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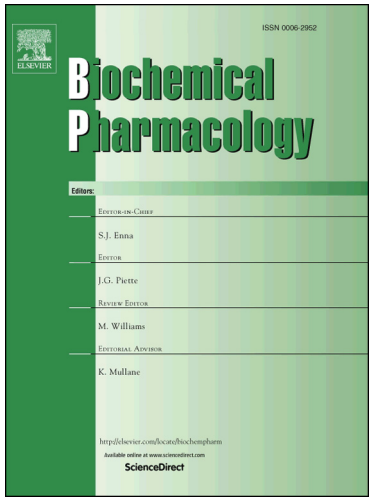

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New tanshinone I derivatives S222 and S439 similarly inhibit topoisomerase I/II but reveal different p53-dependency in inducing G2/M arrest and apoptosis

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Abstract

Tanshinone I (Tanshinone-1), a major active principle of the traditional Chinese medicine *Salvia miltiorrhiza*, possesses excellent anticancer properties, including inhibiting proliferation, angiogenesis and metastasis and overcoming multidrug resistance (MDR). However, its direct anticancer molecular target(s) remain unknown. Here we report that tanshinone-1 and its two new derivatives, S222 and S439, directly inhibit DNA topoisomerase I/II (Top1/2). With significantly improved water solubility, S222 and S439 displayed 12- and 14-times more potent proliferative inhibition than their parent tanshinone-1 in a panel of 15 cancer cell lines. Both retained tanshinone-1's anti-MDR and anti-angiogenesis properties and its capability to reduce the phosphorylation of Stat3 at Tyr705 with apparently enhanced efficacy and in these regards, S439 was also slightly more potent than S222. Both derivatives and tanshinone-1 directly inhibited Top1 and Top2 at molecular and cellular levels; the derivatives displayed similar potency but both were more potent than tanshinone-1. The inhibition of S222 and S439 on Top1 and Top2 was also more potent than that of the Top1 inhibitor hydroxylcamptothecin and the Top2 inhibitor etoposide, respectively. Consistently, tanshinone-1 and its derivatives induced DNA double-strand breaks, G2/M arrest and apoptosis. Unexpectedly, the derivatives demonstrated different p53-dependency in inducing both cell cycle arrest and apoptosis. S222 showed no obvious p53-dependency. In contrast, S439 induced more G2/M arrest in p53-proficient cells than in p53-deficient cells while its apoptotic induction was the opposite. However, their proliferative inhibition was independent of

the p53 status. Due to their structures different from the known Top1, Top2 and dual Top1/2 inhibitors, our results indicate that tanshinone-1 and its derivatives are a new type of dual Top1/2 inhibitors.

Keywords: Tanshinone-1; S222; S439; anticancer targets; DNA topoisomerases; p53

1. Introduction

Tanshinone I (Tanshinone-1) is an active principle of *Salvia miltiorrhiza* (Danshen), a famous traditional Chinese medicine that has been widely used in China for centuries. Tanshinone-1 possesses excellent anticancer properties. It has anticancer and anti-metastasis activities. It also overcomes tumor multidrug resistance (MDR) and inhibits tumor angiogenesis [1-4]. However, tanshinone-1 is characteristic of weak biological activity (IC_{50}: ~40 μM) [1-4], extremely low aqueous solubility (<10^{-4} mg/mL), poor metabolic stability and pharmacokinetic properties ($t_{1/2}$ = 0.17 h; F = ~0%) [3], severely impairing its potential for cancer therapy and hampering the investigations on its anticancer molecular target(s) and mechanisms of action.

We thus conducted broad chemical modifications on tanshinone-1 and obtained 2 new derivatives S222 and S439 (Figure 1a) with improved anticancer activities and drug-like properties [3]. Specifically, their capacity to inhibit the proliferation of cancer cells was enhanced by more than 10-fold (Please refer to Reference 3 and the section of Results). Their water solubility and pharmacokinetic properties also

showed significant amelioration. Moreover, S222 displayed significant *in vivo* anticancer activity at well-tolerated doses [3].

