

Tanshinone-1 induces tumor cell killing, enhanced by inhibition of secondary activation of signaling networks

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Tumor multidrug resistance (MDR) can result from overexpression of drug transporters and deregulation of cellular signaling transduction. New agents and strategies are required for overcoming MDR. Here, we report that tanshinone-1, a bioactive ingredient in traditional Chinese medicine, directly killed MDR tumor cells and their corresponding parental cells, which was potentiated by inhibition of secondary activation of signaling networks. Tanshinone-1 was slightly more potent at inducing cytotoxicity and apoptosis in MDR cells than in corresponding parental cells. Tanshinone-1-induced MDR cell killing was independent of the function and expression of drug transporters but was partially correlated with the phosphatase-dependent reduction of phospho-705-Stat3, which secondarily activated p38-, AKT-, and ERK-involved signaling networks. Cotreatments with p38, AKT, and ERK inhibitors potentiated the anti-MDR effects of tanshinone-1. Our study presents a model for MDR cell killing using a compound of natural origin. This model could lead to new therapeutic strategies for targeting signaling network(s) in MDR cancers as well as new strategies for multitarget design.

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About 90% of tumor treatment failure results from drug resistance.¹ Multidrug resistance (MDR) appears to have a far more important role in treatment failure than individual drug resistance due to its involvement in almost all types of tumors and with many clinical anticancer drugs, including the naturally derived or synthetic drugs used in conventional chemotherapeutics or new molecularly targeted therapies.² There have been only few successes in overcoming MDR despite the enormous amounts of effort devoted toward investigating the development of MDR and possible curative approaches.³ Therefore, explorations of new agents and strategies are required to address this severe therapeutic hurdle.

One of the most common mechanisms that mediates MDR is the expression of ATP-binding cassette drug transporters including P-glycoprotein (P-gp), MDR-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP) in tumor cells.⁴ These transporters function to increase efflux and decrease influx of anticancer drugs to reduce cellular drug accumulation.⁵ It has long been considered that the key to overcoming this type of MDR is to enhance cellular accumulation of the drug. Therefore, MDR reversal

agents, such as verapamil, have been used to inhibit the function of drug transporters,⁴ and other agents have also been investigated for their abilities to suppress drug transporter expression.^{6–9} These approaches could lead to severe toxicity, however, as the majority of drug transporters are also physiologically expressed in important organs or tissues where they have roles in detoxification or protection.²

Deregulation of cellular signaling transduction frequently occurred in MDR tumor cells. For example, signal transducer and activator of transcription 3 (Stat3) is frequently overactivated not only in many types of human cancers¹⁰ but also in MDR tumors.¹¹ Constitutively activated Stat3 is phosphorylated at its Tyr705 (p-705-Stat3). In addition to promoting cell proliferation and survival, p-705-Stat3 may suppress apoptosis by increasing the expression of various antiapoptotic factors.¹¹ The level of Tyr705 phosphorylation is regulated by upstream Janus kinases (Jaks) and phosphatases, including SH-2-containing phosphatase 2 (Shp2) and protein-tyrosine-phosphatase 1B (PTP1B).¹² Reducing p-705-Stat3 either by targeting Stat3 upstream signaling or by activating the phosphatases could result in significant

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Abbreviations: ADR, adriamycin; BCRP, breast cancer resistance protein; ciAP-2, cellular inhibitor of apoptosis 2; EC-Stat3, ectopic Stat3; EGFR, epidermal growth factor receptor; EN-Stat3, endogenous Stat3; ERK, extracellular signal regulated kinase; FGFR1, fibroblast growth factor receptor 1; H3C, constitutively activated human Stat3; IC₅₀, concentration required for 50% inhibition; Jak, Janus kinase; MAPK, mitogen-activated protein kinases; M3C, mouse constitutively activated Stat3; Mcl-1, myeloid cell leukemia 1; MDR, multidrug resistance; MEK, mitogen-activated protein kinase kinase; MMP, mitochondria membrane potential; MRP1, MDR-associated protein 1; mTOR, mammalian target of rapamycin; p38, p38 MAP kinase; PARP, poly(ADP-ribose) polymerase; PDGFR- β , platelet-derived growth factor receptor β ; P-gp, P-glycoprotein; PI3K, phosphatidylinositol 3-kinases; PTP1B, protein-tyrosine-phosphatase 1B; RF, resistance factor; Shp1, SH-2-containing phosphatase 1; Shp2, SH-2-containing phosphatase 2; Stat3, signal transducer and activator of transcription 3; VCR, vincristine

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anticancer activity.^{13–16} Moreover, methyl spongoate, a natural marine product, mediates an apparent killing effect on liver cancer cells overexpressing various drug transporters by depleting Tyr705 phosphorylation of Stat3.¹⁷ Targeting Stat3 with a decoy oligonucleotide has also been recently shown to decrease the expression of its antiapoptotic target genes within cancers in a phase 0 clinical trial.¹⁸ Close relationships between Stat3 and other signaling pathways such as p38, AKT, and ERK^{19–21} also suggest that activation of these signaling pathways may impair the effect of targeting Stat3 in cancer therapy.

