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ORIGINAL ARTICLE

4-Aryl-1,3,2-oxathiazolylium-5-olate: a novel GST inhibitor to release JNK and activate c-Jun for cancer therapy

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Abstract
Purpose The over-expression of glutathion *S*-transferase Pi (GSTπ) in tumors and inhibitory effect of GSTπ to JNK are two possible causes of the development of drug-resistance in chemotherapy. This research is to develop a novel pH-controlled NO donor to inhibit GSTπ(and to activate the JNK/c-Jun pathway (omit "to induce apoptosis").
Methods Four 4-Aryl-1,3,2-oxathiazolylium-5-olate (OZO) derivatives with varying aryl *para*-substitutions (–H, $–CF_3$, –Cl, and $–OCH_3$) were synthesized. Anticancer activity was determined by MTS assay. GST activity was measured with spectrophotometry using 1-chlro-2,4-dinitrobenzene (CDNB) and GSH as substrates. (omit "Apoptosis was evaluated by annexin V staining and flow cytometry"). c-Jun N-terminal kinase 1 (JNK1) association with GSTπ and activation of c-Jun were evaluated with immunoprecipitation and western blot.
Results OZO derivatives showed anticancer effect against leukemia and breast cancer cells by MTS assay. The relative potency of their anticancer effects is OZO-H > OZO-Cl, OZO-OMe > OZO-CF_3. The anticancer activity of these compounds was correlated with their inhibition of GST activity in cancer cells. The immunoprecipitaion result showed that the treatment of OZO-H released JNK1 from GSTπ-JNK1 complex. Consequently, the treatment of OZO-H in cancer cells induced JNK1 phophorylation and activated c-Jun in cancer cells.
Conclusion OZO-H is a novel GST inhibitor to release JNK1 for activation of JNK/c-Jun pathway (original is "c-Jun to trigger apoptosis in cancer cells"). It provides a new class of GST target compound for anticancer therapy.

Keywords 4-Aryl-1,3,2-oxathiazolylium-5-olates · Glutathion *S*-transferases · c-Jun H2-terminal kinase

Abbreviations

OZO	4-Aryl-1,3,2-oxathiazolylium-5-olate
GST	Glutathion S-transferases
JNK	c-Jun H2-terminal kinase
MAP kinase	Mitogen-activated protein kinase
GSH	Glutathione
SAR	Structure activity relationship

Introduction

Glutathione-*S*-transferases (GSTs) are a family of Phase II detoxification enzymes that catalyze the conjugation of GSH to electrophilic xenobiotics. Eight GST isozymes have been identified and each of them exhibited various functions [2]. Of the GST isozymes, GSTπ has attracted more attention in the recent anticancer studies. GSTπ is over-expressed in various tumors [26, 38, 41] and over-expression of GSTπ in tumors can confer drug resistance [37]. Two mechanisms were proposed for the role of GSTπ

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in drug resistance. First, GSTπ detoxifies anticancer agents via the formation of GSH-conjugate, which is subsequently exported outside the cancer cell [17]. Second, GSTπ is an endogenous inhibitor of c-Jun N-terminal kinase 1 (JNK1) [3, 27, 50]. Inhibition of JNK blocks the signal transduction of MAP kinase pathway, resulting in cancer cell survival [9, 10, 29, 40]. Therefore, GSTπ is identified as a therapeutic target to overcome drug resistance in chemotherapy [6, 21, 48, 51]. Several drug candidates targeting GST were studied for cancer therapy (Fig. 1). These inhibitors were classified into four groups. The first group consisted of GSH conjugates or the peptidomimics [5, 7, 12]. They were competitive inhibitors of GSH's binding site. The modification on the peptide moiety has been extensively studied to either enhance the GST isozyme selectivity [7, 12] or to increase the drug's stability [5, 7]. TLK 199 (or TER 199) [8, 12, 15, 16, 23, 25, 28, 36]) is currently studied in clinical trial. The second group of inhibitors had an electrophilic functional group, which can generate the GSH-conjugate in vivo [31, 33, 42, 43]. A potential problem for the second group of inhibitors is that they react with GSH in the absence of GST enzymes. The third group of inhibitors are suicide inhibitors [47, 52], which generate an active intermediate upon the activation by GSH in the presence of GST. The active intermediate reacts with GST residues in situ to irreversibly inhibit the GST activity. The fourth group inhibitors are prodrugs that release toxin molecules upon the activation by GST [20, 35, 40]. TLK 286 is currently investigated in clinical trials [24, 35].