Deregulation of cellular signaling transduction such as signal transducer and activator of transcription 3 (Stat3) frequently occurs in MDR tumor cells. Constitutively activated Stat3 is phosphorylated at its Tyr705 (p-Y705-Stat3), which promotes cell proliferation and survival, and has been revealed to drive tumor drug resistance, angiogenesis and metastasis [1, 2]. In this study, we first examined the anti-proliferation, anti-MDR, reducing p-Y705-Stat3 and anti-angiogenesis activities of S222 and S439. Then we focused on the discovery of their primary anticancer molecular target(s) and mechanisms of action. We found that tanshinone-1 and its two derivatives S222 and S439 concurrently inhibited DNA topoisomerase (Top)1 and Top2, two well-known anticancer molecular targets [5], in both cell-free and cellular systems. Consistently, both S222 and S439 induced DNA double-strand breaks (DSB). S439 induced G2/M arrest only in p53-proficient cells but caused much more apoptosis in p53-deficient cells than in p53-proficient cells. In contrast, S222 induced both G2/M arrest and apoptosis in a p53-independent manner. However, tanshinone-1, S222 and S439 all exerted proliferative inhibition independently of the p53 status. These results indicate that the tanshinone-1 derivatives S222 and S439 are dual inhibitors of Top1 and Top2.

2. Materials and Methods

2.1. Reagents and Antibodies

S439 and S222 were prepared according to our previous procedure [3] with the purity of over 98% determined by high performance liquid chromatography. Tanshinone-1 was purchased from Selleck (Houston, TX). Doxorubicin, vincristine, cisplatin, oxaliplatin, temozolomide and cyclophosphamide were obtained from Melonepharma (Dalian, China). All tested compounds were dissolved at 10 mM in 100% dimethyl sulfoxide (DMSO) as stock solutions, aliquoted, stored at -20°C and diluted to desired concentrations in normal saline immediately prior to each experiment. Antibodies against Histone H3 (sc-8654), γH2AX (sc-101696), p-S1981-ATM (sc-47739), ATM (sc-23921) and Chk1(sc-8408) were from Santa Cruz Biotechnology (Santa Cruz, CA), Stat3 (#9139), p-Y705-Stat3 (#9145), p-S727-Stat3 (#9134), Top2α (4733S), p-S428-ATR (#2853), ATR (#13934), p-S317-Chk1 (#2344), p-T68-Chk2 (#2661) and Chk2 (#6334) from Cell Signaling Technology (Danvers, MA), β-Actin (AF0003) was from Beyotime (Shanghai, China), and Top1 from BD Pharmingen (Franklin Lakes, NJ). RNase A (ST576) and propidium iodide (PI, ST511) were from Beyotime. The AnnexinV-FITC/PI apoptosis detection kit was from Keygen (Nanjing, China).

2.2. Cell Culture

Human cancer KB, UWB1.289, UWB1.289+BRCA1, HCT116 (p53-proficient), HCT15, HT29, MDA-MB-436, MDA-MB-231, MDA-MB-468, MCF7 and A549 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SW620, MX-1, BEL-7402, SPC-A4, SMMC-7721 cell lines were kept in the Shanghai Institute of *Materia Medica* of the Chinese Academy of Sciences (Shanghai,

China). CAL-51 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). SKOV-3 cells were from the Japanese Foundation of Cancer Research (Tokyo, Japan). The vincristine-selected resistant cell line KB/VCR was obtained from Zhongshan University of Medical Sciences (Guangzhou, China). Human leukemia K562 cells and adriamycin-selected resistant K562/A02 cells were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). HCT116/p53$^{-/-}$ (p53-deficient) cells was kindly provided by Dr. Ling-Hua Meng (Shanghai Institute of *Materia Medica*, Shanghai, China.). During this study, all the cell lines were authenticated using the short tandem repeat (STR) profiling at Shanghai Genesky Bio-Tech CO (Shanghai, China). Cells were also periodically authenticated with morphologic inspection and tested for mycoplasma contamination. Cells were cultured according to the supplier's instructions.

2.3. Proliferative inhibition assays

Proliferative inhibition of different compounds in adherent and suspended cells was measured by sulforhodamine B (SRB; Sigma, St. Louis, MO) assays and Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) assays, respectively. Cells were seeded into 96-well plates, cultured overnight, and treated with gradient concentrations of the tested compounds for 72 hours. Optical density for both assays was read with a SpectraMax 190 (Molecular Devices, San Jose, CA). Averaged IC_{50} values were calculated using the Logit method from three independent experiments and expressed as mean ± SD.

2.4. Western blotting analyses

Standard Western blotting analyses were performed as described previously [1].