Salvia miltiorrhiza (Danshen) is a famous traditional Chinese medicine that has been used to treat various diseases, including cardiovascular diseases, for centuries.^{22,23} Several preparations that contain its major bioactive ingredients still have important clinical roles, especially in the treatment of angina pectoris in China.²⁴ Danshen contains two types of major constituents: water-soluble phenolic acids and lipophilic tanshinones.²³ Tanshinones, including tanshinone-1 (Supplementary Figure S1) and tanshinone-2A, are abietanediterpenes characterized by an *ortho*-quinone C-ring.²² Tanshinone-1 accounts for ~2% of the ethanolic fraction of crude Danshen,²⁵ indicating its high content in this plant as well as its clinical safety. Tanshinones have also been shown to elicit anticancer effects.^{22,23,26–29} Nevertheless, the mechanisms of action and their molecular target(s) remain unknown.

In the search for MDR-overcoming drugs, we found that tanshinone-1 exerted more potent killing effects on MDR tumor cells than tanshinone-2A with resistance factor (RF) 0.9 *versus* 3.6 (unpublished data). In this study, we compared the capability of tanshinone-1 for inducing cytotoxicity and apoptosis to its impact on the function and expression of important drug transporters in MDR and corresponding parental tumor cell lines. To investigate its potential mechanism of anticancer action, we further explored whether and how tanshinone-1 changed the phosphorylation levels of Stat3, p38, AKT, and ERK in these cells. Finally, we examined how cotreatments with p38, AKT, and ERK inhibitors affected the anticancer and anti-MDR activities of tanshinone-1. Our results reveal that tanshinone-1 has a potent capability for directly killing MDR tumor cells, independent of drug transporters but partially dependent on reduced Tyr705 phosphorylation of Stat3. Moreover, inhibiting the secondary effects of increased phosphorylation of other signaling molecules, especially p38 and AKT, potentiates its cytotoxicity in both MDR and parental tumor cells.

Results

Tanshinone-1 kills MDR cells in a drug-transporter-independent manner. To determine whether tanshinone-1 could kill MDR tumor cells, we used three MDR sublines, K562/A02, KB/VCR, and MCF-7/ADR, that express drug transporters, including P-gp and MRP1.^{6,7,17} Tanshinone-1 elicited a more potent cytotoxicity against MDR cells than the respective parental cells with an average RF of 0.83 (Table 1). In contrast, the average RF of adriamycin and vincristine reached 162.7 (Table 1). However, tanshinone-1 was less toxic to the normal cells (human liver QSG7701 and HL7702 cells and mouse fibroblast NIH3T3 cells) (Table 1 and Table 2). Tanshinone-1 induced more apoptosis (Figure 1a and Supplementary Figure S2a) by triggering increased loss of mitochondria membrane potential (MMP) (Figure 1b) and stronger activation of caspase-3 and caspase-9 (Figure 1c) in KB/VCR cells than in KB cells in a concentration-dependent manner. However, tanshinone-1 did not seem to affect caspase-8 in either MDR or parental cells (Figure 1c). The data indicate that tanshinone-1 activates the intrinsic, rather than extrinsic, apoptosis pathway, which leads to the killing of both MDR and parental cells.

The results suggest that the expression of drug transporters in MDR cells does not impair the biological effect of tanshinone-1. To clarify this point, we analyzed the efflux of rhodamine 123 (Rh123, a fluorescent dye known as a substrate of P-gp).³⁰ Rh123 stayed in the parental KB cells but was transported out of KB/VCR cells (Figure 1d). This was prevented by treating with the well-known P-gp blocker verapamil^{31,32} instead of tanshinone-1. Similarly, verapamil, but not tanshinone-1, significantly sensitized KB/VCR cells to vincristine (Figure 1e, left) but did not affect the sensitivity of KB/VCR cells to tanshinone-1 (Figure 1e, right). KB/VCR cells were also observed to accumulate slightly more tanshinone-1 than the parental cells (Supplementary Figure S2b), and tanshinone-1 did not change the expression of the *mdr-1* and *MRP1* genes at either protein (Figure 1f) or mRNA (Supplementary Figure S2c) levels. These data indicate that tanshinone-1-induced killing of MDR cells is independent of drug transporters.

Tanshinone-1 depletes the Tyr705 phosphorylation of cellular Stat3. As the tanshinone-1-induced cytotoxicity and apoptosis in MDR cells occurs in a drug-transporter-independent fashion, additional factor(s) most likely contribute to the sensitivity of those cells to this natural product.