In this report, we intend to develop a novel class of GSTπ inhibitor through NO donors and study their inhibition mechanism to GSTπ for activation of JNK/c-Jun pathway. Recently, we synthesized several 4-aryl-1,3,2-oxathiazolylium-5-olate (OZO) derivatives (Fig. 2) [19] as novel caged NO donors. OZO was a masked and stabilized form of *S*-nitrosothiol (RSNO) NO donor. OZO was converted to RSNO intermediate through acid-catalyzed hydrolysis. The NO-releasing rate was adjusted by the *para*-substitution on the benzene ring. Unlike other NO donors, OZO did not react with GSH to release NO in the absence of GST. However, GST triggered the NO release from OZO in vitro. The catalysis capacity of GST was completely lost within minutes in the reaction of OZO and GSH. These data suggest that OZO is a novel GST inhibitor (unpublished data). In this paper, we intend to study the following questions: (1) Can OZO derivatives exhibit anticancer effect? (2) Can OZO derivatives inhibit GST activity and regulate JNK/c-Jun pathway in cancer cell? (3) How does structure modification of OZO influence their GST inhibitory effect? and (4) what is the possible molecular mechanism for OZO's GST inhibition?

Materials and methods

Cell lines and chemicals

Drug-sensitive leukemia cells K562 and drug-resistant leukemia cells K562/Dox were grown in RPMI 1640. Adherent cell lines including Panc-1 (human pancreatic carcinoma, epithelial-like cell line), BxPC-3 (human Pancreatic Cancer Cell Line), MCF7 (human breast cancer cell line), MCF7/ADR (human breast cancer cell line resistant to adriamycin) were grown in DMEM/F12. Both RPMI1640 and DMEM/F12 were supplemented with 10% FBS, 100 units/ml of penicillin and 100 mg/ml streptomycin (Invitrogen Gibco Co., Carlsbad, CA, USA). Cells were grown in a humidified 5% CO_2 atmosphere at 37°C.