2.5. Migration assays [2]

The migration assay was done in the transwell Boyden chamber (Corning Costar, NY). HMEC-1 cells (2×10^5 cells per well) in the serum-free MCDB131 medium containing the indicated tested compounds were added to the upper compartment of the chamber. The lower compartment contained MCDB131 medium supplemented with 20% fetal bovine serum (FBS; Gibco™, Thermo Scientific, Waltham, MA) and the same concentration of the corresponding compounds. After 6 h incubation at 37℃, cells still staying on the upper face of the transwell membrane were removed with a cotton swab. The migrated cells on the lower side of the transwell membrane were fixed with 90% ethanol and then stained with 0.1% crystal violet in 0.1 M borate and 2% ethanol (pH 9.0). After being photographed, the stained cells on the lower side were subsequently extracted with 10% acetic acid. The absorbance values were read with a SpectraMax 190 (Molecular Devices, San Jose, CA) at 600 nm.

2.6. Tube formation assay [2]

Chilled liquid Matrigel (BD Biosciences, Franklin lake, NJ) was dispensed onto 96-well plates (50 μL per well) and allowed to solidify (37℃, 1 h). Then HMEC-1 cells (2×10^4 cells per well) were seeded onto the gel and cultured in the medium containing the indicated tested compounds at 37℃ for 6 h. The enclosed networks of complete tubes from randomly chosen fields of three independent experiments were counted and photographed under a microscope (Olympus, Tokyo, Japan).

2.7. Top1 or Top2-mediated supercoiled pBR322 plasmid relaxation

DNA relaxation assays were conducted as described previously [6]. For Top1 assays, the reaction buffer contained 0.5 μg supercoiled pBR322 DNA (Takara, Japan), 1 unit of Top1 (Takara, Japan), 2 μl 10× DNA Top1 buffer (350 mM TrisHCl, pH8.0; 720 mM KCl; 50 mM $MgCl_2$; 50 mM DTT; 50 mM spermidine), 2 μl 0.1% BSA and sterilized distilled water up to 20 μl. For Top2 assays, the reaction buffer contained 0.25 μg supercoiled pBR322 DNA, 2 units of Top2 (TopoGEN, Port Orange, FL), 4 μl 10× DNA Top2 buffer (mixed by buffer A and B in the Top2 assay kit, TopoGEN, Port Orange, FL) and sterilized distilled water up to 20 μl. Relaxation reaction was done at 37°C for 30 min and stopped by adding 5 μl stop buffer (TopoGEN, Port Orange, FL). DNA electrophoresis was carried out on a 1% agarose gel in TAE buffer at 100 V for 1 h. DNA bands were stained in 1 μg/ml of GelRed solution (BioTium, Hayward, CA) and photographed with a Gel Document System (GELDOCXR+, Biorad, Berkeley, CA).

2.8. Top1/2-DNA cleavable complex trapping assays [7]

Cells (1×10^6) were pretreated with the indicated tested compounds for 1 h. Then the cells were collected and washed by PBS. A Subcellular Protein Fractionation Kit (#78840) from Thermo Scientific (Waltham, MA) was used to separate and obtained the nuclear-soluble and chromatin-binding components following the manufacturer's instructions. Top1/2-DNA cleavable complex was analyzed by Western blotting and histone H3 was used as the loading control.

2.9. Cell cycle assays [8]

Cells treated with the tested compounds for the indicated time were collected and washed with PBS, fixed with precooled 70% ethanol at 4°C overnight. Staining went along in PBS containing 40 μg/ml RNase A and 10 μg/ml propidium iodide in the dark for 15 min. Cells (at least 1×10^4 cells per sample) were detected with FACS Calibur (BD Biosciences, Franklin Lakes, NJ).

2.10. Annexin V-FITC apoptosis detection [8]

Cells treated with the tested compounds for the indicated time were collected and washed with PBS. Then the cells were co-stained using an AnnexinV-PI apoptosis detection kit (Keygen, Nanjing, China) as indicated. Fluorescence of the cells was immediately determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.11. Statistical analysis

Data were expressed as mean ± SD. Comparison between two groups was analyzed using the Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Both S439 and S222 display apparently improved proliferative inhibition while retaining the anti-MDR activity of tanshinone-1

Both S439 and S222 are chemically modified from tanshinone-1 (Figure 1a) and show significantly improved drug-like properties [3]. In a panel of 15 cancer cell lines with different tissue origins, S439 and S222 displayed apparent proliferation inhibition with averaged IC_{50} values of 1.56 μM (0.67~4.51 μM) and 1.79 μM (0.53 ~6.11 μM), respectively. The anti-proliferative efficacy of S222 and S439 was

approximately 12 and 14 times that of their parent tanshinone-1 (its averaged IC_{50} value was 21.49 μM, ranging from 6.30 μM to 74.71 μM), respectively (Figure 1b). The derivatives caused roughly equal cytotoxicity in those tumor cells although S439 appeared to be slightly more potent than S222. Similar to their parent tanshinone-1, both showed no apparent selectivity except that liver cancer cells were approximately 4-fold lower than the average sensitivity.