Table 1 Cytotoxicity of tanshinone-1 in MDR and corresponding parental tumor cells

Compound	IC ₅₀ ^a (μM)		RF ^b	IC ₅₀ (μM)		RF	IC ₅₀ (μM)		RF
	KB	KB/VCR		MCF-7	MCF-7/ADR		K562	K562/A02	
Adriamycin	—	—	—	0.11 ± 0.06	9.7 ± 2.9	88.2	0.05 ± 0.03	7.3 ± 3.3	146.0
Vincristine	0.002 ± 0.0007	0.5 ± 0.2	254.0	—	—	—	—	—	—
Tanshinone-1	8.7 ± 1.2	8.3 ± 0.1	0.9	7.7 ± 1.2	5.6 ± 1.0	0.7	21.2 ± 6.4	20.1 ± 5.5	0.9

^aThe drug concentration required for 50% growth inhibition (IC₅₀) of tumor cells was determined from three separate experiments and expressed as mean ± S.D. Each drug concentration was tested in triplicate for 72 h

^bThe resistance factor (RF) was calculated as the ratio of the IC₅₀ value of the MDR cells to that of corresponding parental cells

Table 2 Cytotoxicity (IC₅₀^a, μ M) of tanshinone-1 in normal cell lines

Cell lines	QSG7701	HL7702	NIH3T3
Tanshinone-1 (μ M)	> 80	40.1 \pm 4.3	28.6 \pm 5.5

^aThe drug concentration required for 50% growth inhibition (IC₅₀) of the tested cells was determined from three separate experiments and expressed as mean \pm S.D. Each drug concentration was tested in triplicate for 72 h

Stat3 has been reported to be involved in tumor drug resistance.^{33–37} Therefore, we examined Stat3 protein expression and phosphorylation levels of Tyr705 and Tyr727; phosphorylation of these residues differentially regulates the transcriptional activity of Stat3.^{38,39} There was no apparent difference in the levels of total Stat3 and p-727-Stat3 in all three tested MDR sublines when compared with their respective parental cell lines (Figures 2a–d). However, a significant enhancement of Tyr705 phosphorylation of Stat3 was observed in the MDR cells relative to the parental cells (Figures 2a–d). Importantly, treating both MDR and parental cells with tanshinone-1 resulted in a quick depletion of p-705-Stat3 but only a slight change in the p-727-Stat3 levels and no discernible change in total Stat3 protein levels (Figures 2a–d). Similarly, MDR KB/VCR cells had higher protein expression of Stat3-target genes, including Mcl-1, clAP-2, cyclin D1, c-Myc and Survivin than KB cells (Figure 2e, left). Tanshinone-1 also significantly reduced these proteins in KB/VCR cells (Figure 2e, right). Additionally, downregulating Stat3 expression partially rescued apoptotic induction and reversed the tanshinone-1-induced cleavage of caspase-3 and PARP in KB/VCR cells (Figures 2f–h). These results suggest that the depletion of p-705-Stat3 caused by the treatment with tanshinone-1 could partially contribute to its killing of MDR cells. Unexpectedly, however, reduction of Stat3 expression in MDR MCF-7/ADR and KB/VCR cells did not enhance the cellular sensitivity to the anticancer drugs vincristine and adriamycin (Supplementary Figures S3a and b). Moreover, constitutive activation of Stat3 in parental KB cells did not significantly change their sensitivity to tanshinone-1 (Supplementary Figures S3c–h). The data indicate that other mechanism(s) may be involved in the response to tanshinone-1 in these cells.

Tanshinone-1 activates cellular phosphatases. We next examined how tanshinone-1 causes depletion of p-705-Stat3 in these cells. One possibility is that tanshinone-1 reduces upstream kinase(s) of Stat3, such as Jak1, Jak2, and Src,^{10,21} at either protein levels or the levels of their phosphorylated (active) counterparts. However, no apparent changes were observed in either their total protein or phosphorylation levels in both KB/VCR and KB cells (Figure 3a). Tanshinone-1 did not inhibit the kinase activity of the Jak1, Jak2 and Src (Supplementary Table S1). Therefore, we further examined phosphatases, as they also regulate the phosphorylation levels of cellular proteins.¹² Pretreatments of KB/VCR cells for 30 min with Na₃VO₄, a pan phosphatase inhibitor, prevented the reduction of p-705-Stat3 (Figure 3b), proliferation inhibition and apoptotic induction mediated by tanshinone-1 (Figures 3c and d),