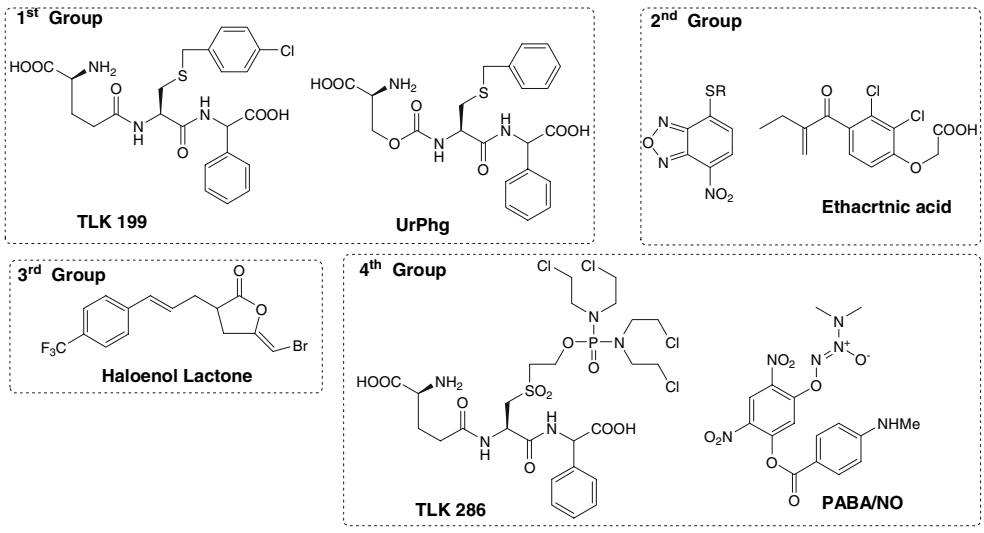

Fig. 1 GST target drug candidates

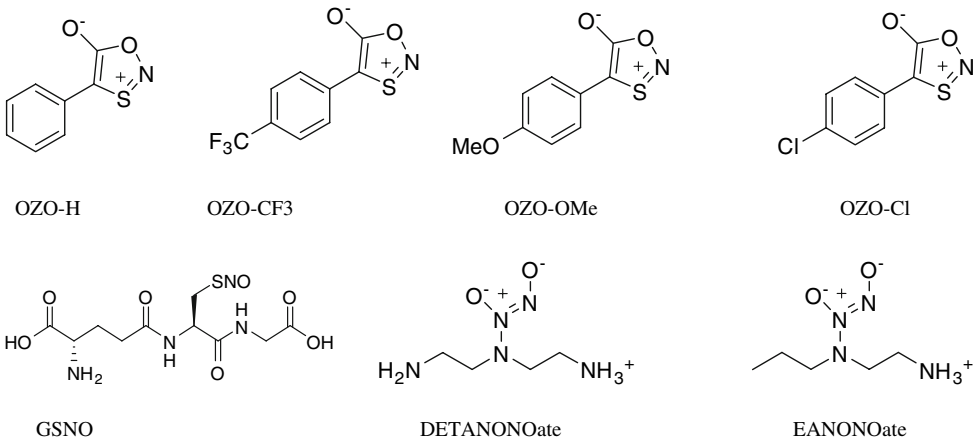

Fig. 2 Chemical structures of OZO derivatives and traditional NO donors

Four OZO derivatives with varying aryl *para*-substituents (–H, $–CF_3$, –Cl, and $–OCH_3$) were synthesized as described previously [19]. GSNO, DETANONOate were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). EANONOate was synthesized in our lab. Chemical structures of the derivatives studied are shown in Fig. 2. Stock solutions of these compounds (20 mmol/l) were prepared in DMSO. The compounds were freshly diluted to the appropriate concentrations in medium with final DMSO concentration not exceeding 0.1%.

Cytotoxicity assays

Cytotoxicity of compounds was determined using the MTS assay. Briefly, cells were seeded in 96-well plates at a density of 3×10^3 cells per well, in 100 µl of medium. After 24 h, the cells were incubated with each of the above compounds, at various concentrations for 72 h. Then MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium) and phenazine methosulfate (PMS), were added directly to the cell culture and incubated for 4 h at 37°C in a humidified, 5% CO_2 atmosphere. The MTS/PMS mixture was metabolized by living cells into formazan. The absorbance of formazan (metabolite of MTS by viable cells) was measured at 490 nm. The absorbance value was used to calculate the surviving cell number. The IC50 values (concentration of a compound that is required to inhibit 50% of cell growth) were calculated with dose-response curves using WinNonlin software.

GST inhibitory effect assays

GST activity was measured using 1-chlro-2,4-dinitrobenzene (CDNB) and GSH as substrates [14]. The GST assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). K562 cells were plated onto six-well dishes (1×10^5 cells/dish). They were then treated in the absence (control) or presence of OZO derivatives (at their IC50 dose on K562 cells) for 72 h. After the indicated time, cells were harvested. The cell pellets were washed with cold PBS, and then resuspended in 100 mmol/l potassium phosphate buffer, pH 6.8. Pellets were then sonicated for 10 s on ice, followed by centrifugation at 10,000 rpm for 15 min at 4°C. Supernatant was used for GST measurement according to the manufacturer's instructions. The absorbance at 340 nm was continuously recorded for 5 min, and the lysate protein content was assayed by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The GST activity was expressed in terms of nanomoles per milligram protein per minute.

JNK/c-Jun pathway signal transduction assays

Immunoprecipitation was performed according to literature [11]. Briefly, 500 µg of protein from total cell lysates was incubated in lysis buffer with 15 µl of anti-JNK1 antibody to a total volume of 500 µl for 2 h at 4°C. Immunocomplexes were absorbed with 25 µl of protein A-Sepharose for 30 min at 4°C. Immune pellets were boiled in SDS sample buffer. The beads were washed thrice with the lysis buffer, separated by SDS-PAGE, and immunoblotted with Polyclonal anti-GSTPi (1:1,000; Assay Designs Inc., Ann Arbor, MI, USA) and anti-JNK1 (1:200; Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) antibodies. The proteins were detected with the ECL system (Amersham Biosciences). The ECL signal was quantified using a scanner and a densitometry program (Scion Image, Scion, Frederick, MD, USA).