We previously reported that tanshinone-1 killed MDR cells independently of drug transporters [1]. Encouragingly, both S439 and S222 basically retained this prominent activity of tanshinone-1 (Figure 1c and d). Three MDR sublines (KB/VCR established with the tubulin inhibitor VCR and both MCF7/ADR and K562/A02 established with the Top2 inhibitor ADR), which express drug transporters including P-glycoprotein (P-gp) and MDR-associated protein 1 (MRP1) [1], displayed 91, 398 and 203-fold resistance to vincristine (KB/VCR) or adriamycin (MCF7/ADR and K562/A02), respectively (Figure 1c). In contrast, S439 and S222 had an averaged resistance factor (RF) of 0.85 and 1.68 in these MDR sublines, respectively (Figure 1d). Therefore, S439 was approximately 1-fold more potent than S222 regarding their anti-MDR activity.

3.2. Both S439 and S222 reduce the phosphorylation of Stat3 at Tyr705 and inhibit migration and tube formation of microvascular endothelial cells

Tanshinone-1 has been shown to reduce p-Y705-Stat3, which contributes to its anti-MDR [1] and anti-angiogenesis activities [2]. Similarly, the two derivatives S439

and S222 reduced the levels of p-Y705-Stat3 in a concentration-dependent manner while not altering the levels of p-S727-Stat3 and total Stat3 in MDR KB/VCR cells and corresponding parental KB cells (Figure 2a). However, S439 resulted in apparently more reduction of p-Y705-Stat3 than S222 at same concentrations, and even the treatment with 10 μM of S222 caused more remaining p-Y705-Stat3 than the treatment with 2.5 μM of S439 (Figure 2a). The data indicate that S439 is stronger than S222 regarding their abilities to reduce p-Y705-Stat3, which is consistent with their anti-MDR activity and anti-angiogenesis efficacy. In addition, tanshinone-1 and its two derivatives had similar trends of reducing p-Y705-Stat3 in HCT116 cells with or without the wild-type p53 although the base level of p-Y705-Stat3 in the p53-deficient cells was lower than in the p53-proficient cells (Figure 2b). The data indicate that their reduction of p-Y705-Stat3 is independent of the p53 status.

Consistent with their reduction of p-Y705-Stat3, both S439 and S222 inhibited migration and tube formation of the microvascular endothelial HMEC-1 cells in a concentration-dependent manner and both the derivatives were significantly more potent than the parent tanshinone-1 (Figure 2c-f). Moreover, S439 was significantly more potent than S222, especially at lower concentrations.

Together, the two derivatives S439 and S222 retain the primary anticancer effects of their parent tanshinone-1, including reduction of p-Y705-Stat3, anti-MDR and anti-angiogenesis activities, and have an apparent increase in their efficacy. Moreover, S439 is more potent than S222 in all these tested aspects.

3.3. Tanshinone-1 and its two derivatives inhibit Top1 and Top2 concurrently

Like many natural products, the direct anticancer target(s) of tanshinone-1 remain unknown, partly because of its poor water solubility and weak anticancer activity [1-4]. Due to the apparent improvements in water solubility [3] and anti-proliferative activity of S439 and S222, we endeavored to examine their effects against multiple known anticancer molecular targets. Consequently, we found that both S439 and S222 and their parent tanshinone-1 directly inhibited Top1 and Top2 (Figure 3a and 3b). The classical Top1 inhibitor hydroxylcamptothecin (HCPT) and Top2 inhibitor etoposide (VP-16) obviously suppressed Top1 (Figure 3a) and Top2 (Figure 3b)-mediated pBR322 supercoil relaxation, respectively. Similarly, S439 and tanshinone-1 inhibited the pBR322 supercoil relaxation driven by both Top1 and Top2 in a concentration-dependent manner. Moreover, S439 inhibited Top2 more potently than Top1 because at 25 μM, it almost completely inhibited the enzymatic activity of Top2 but had not any observable effect on that of Top1 (Figure 3a and 3b). In inhibiting Top1, S439 and tanshinone-1 were weaker than HCPT at the same concentration (200 μM) (Figure 3a). In contrast, S439 was a more potent Top2 inhibitor compared with VP-16 because 25 μM of S439 inhibited the Top2-mediated pBR322 supercoil relaxation more potently than 200 μM of VP-16 (Figure 3b).