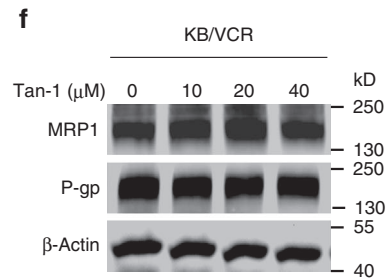
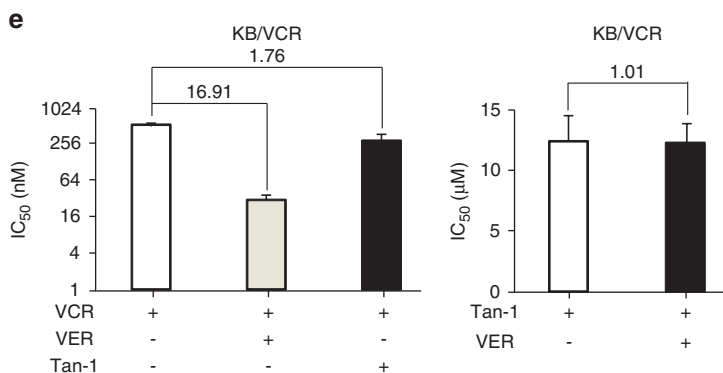
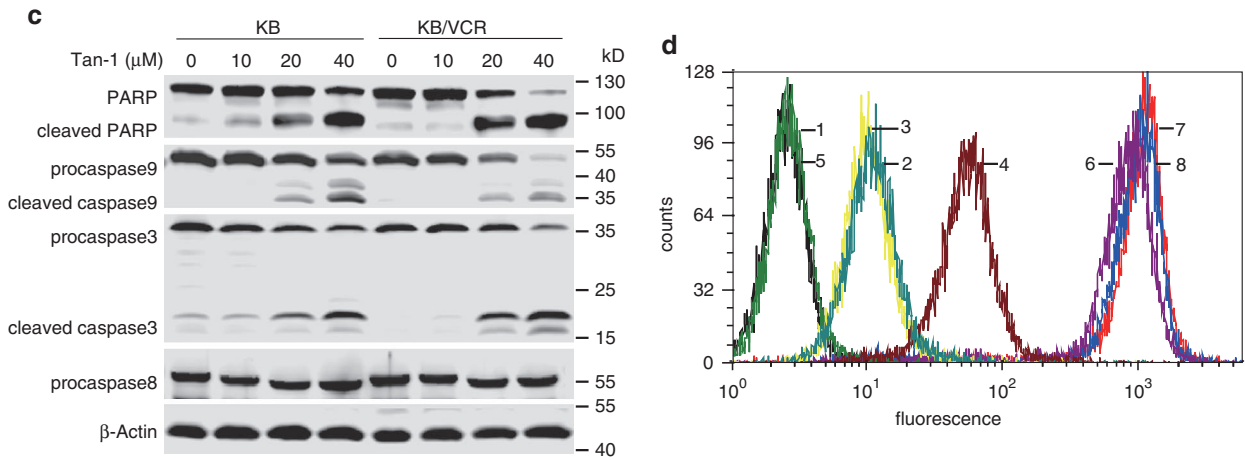
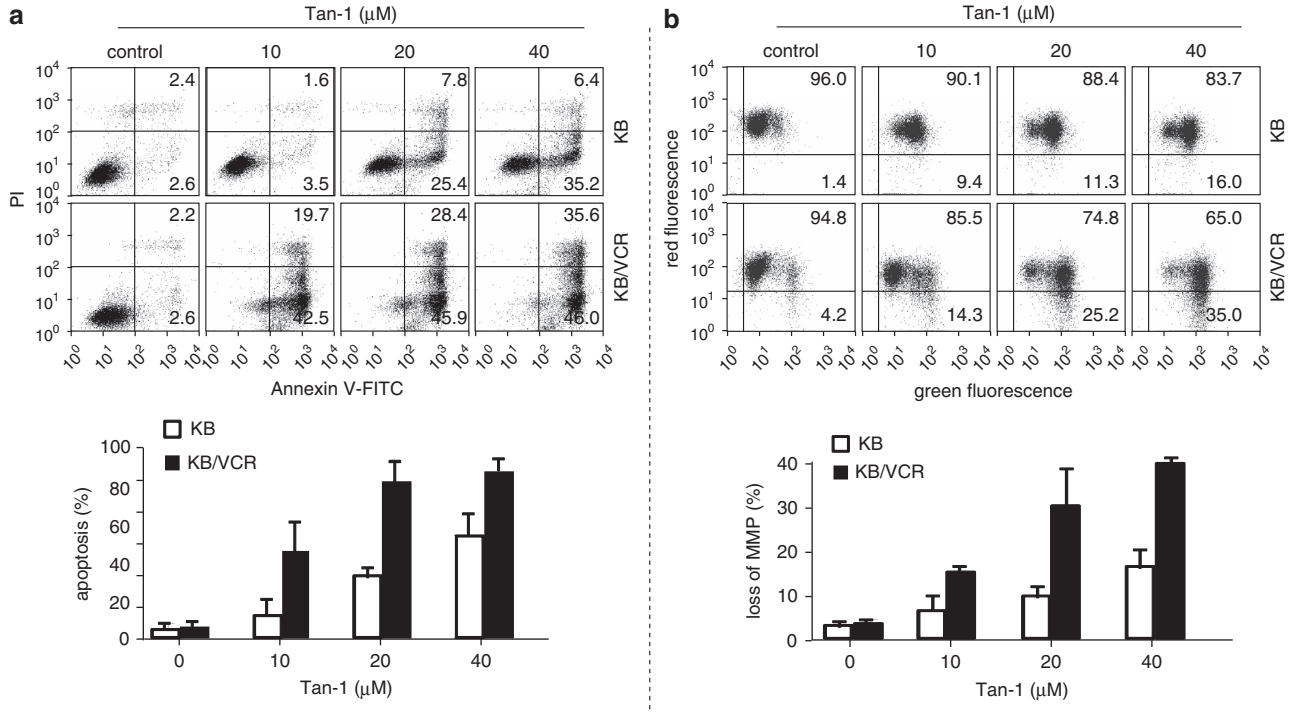
suggesting that phosphatases may participate in the biological effects of tanshinone-1.