Western blot analysis

K562 and MCF7 cells were treated with OZO-H at a concentration double that of their IC50 doses. At each time point analyzed, the cell pellet was washed in cold PBS and resuspended in lysis buffer containing 20 mmol/l Tris−HCl (pH 7.5), 1 mmol/l EDTA, 150 mmol/l NaCl, 1 mmol/l

EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l beta-glycerophosphate, 1 mmol/l Na_3VO_4, 1 μg/ml leupeptin. One-milli moles per litter protease inhibitors (Sigma) were added immediately before use. After a 30-min incubation on ice, cells were disrupted by a 10-s sonication. Lysates were then centrifuged at 13,000×*g* for 20 min at 4°C and supernatants were removed and stored at −80°C. Proteins (40 μg) were loaded on 4–15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Polyclonal anti-c-Jun and anti-JNK1, anti-phospho-activated c-Jun and JNK isoforms (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies. Detection of immunoreactive bands was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

All of the experiments were repeated at least three times. The data were expressed as mean ± SD, and significance was assessed by Student's *t* test. The criterion for statistical significance was $P < 0.05$.

Results

Cytotoxicity of OZO derivatives

The cytotoxicity of OZO derivatives was evaluated against two cancer cell lines, K562 and Panc-1. The IC50 values obtained after 72 h of treatment with each compound are reported in Table 1. Among the compounds tested, OZO derivatives, except OZO-CF_3, show more potent activity than traditional NO donors. Of the OZO derivatives, OZO-H had the lowest IC50. The potency of anticancer activity of these compounds is OZO-H > OZO-Cl, OZO-OMe > OZO-CF_3. These data suggest that either strong electron donating or strong electron-withdrawing substitution on the benzene ring could reduce the activity.

To test if OZO-H exhibit anticancer effects on both drug-sensitive and drug-resistant cancer cells, MTS assay was used to test the cytotoxicity of OZO-H on drug-sensitive leukemia K562, drug-resistant leukemia K562/Dox, drug-sensitive breast cancer cells MCF7 and drug-resistant breast cancer cells MCF7/ADR and BxPC-3. The results are shown in Fig. 3. The IC50 values of OZO-H on K562, MCF7, and BxPC-3 cells were 48.2 μmol/l, 100.1 μmol/l, and 140.2 μmol/l. However, an IC50 value of OZO-H was 240.3 μmol/l against both K562/dox and MCF7/ADR cells. These data suggest that OZO-H exhibit stronger anticancer effect against drug-sensitive cancer cells than against drug-resistant cancer cells (this paragraph was omitted in the revised reversion).

GST inhibitory effect

To test if OZO derivatives inhibit GST activity, we determined the GST activity in leukemia cells after treatment with these compounds at their IC50 for 72 h in K562 cells. OZO-H, OZO-Me, and OZO-Cl significantly decreased the intracellular GST activity, while OZO-CF_3 did not show any effects (Fig. 3). OZO-H was the most potent GST inhibitor, which decreased the intracellular GST activity level from 32.1 to 7.2 nmol/min mg protein. The inhibition of GST activity by OZO derivatives correlated with their cytotoxicity. These suggest that the anticancer activity of OZO derivatives may be through the inhibition of GST activity (Table 1).

Activation of the JNK/c-Jun pathway

JNK is one of the key MAP kinases that trigger apoptosis. GSTπ was reported to be the inhibitor of JNK by

Table 1 Cytotoxicity of OZO derivatives

Compound	IC50 (μmol/l)	
	Panc-1	K562
OZO-H	62 ± 3.6	42 ± 4.3
OZO-Cl	150 ± 11.3	120 ± 8.4
OZO-OMe	126 ± 9.3	120 ± 10.2
OZO-CF_3	>200	>200
GSNO	>200	>200
DETANONOate	>200	>200
EANONOate	>200	>200

IC50 value (μmol/l) was determined by the MTS assay 72 h after the treatment. The percentage of cell survival rate at different derivative concentrations, is used to determine the IC50 value for each compound