Similar to S439, S222 at 25 μM also caused approximately complete inhibition of Top2 enzymatic activity (Figure 3b). Curiously, however, the DNA bands of the samples treated with S222 at 25-200 μM obviously lagged behind all others in the Top1 inhibition assays (Figure 3a), making difficult to judge whether S222 inhibited

Top1 or not. One of possible reasons for this phenomenon is that S222 might intercalate into DNA (pBR322). So we compared S222 with several DNA intercalators in the presence and/or absence of Top1 and/or the corresponding buffer (Figure 4a). The result strongly suggests the Top1 buffer as the causal factor. When comparing the two buffers for Top1 and Top2 assays. We thus conducted experiments with different components at different conditions. The results showed that except for ATP, the only difference was spermidine (Figure 4b). In the presence of DNA (pBR322), only addition of spermidine indeed led to the S222-mediated lagging of DNA migration (Figure 4b). However, the cause remains to be further clarified.

Nuclear Top1 and Top2 can act on DNA and form transient, generally undetectable enzyme-DNA cleavable complexes (Topcc), which can be transformed into persistent, easily detectable Topcc by their corresponding inhibitors [5]. Using this property, we treated SW620 cells separately with tanshinone-1, its two derivatives and positive controls (HCPT and VP-16) and then made subcellular protein fractionation to fractionate proteins into nuclear-soluble and chromatin-bound fractions as described previously [7] followed by Western blotting. As expected, the Top1 inhibitor HCPT and the Top2 inhibitor VP-16 caused the formation of Top1cc and Top2cc, respectively (Figure 3c). S439 and S222 resulted in large amounts of both Top1cc and Top2cc concurrently at 2-4 μM (Figure 3d), indicating that both S439 and S222 can inhibit Top1 and Top2 simultaneously. In contrast, tanshinone-1 led to only a trace of Top1cc and Top2cc, even at 20 μM (Figure 3c), showing that the two derivatives are much more potent than tanshinone-1 in inhibiting nuclear Top1

and Top2. Consistently, treatments with either S439 or S222 increased the accumulation of γH2AX, a biomarker for DSB, in a time-dependent manner. Moreover, S222 caused more increase in the levels of γH2AX than S439 and tanshinone-1 at the same concentration (1 μM) and exposure time (Figure 3e).

Together, the data indicate that tanshinone-1 and its two derivatives are dual Top1/2 inhibitors; in inhibiting Top1 and Top2, both S439 and S222 are significantly more potent than their parent tanshinone-1 and S222 appears to be slightly more potent than S439.

3.4. S439 induces significantly more G2/M arrest in p53-proficient cells than in p53-deficient cells while S222 induces approximate degrees of G2/M arrest in both p53-proficient and deficient cells

We then determined the effects of S439 and S222 on the cell cycle progression of SW620 cells. S222 induced typical G2/M arrest even at 0.5 μM (Figure 5a and 5b). Unexpectedly, however, S439 did not induce significant G2/M arrest even at 2 μM (Figure 5a and 5b), at which S439 inhibited both Top1 and Top2 (Figure 3d) and led to DSBs (Figure 3e) in the same SW620 cells. When examining the genetic background of the SW620 cell line, we learned it was p53-mutated (R273H; P309S) [9]. Therefore, we used a pair of HCT116 cells that are p53-proficient (wild-type) and p53-deficient, respectively, to confirm the possible p53-dependency. The result showed that S222 induced significant G2/M arrest in both p53-proficient and deficient HCT116 cells while S439 did only in p53-proficient cells (Figure 5a and 5c).

Moreover, S439 was weaker than S222 in inducing G2/M arrest even in the p53-proficient HCT116 cells at the same concentrations (Figure 5a and 5c; Supplementary Figures 1a and 1b). Similar phenomena were also observed in other p53-proficient cells (MCF7) and p53-deficient cells (HeLa and KB cells) treated with S439 and S222 (Data not shown).

However, the difference of p53-dependency in inducing G2/M arrest by S439 and S222 was not reflected in their DNA damage responses. At the same treatment conditions (*i.e.*, 4 μM for 6 h), S439 and S222 caused similar degrees of increases in DSBs, the phosphorylation of ataxia-telangiectasia mutated (ATM) at Ser1981 (p-S1981-ATM), checkpoint kinase (Chk1) at Ser317 (p-S317-Chk1) and Chk2 at Thr68 (p-T68-Chk2) in both p53-proficient and deficient HCT116 cells. Neither S439 nor S222 affected the levels of p-S428-ataxia-telangiectasia mutated and Rad3 related (p-S428-ATR) (Figure 5e). In addition, tanshinone-1 elicited similar but much weaker DNA damage responses (Figure 5e). Moreover, similar results were observed in p53-mutated SW620 cells and p53-wildtype MCF7 cells (Data not shown).