There are at least three phosphatases, Shp1, Shp2, and PTP1B, which have been reported to regulate the phosphorylation levels of cellular Stat3 at its Tyr705.^{14,40,41} Compared with Shp2 and PTP1B, Shp1 was expressed at very low levels in the MDR KB/VCR and K562/A02 sublines and their respective parental cell lines, whereas higher expression was found in the MCF-7 cell lines (Supplementary Figure S4). The differential expression of Shp1 indicates only a slight possibility that Shp1 mediates the effect of tanshinone-1. Therefore, we focused on Shp2 and PTP1B. Treatments with tanshinone-1 did not significantly change their protein levels in KB/VCR cells (Figure 3e). However, reduction of either Shp2 or PTP1B with specific siRNA against Shp2 (siShp2) or PTP1B (siPTP1B) reversed the tanshinone-1-mediated depletion of p-705-Stat3 (Figures 3e and f). Additionally, both siShp2 and siPTP1B mitigated the apoptotic induction by tanshinone-1 in KB/VCR cells (Figures 3g and h). In a cell-free system, however, tanshinone-1 did not cause significant alterations in the enzymatic activities of Shp2, PTP1B, and four other phosphatases (Supplementary Table S2). The data indicate that tanshinone-1 likely activates cellular Shp2 and PTP1B indirectly, resulting in dephosphorylation of p-705-Stat3.

Tanshinone-1 enhances phosphorylation levels of AKT, ERK, and p38. The Stat3 signaling pathway has been shown to interact with the PI3K-AKT-mTOR, MAPK, and p38 pathways^{20,42–46} (Figure 4a). Therefore, we examined the effects of tanshinone-1 on the key signaling molecules, AKT, ERK, and p38, of these pathways. Treatments with tanshinone-1 led to increased phosphorylation of AKT, ERK, and p38 in distinct patterns but did not change their total protein (Figure 4b). In both MDR KB/VCR and parental KB cells exposed to tanshinone-1, AKT phosphorylation increased progressively; in contrast, both ERK and p38 phosphorylation rose relatively rapidly at first, before returning to basal levels (Figure 4b). A similar phenomenon was observed in MDR K562/A02 and parental K562 cells (Figure 4c). However, tanshinone-1 did not change the enzymatic activities of the upstream tyrosine kinases of these pathways, including EGFR, FGFR1, PDGFR- β , and c-Src (Supplementary Table S1). Pretreatments with the respective phosphorylation inhibitors of AKT (PI103), ERK (AZD6244), and p38 (SB203580) eliminated the increased phosphorylation but did not reverse the reduction of p-705-Stat3 in cells exposed to tanshinone-1 (Figure 4d and Supplementary Figure S5). Moreover, specific siRNA against *stat3* slightly increased phosphorylation of AKT, ERK, and p38 in MDR KB/VCR cells and decreased changes in ERK and p38 phosphorylation mediated by tanshinone-1 (Figure 4e). The results suggest that the increased phosphorylation of AKT, ERK, and p38 may be due to the decreased Tyr705 phosphorylation of Stat3. The secondary activation of the pathways containing AKT, ERK and p38 could alleviate, to a certain degree, the deleterious effects of tanshinone-1 on both MDR and parental cells, thereby acting as a compensatory mechanism.

Phosphorylation inhibitors of AKT, ERK, and p38 potentiate cell killing by tanshinone-1. We further examined whether suppressing the tanshinone-1-induced increase

in AKT, ERK, and p38 phosphorylation affected cell killing. Cotreatments with phosphorylation inhibitors PI103, SB203580, or AZD6244 significantly enhanced the apoptotic



induction by tanshinone-1 in both MDR KB/VCR and parental KB cells. The ERK phosphorylation inhibitor AZD6244 showed a relatively weak potency compared with PI103 and SB203580 (Figures 5a–c). Each inhibitor also sensitized MDR and parental cells to tanshinone-1 to different degrees (Figure 5d). The p38 inhibitor SB203580 resulted in the most potent sensitization to tanshinone-1; the inhibitor PI103 had slightly weaker potentiation; and the inhibitor AZD6244 had the weakest or no potentiation. Notably, the combination of SB203580 with PI103 displayed nearly equivalent (in the parental KB cells) or even slightly more potent (in MDR KB/VCR cells) sensitization than a combination of all three inhibitors (Figure 5d). These data suggest that, although tanshinone-1 may increase the phosphorylation levels of AKT, ERK, and p38, the PI3K-AKT and p38 signaling pathways likely have more important roles in potentiating the cellular sensitivity to tanshinone-1 than the ERK-involving MAPK pathways. Moreover, a combination of phosphorylation inhibitors of AKT and p38 could be the primary choice for the future investigations of experimental therapeutics related to tanshinone-1.