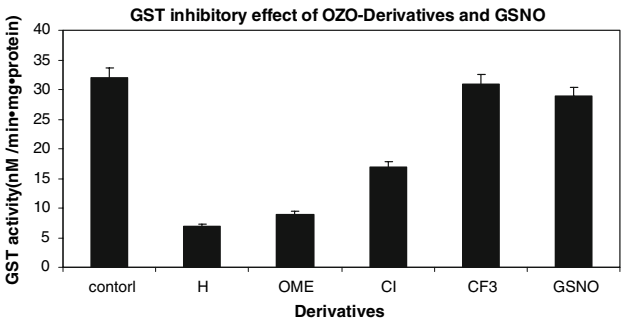

Fig. 3 OZO derivatives inhibit GST activity. The GST inhibitory effect was measured spectrophotometrically at 340 nm in total cell lysates as described in "Materials and methods". The enzymatic activity was normalized by protein concentration (nmol/l min mg protein)

forming the GSTπ-JNK complex via protein–protein interaction. Specific inhibitors of the GSTπ may induce dissociation of the GSTπ-JNK complex, activate JNK and phosphorylate the downstream transcription factor c-Jun [3] to induce apoptosis. To verify whether OZO-H can inhibit GST and activate the JNK pathway to induce apoptosis, we performed the immunoprecipitation and western blot analysis in K562 cells after treatment with OZO-H (Fig. 4). The results showed that OZO-H treatment from 1 to 16 h decreased the amount of GSTπ that is coprecipitated with JNK1 in K562 cells compared with control cells. As shown in Table 2, the ratios of GSTπ/JNK were reduced after treatment with OZO-H; the lowest ratio was 0.64 at 4 h compared to 1.31 of the control (Table 2). This indicates that OZO-H triggers dissociation of the GSTπ from the GSTπ-JNK1 complex (Fig. 4a, Table 2).

In order to test the down stream effects of released JNK1 that released rom GSTπ, we tested if the released JNK1 is phosphorylated to activate the downstream transcritption factor (c-Jun) and western blot analysis was used to detect the phosphorylated JNK1 and c-Jun (Fig. 4b). The data showed that OZO-H treatment for 1–24 h induced phosphorylation of JNK1. As a result, the concentration of phospho-c-Jun was also increased after treatment with OZO-H.

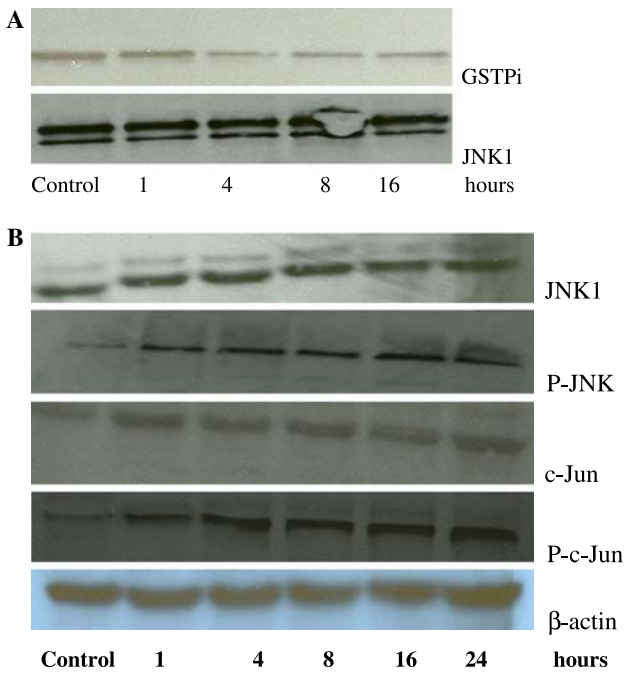

Fig. 4 OZO compound inhibit GST to release JNK for activation of c-Jun. **a** Immunoprecipitation of JNK after treatment with OZO-H. **b** Immunoblotting analysis of JNK1, c-Jun and their phosphoralation (p-JNK, p-c-Jun) *β*-actin was used as control

Table 2 Changes of GSTπ and JNK after drug treatment

Experimental conditions	GSTπ (%)	JNK1 (%)	GSTπ/JNK1
Control	30.3	23.2	1.31
1 h	23.6	20.3	1.16
4 h	12.6	19.8	0.64
8 h	15.4	18.0	0.85
16 h	18.1	19.2	0.94

The percentage of GSTπ and JNK1 at indicated experimental conditions was the percentage of their integral optical density, the sum of their integral optical density as 100%

Discussion

Our data showed that OZO-H was a novel GST inhibitor. It dissociates GSTπ-JNK complex, and then activates the JNK/Jun pathway in cancer cells. The cytotocixity of OZO compounds was correlated to their levels of GST inhibition and activation of JNK and c-Jun.