3.5. Tanshinone-1 and S439 induce significantly more apoptosis in p53-deficient cells than in p53-proficient cells while S222 causes similar degrees of apoptotic induction in those cells

The majority of Top inhibitors exert cytotoxicity by inducing apoptosis [10, 11]. Similarly, tanshinone-1 and its two derivatives were able to induce apoptosis (Figure 6a-d). However, their apoptotic induction showed different p53-dependency from

their arresting G2/M phase. In p53-mutated SW620 cells, both S439 and S222 induced apoptosis in a concentration-dependent manner but S439 was significantly more potent than S222 (Figure 6a and 6b). Moreover, S439 induced significantly more apoptosis in p53-deficient HCT116 cells than in the p53-proficient ones while S222 elicited similar degrees of apoptosis in those cells (Figure 6c and 6d). Tanshinone-1 was similar to S439 in inducing apoptosis though less potent.

To further learn the effect of the p53 status on their cytotoxicity, we determined their proliferative inhibition in p53-proficient and deficient HCT116 cells. S439 and S222 displayed approximate IC_{50} values of 0.62 μM and 0.97 μM (for S439) and 0.62 μM and 1.04 μM (for S222) in the p53-proficient and deficient cells, respectively. The IC_{50} values of tanshinone-1 had no significant difference in those cells either (Figure 6e). The data reveal that tanshinone-1 and its two derivatives are independent of the cellular p53 status in their anti-proliferative activity.

4. Discussion

We previously demonstrated that tanshinone-1 had prominent anti-MDR and anti-angiogenesis activities, both of which were associated with its ability to reduce the phosphorylation of Stat3 at Tyr705 [1, 2]. In this study, we identified its potential direct anticancer molecular targets and investigated the possible mechanisms of action by using its two new derivatives, S222 and S439 [3]. Our data show that the proliferative inhibition of S222 and S439 is approximately 12- and 14-times more potent than that of tanshinone-1, respectively. Moreover, both derivatives retain

tanshinone-1's anti-MDR and anti-angiogenesis properties and its capability to reduce p-Y705-Stat3 with apparently improved potency. In all these regards, S439 appears to be more potent than S222. These results indicate that both the derivatives keep the predominant anticancer characteristics of tanshinone-1, which allows us to use them to probe the common, direct molecular targets.

Our cell-free and cellular experimental results reveal that tanshinone-1, S222 and S439 directly target Top1 and Top2 concurrently. In inhibiting Top1 and Top2, S222 and S439 are similar but more potent than tanshinone-1. Both S222 and S439 inhibit Top2 more potently than Top1 in the cell-free system although they exert similar degrees of suppression on both enzymes in the cellular systems. In addition, the inhibition of both derivatives on Top2 appears to be more potent than the classical Top2 inhibitor VP-16. However, tanshinone-1 and its derivatives are structurally different from the known Top1 inhibitors [10, 12-14], Top2 inhibitors [5, 15-19] and dual Top1/2 inhibitors [20-25]. Therefore, they are a new type of dual Top1/2 inhibitors. Different from tanshinone-1 and S439, notably, S222 might intercalate into DNA in the presence of spermidine, an important metabolite in animals. However, its biological implication remains to be further clarified.

As other topoisomerase inhibitors [10, 11], tanshinone-1 and its derivatives induce DSBs, G2/M arrest and apoptosis. Unexpectedly, they show different dependency on p53. S222 displays no significant p53-dependency but S439 shows the opposite p53-dependency in inducing cell cycle arrest and apoptosis. S439 induces significantly more G2/M arrest in p53-proficient cells than in p53-deficient cells

while it elicits significantly more apoptosis in p53-deficient cells than in p53-proficient cells. In this aspect, tanshinone-1 is similar to S439 rather than S222. However, such different p53-dependency does not affect their induction of DSBs, DNA damage signaling transduction or proliferative inhibition. Thereby, the importance of p53-dependency in inducing cell cycle arrest and apoptosis remains to be established for tanshinone-1 and its derivatives.