Discussion

As a traditional Chinese medicine, *Salvia miltiorrhiza* has been shown to have reliable therapeutic effectiveness and excellent safety based on its long-term use in clinics.²² In this study, tanshinone-1, one of its major ingredients, was shown to possess the ability to kill MDR tumor cells as shown by six prominent characteristics: 1) tanshinone-1 can directly kill human MDR tumor cells derived from different tissues and established with different anticancer drugs, indicating its potentially wide spectrum of action. 2) Tanshinone-1 is more potent at inducing cytotoxicity and apoptosis in MDR tumor cells than in the corresponding parental cells; one possible explanation of this may be more cellular accumulation of the drug in the former than in the latter. 3) Tanshinone-1 kills MDR cells independently of drug transporters; it is not affected by their drug-transporting function and also does not alter their expression. 4) Unlike classical approaches that target drug transporters, tanshinone-1 kills MDR tumor cells by other mechanism(s) of action. For example, tanshinone-1 reduces the Tyr705 phosphorylation levels of Stat3 and subsequently decreases the expression of various antiapoptotic/prosurvival factors. 5) The tanshinone-1-induced reduction of p-705-Stat3 is likely associated with activating phosphatases of Stat3 (Shp2 and PTP1B) but is independent of Stat3 upstream signaling molecules such as Jaks, Src, or receptors. And finally 6) tanshinone-1-induced transcriptional inactivation of Stat3

may result in compensatory activation of other related signaling networks, as evidenced by the increased phosphorylation of p38, AKT, and ERK; additionally, a combination of the phosphorylation inhibitors for p38, AKT, and ERK potentiates tanshinone-1-induced cytotoxicity (Supplementary Figure S6a).

These features make tanshinone-1 stand out as a potential candidate of natural origin that can kill MDR tumor cells. The majority of small-molecule anticancer drugs derived from natural products, including taxol and, recently reported, trabectedin, are substrates of drug transporters such as P-gp, MRP1, and/or BRCP, and their therapeutic effectiveness could be severely impaired by MDR mechanisms.^{47,48} One advantage of tanshinone-1, compared with those clinical drugs, is that its anticancer effect is independent of the MDR mechanism. Therefore, the compound is toxic to any cancer cells, even those with the expression of the MDR phenotype. Another advantage of tanshinone-1, compared with MDR reversal agents such as verapamil,⁴ is that it does not interfere with either the expression or function of drug transporters. Therefore, tanshinone-1 kills MDR tumor cells directly while sparing normal tissues that also express drug transporters.

Tanshinone-1-induced cell killing in the MDR and parental cells might partially be dependent on its reduction of p-705-Stat3, an active form of Stat3. Stat3 functions as a transcription factor that promotes survival and prevents apoptosis by regulating the expression of prosurvival/anti-apoptotic genes. In MDR KB/VCR cells, Stat3 siRNA led to the decrease in tanshinone-1-induced apoptosis, indicating that its effect is partially dependent on Stat3 signaling. Compared with parental tumor cells, MDR cells have enhanced levels of p-705-Stat3 and its downstream gene products, including Mcl-1, cIAP-2, cyclin D1, c-Myc, and Survivin, suggesting that the Stat3 signaling might have more important roles in MDR cells. Tanshinone-1 elicits significant reduction of these proteins in MDR tumor cells, and this reduction might be another possible cause for the stronger potency of tanshinone-1 for inducing cytotoxicity and apoptosis in MDR tumor cells. However, *Stat3* siRNA did not affect the sensitivity of MDR cells to vincristine or adriamycin and constitutively activated Stat3 did not significantly change the sensitivity of parental KB cells to tanshinone-1 either, suggesting that Stat3 is not the common mediator of cellular responses to tanshinone-1 in parental and MDR cells. Therefore, other unknown mechanism(s) may be involved, which deserves further investigating.

On the other hand, tanshinone-1-triggered reduction of p-705-Stat3 may lead to secondary activation of related signaling networks. In the cells, Jak-Stat, p38MAPK,

Figure 1 Tanshinone-1 induced apoptosis independent of drug transporters. (a) Tanshinone-1 (Tan-1) increased Annexin V-positive cells. Cells were treated with Tan-1 for 24 h, then stained with Annexin V/propidium iodide (PI) and analyzed by flow cytometry. Upper panel, representative histograms; lower panel, mean \pm S.D. (b) Tan-1 disrupted the mitochondria membrane potential. Cells were exposed to Tan-1 for 18 h, stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide, and then detected by flow cytometry. Upper panel, representative histograms; lower panel, mean \pm S.D. (c) Tan-1 induced the cleavage of procaspase 3/9 and PARP. Cells were treated with Tan-1 for 18 h and analyzed by western blotting. (d) Tan-1 did not affect Rh123 export. Cells were pretreated with Tan-1 or verapamil for 90 min, cultured in medium with or without 1 μ g/ml Rh123, and then followed by flow cytometry detection. 1–4: KB/VCR, blank; Rh123; Rh123 and 40 μ M Tan-1; Rh123 and 5 μ M verapamil; 5–8: KB, blank; Rh123; Rh123 and 40 μ M Tan-1; Rh123 and 5 μ M verapamil. (e) Verapamil did not significantly enhance the sensitivity of KB/VCR cells to Tan-1. Cells were treated with 5 μ M verapamil or 5 μ M Tan-1 in combination with vincristine (left panel), or treated with 5 μ M verapamil plus Tan-1 for 72 h (right panel). The IC₅₀ was expressed as mean \pm S.D. (f) Tan-1 did not decrease expression of drug transporter proteins. Cells were treated with Tan-1 for 12 h and analyzed by western blotting. All the data were obtained from at least three independent experiments