The MTS assay also demonstrated the structure−activity relationship (SAR) for OZO compounds. Either strong electron-donating or strong electron-withdrawing substitution on the benzene ring could reduce the inhibitory potency of OZO derivatives to GST. Of the four OZO derivatives, OZO-H was the most active, implying that an alkyl group should be employed in the future structure modification of OZO derivatives to improve their ability to inhibit GST. It is worth noting that OZO derivatives exhibited much more cytotoxic effects than traditional NO donors. In addition, the SAR of GST inhibition is different from the SAR of NO-releasing [42]. These findings indicate that OZO's GST inhibition might not depend on NO release.

It was reported that OZO consumes three equivalent thiol anions (thiol anion is an active thiol, similar to the GST-activated GSH) [1]. In addition to the homo-disulfide derived from the thiol agent, a thiol-OZO conjugate was generated (Fig. 5). Computational analysis [1, 33] indicated two electrophilic sites (C-5 and S) on OZO's hetero-ring. Whereas the NO-releasing pathway was initiated via the H_2O-attack at C-5, the thiol/OZO interaction might begin with the thiol-attack at either S atom or C-5 of the hetero-ring [1]. Based on these data, we propose two possible inhibition mechanisms. (1) GST inhibition comes from the generation of GSH-OZO conjugate. Unlike other GST inhibitors, negligible reaction occurs between OZO and GSH alone at physiological conditions in the absence of GST activation (unpublished data). In view of this property, OZO is similar to organic nitrate substrates for anticancer activities [4, 13, 36, 39, 49]. (2) OZO is activated by GSH/GST complex to generate a reactive intermediate, which is

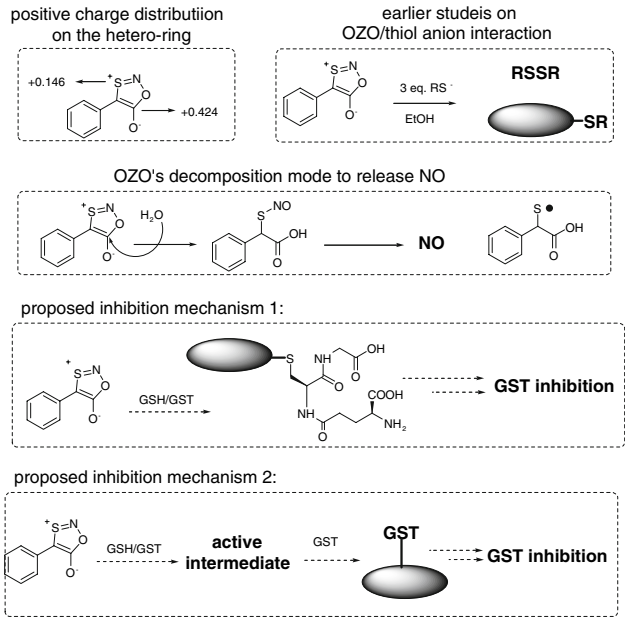

Fig. 5 Possible mechanism of GST inhibition by OZO derivatives

captured by the GST residue in situ. Cys47 of GSTπ is one of the candidate residues, which is highly reactive [22] towards electrophiles [18, 30, 34, 44–46] and located on a loop near the GSH binding site [32]. Enzymatic experiments to elucidate the true inhibition mechanism are currently underway. This hypothesized mechanism can be employed to explain the SAR observed in this work. The electron-donating substitution (e.g., OMe) could deactivate the OZO substrate, resulting in reduced GST inhibition efficacy. The electron-withdrawing substitution (e.g., CF_3) could destabilize the OZO substrate and OZO-CF_3 substrate is susceptible to water media and prefers the NO-releasing decomposition pathway at physiological conditions. Therefore, the NO-releasing decomposition may contribute little to the GST inhibition.

In summary, the current study identifies OZO-H as a novel GST inhibitor to release JNK1 for activation of JNK/c-Jun pathway. It provides a new class of compound to inhibit GST from anticancer activity.

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