Nevertheless, S222 and S439 display apparent difference in their *in vivo* anticancer activity. S222 at 10 mg/kg (*i.v.*) or 30 mg/kg (*i.p.*) caused similar, significant growth inhibition of HCT116 xenografts in nude mice ($p<0.01$) [3]. However, S439 even at 100 mg/kg (*i.p.*), a lethal dose that can cause the death of nude mice (specifically, one died among 5 treated animals), produced only 10.08% tumor growth inhibition ($p>0.05$). The data indicate that S222 is much more potent than S439 in their *in vivo* anticancer activity. The possible reason for this might be that S222 has better aqueous solubility and metabolic stability than S439 [3] because both have similar Top1/2 suppression and S439 appears to possess a little more potent proliferative inhibition against cancer cell lines than S222.

Together, our study demonstrates that tanshinone-1 and its derivatives (S222 and S439) are new dual Top1/2 inhibitors for the first time. We also show their different p53-dependency in inducing cell cycle arrest and apoptosis. These findings will facilitate the further investigations on the anticancer mechanisms of tanshinones on the one hand and promote the tanshinone-1-based new anticancer drug development on the other.

Author contributions

Conception and design of the study: Qian-Ting Tian, Ying-Qing Wang, Ao Zhang and Ze-Hong Miao; performing the experiments: Qian-Ting Tian, Chun-Yong Ding, Shan-Shan Song; analysis and interpretation of data: Qian-Ting Tian, Chun-Yong Ding, Shan-Shan Song, Ying-Qing Wang, Ao Zhang and Ze-Hong Miao; drafting and revising the manuscript: Qian-Ting Tian, Ying-Qing Wang and Ze-Hong Miao. All authors read and approved the submitted manuscript.

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Conflicts of interest

The authors declare no conflicts of interest related to this work.

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Figure Captions

Figure 1. Tanshinone-1 (Tan-1) and its derivatives inhibit proliferation of tumor cells.

(**a**) Chemical structures of Tan-1, S439 and S222. The structures of S439 and S222 contained in the rectangles with broken line are those that were modified from Tan-1. (**b**) Proliferative inhibition of Tan-1, S439 and S222 against 15 tumor cell lines derived from different tissues. (**c** and **d**) Resistance of three MDR sublines (KB/VCR, MCF7/ADR and K562/A02) to the drugs that were used to generate the corresponding sublines and to the Tan-1 derivatives S439 and S222. Cells were treated with gradient concentrations of drugs for 72 h. IC_{50} values were calculated from three independent experiments and expressed as mean ± SD (**b**-**d**). The resistance factor (RF) was calculated as the ratio of the IC_{50} value of the MDR cells to that of the corresponding parental cells (**c** and **d**).

Figure 2. Tanshinone-1 (Tan-1) and its derivatives decrease the levels of p-Y705-Stat3 in tumor cells and inhibit migration and tube formation of endothelial cells.

(**a** and **b**) Tan-1, S439 and S222 reduced the levels of p-Y705-Stat3 in KB and KB/VCR cells (**a**) and in p53-proficient and deficient HCT116 cells (**b**). Cells were treated with different concentrations of Tan-1 and derivatives for 4 h, and then the levels of Stat3, p-Y705-Stat3 and p-S727-Stat3 were detected by Western blotting. Representative blot densitometry measurements, normalized to β-actin and then

compared to vehicle-treated control, are indicated below the corresponding blot. (**c**-**f**) Tan-1, S439 and S222 inhibited migration (**c** and **d**) and tube formation (**e** and **f**) of HMEC-1 cells. Cells at 2×10^5 cells/well for migration (**c**, 100×) and 2×10^4 cells/well for tube formation (**e**, 200×) were treated with Tan-1, S439 or S222. All the corresponding data from three independent experiments were expressed as mean ± SD (**d** and **f**). **, $p < 0.01$.

Figure 3. Tanshinone-1 (Tan-1) and its derivatives inhibit Top1 and Top2.