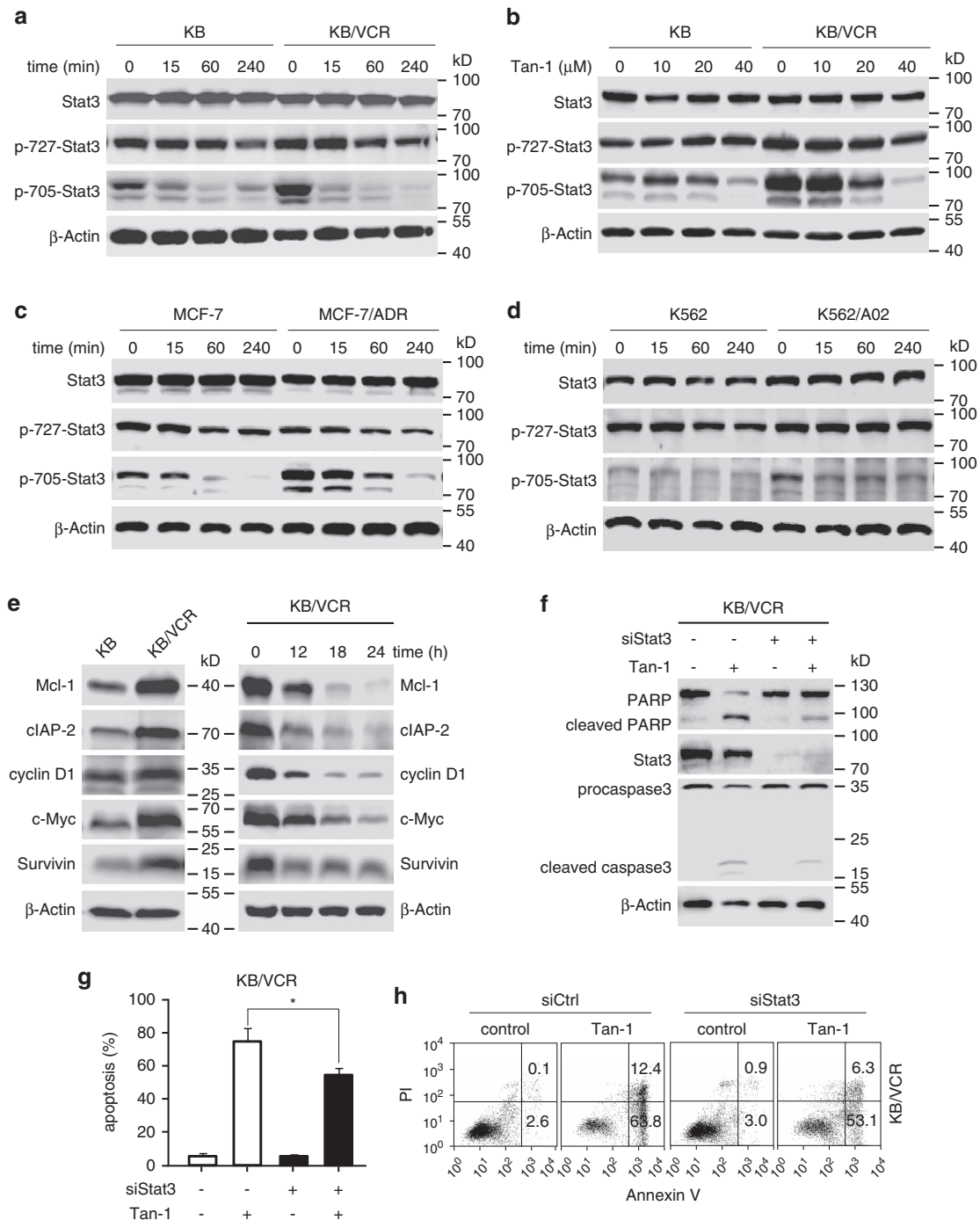


Figure 2 Tanshinone-1 reduced Tyr705 phosphorylation of cellular Stat3, which contributed to apoptotic induction. (a–d) Tanshinone-1 (Tan-1) led to the dephosphorylation of Stat3 at Tyr705 in a time- and concentration-dependent manner in MDR and corresponding parental cells. The cells were treated with 40 μ M tanshinone-1 for the indicated time (a, c and d) or with gradient concentrations of tanshinone-1 for 4 h (b), and then detected by western blotting. (e) Tanshinone-1 reduced the protein expression of the Stat3-targeted genes. KB and KB/VCR cells were treated with or without 20 μ M tanshinone-1 for the indicated time and then western blotting for cellular levels of Mcl-1, cIAP-2, cyclin D1, c-Myc, and Survivin proteins was done. Basal levels (left) and reduced levels (right) of the examined proteins. (f) The siRNA for Stat3 (siStat3) decreased Stat3 expression and reversed the cleavage of procaspase 3 and PARP induced by tanshinone-1. KB/VCR cells were transfected with siStat3 for 48 h and then treated with 20 μ M tanshinone-1 for another 24 h. The cell lysates were subjected to western blotting. (g and h) siStat3 rescued apoptotic induction by tanshinone-1. KB/VCR cells were transfected with siStat3 for 48 h and then treated with 20 μ M tanshinone-1 for another 18 h. The resulting cells were stained with Annexin V/PI and analyzed by flow cytometry. The apoptosis rates from three independent experiments were expressed as mean \pm S.D.; * P < 0.05 (g). Representative histograms are shown in h

PI3K-AKT-mTOR, and Ras-Raf-MEK-ERK pathways are interdependent, interconnected signaling pathways that are responsible for regulating, integrating, and balancing various

signals from exogenous or endogenous stimulators, including growth factors, cytokines, and stress stimuli (Figure 4a). These interaction networks precisely control cell survival,

proliferation, growth, apoptosis, and the cell's stress response to ensure that biological systems function normally. However, the robustness and redundancy of such networks could impair the therapeutic sensitivity of, for example, cancers. Under these conditions, network pharmacology may provide solutions, particularly for drug discovery.^{49,50} Our data show that tanshinone-1 activates p38, AKT, and ERK signaling networks as a secondary effect of inhibiting p-705-Stat3 signaling. Phosphorylation inhibitors of these proteins could enhance cytotoxicity and apoptotic induction by tanshinone-1, though to differing degrees. Our results provide important data with regards to combining tanshinone-1 (and its derivatives) with these inhibitors, especially p38 inhibitors and AKT inhibitors. They also lay the foundations for future multitarget drug design targeting p-705-Stat3-involved signaling networks (Supplementary Figure S6a).

