. Med.* **2011**, *52*, 436−443.
(45) Cernuda-Morollon, E.; Pineda-Molina, E.; Canada, F. J.; Perez-Sala, D. *J. Biol. Chem.* **2001**, *276*, 35530−35536.
(46) Yang, J.; Ding, L.; Jin, S.; Liu, X.; Liu, W.; Wang, Z. *J. Chromatogr. B* **2010**, *878*, 659−666.