(**a**) Tan-1, S439 and S222 inhibited Top1-mediated supercoiled DNA relaxation. Plasmid pBR322 was incubated with Top1 (1 U) in the absence or presence of the indicated compounds at 37°C for 30 min. The Top1 inhibitor hydroxylcamptothecin (HCPT; 200 μM) was used as the positive control. DNA samples were separated by electrophoresis on a 1% agarose gel. RLX, the relaxed form of pBR322 DNA; SC, the supercoiled form of pBR322 DNA. (**b**) Tan-1, S439 and S222 inhibited Top2-mediated supercoiled DNA relaxation. Plasmid pBR322 was incubated with Top2 (2 U) in the absence or presence of the indicated compounds at 37°C for 30 min. The Top2 inhibitor etoposide (VP-16; 200 μM) was used as the positive control. (**c** and **d**) Tan-1 and its derivatives induced formation of Top1-DNA cleavable complexes (Top1cc) and Top2cc. SW620 cells were treated with different concentrations of drugs for 1 h. HCPT (100 μM) and VP-16 (100 μM) were used as positive controls. Subcellular protein fractionation were collected and detected by Western blotting. Representative blot densitometry measurements, normalized to

Histone H3 and then compared to vehicle-treated control, are indicated below the corresponding blot. (**e**) Tanshinone -1, S439 and S222 increased the accumulation of γH2AX. SW620 cells were treated with 1 μM S439, S222 and tanshinone-1 or 10 μM tanshinone-1 for the indicated time, and the levels of γH2AX were detected by Western blotting. Densitometry measurements, normalized to β-actin and then compared to vehicle-treated control, are indicated below the corresponding blot. The results were confirmed in two independent experiments.

Figure 4. Spermidine (spd) in the Top1 buffer leads to the migration lagging of the DNA bands of the S222-treated samples in the Top1-mediated supercoiled DNA relaxation assay.

(**a**) Supercoiled pBR322 (0.5 μg) was incubated with the indicated drugs at 37°C for 30 min in the absence or presence of Top1 (1 U) or the Top1 buffer followed by standard DNA electrophoresis. (**b**) Supercoiled pBR322 (DNA; 0.5 μg) was incubated at 37°C for 30 min with different combinations of S222 and spd as indicated. Samples were subjected to standard DNA electrophoresis. The reaction conditions were as follows: 1, 0.5 μg supercoiled pBR322 (DNA); 2, DNA + 100 μM S222; 3, DNA + 1 mM spermidine (spd); 4, DNA + 100 μM S222 + 1 mM spd; loading immediately after mixing; 5, DNA + 100 μM S222 + 1 mM spd; incubating at 37°C for 1 h after mixing and then loading; 6, 100 μM S222 + 1 mM spd; 7, DNA + 100 μM S222 + 5 mM spd; loading immediately after mixing; 8, DNA + 1 mM S222 + 1 mM spd; loading immediately after mixing. All the images were representative of at least 3

separate experiments.

Figure 5. Tanshinone-1 (Tan-1) and its derivatives induce G2/M arrest.

(**a**) SW620 cells were treated separately with Tan-1 and its derivatives for 12 h. The cell cycle progression was analyzed by flow cytometry. (**b**) SW620 cells in G2/M phase (%) were statistically analyzed and expressed as mean ± SD from three independent experiments. (**c**) HCT116/p53$^{-/-}$ and HCT116/p53$^{+/+}$ cells were treated with the indicated drugs for 12 h and then analyzed by flow cytometry. (**d**) Cells in G2/M phase (%) were statistically analyzed and expressed as mean ± SD from three independent experiments. (**b** and **d**) *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. (**e**) Effects of Tan-1 and its derivatives on signaling proteins in DNA damage responses. HCT116/p53$^{-/-}$ and HCT116/p53$^{+/+}$ cells were separately treated with the indicated drugs for 6 h. Then the cells were lysed and detected by Western blotting. Representative blot densitometry measurements, normalized to β-actin and then compared to vehicle-treated control, are indicated below the corresponding blot. The results were confirmed in two independent experiments.

Figure 6. Tanshinone-1 (Tan-1) and its derivatives induce apoptosis.

(**a-d**) Tan-1, S439 and S222 induced apoptosis in SW620 cells (**a** and **b**) and HCT116/p53$^{-/-}$ and HCT116/p53$^{+/+}$ cells (**c** and **d**). Cells were treated with the indicated drugs for 48 h. Apoptosis was detected by the Annexin V-FITC and PI co-staining-based flow cytometry. The percentage of Annexin V-positive cells [right

upper (PI positive) and right lower (PI negative)] was indicated. (**b** and **d**), the data were statistically analyzed and expressed as mean ± SD from three independent experiments for SW620 (**b**) and for HCT116 cells (**d**). ***, $p < 0.001$. (**e**) Proliferative inhibition of Tan-1, S439 and S222 against HCT116/p53$^{-/-}$ and HCT116/p53$^{+/+}$ cells. Cells were treated with the indicated drugs for 72 h. IC_{50} values were calculated from three independent experiments and expressed as mean ± SD.