These findings on tanshinone-1 may lead to new strategies for overcoming MDR. Typically, the agents used to sensitize cells to anticancer drugs have targeted drug transporters by either interfering with transporter functions or reducing transporter expression. These agents themselves generally lack anticancer activities and may reduce the physiological functions of normal tissues such as the small intestine, liver, and blood-brain barrier⁴ which would likely lead to severe toxicity. In contrast, agents such as tanshinone-1 can directly kill MDR tumor cells or inhibit their growth and proliferation but are not substrates of MDR transporters and do not interfere with either function or expression of the transporters, resulting in much lower risks for toxicity (Supplementary Figure S6b). Here, we define these agents as anti-MDR drugs. Another potential advantage of anti-MDR drugs is that they are likely to have enhanced oral bioavailability and increased permeability into the brain and other sites where penetration is prevented by drug transporters.⁴ Therefore, developing anti-MDR drugs could be a feasible strategy for treating MDR cancers in the future.

This study characterizes several important features of tanshinone-1, presents a model compound for MDR cancer therapy, and advances a strategy for overcoming MDR through the use of anti-MDR drugs. Several important issues remain to be resolved, however. Tanshinone-1 has very low solubility, and additionally, its overall anticancer activity is relatively weak. Therefore, chemical modifications are required to allow tanshinone-1 to enter drug development. For this purpose, it is necessary to further investigate and validate the precise molecular target(s) of tanshinone-1 to establish exact structure–effect relationships that can guide chemists in their manipulations to obtain drug candidates with ideal druggable properties. Additionally, more detailed mechanistic studies are required to provide a more in-depth analysis of the impact of tanshinone-1 on cellular signaling networks. These studies may also provide critical data for potential multitarget drug designs.

Materials and Methods

Cell culture. The KB, K562, NIH3T3, and MCF-7 parental cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). The QSG7701 and HL7702 cell lines were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The MDR KB/VCR and MCF-7/ADR sublines were obtained from the Zhongshan University of Medical Sciences (Guangzhou, China) and the MDR K562/A02 subline was

from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). All the cells were cultured and used as described previously.^{6,7,51}

Cytotoxicity assays. Cytotoxic effects on the cells were assessed by sulforhodamine B (SRB) assays for solid tumor cells or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays for suspension cells as reported previously.^{6,17,52} The concentration required for 50% inhibition (IC₅₀) of the tested cells was calculated using the Logit method. The RF for each examined drug was calculated as the ratio of the IC₅₀ value in a MDR subline to that in its parental cell line.

Materials. Tanshinone-1 and Tanshinone-2A was purchased from Shanghai Tauto Biotech Co., Ltd (China). Vincristine (VCR), verapamil (VER), SRB, and MTT were purchased from Sigma (St. Louis, MO). Adriamycin (ADR) was obtained from Dalian Meilun Biology Technology Co., Ltd (Dalian, China). Antibodies against phospho-Stat3 (Tyr705), phospho-Stat3 (Ser727), Stat3, Src, phospho-Src (Ser416), caspase-3, caspase-9, phospho-AKT, AKT, phospho-ERK, ERK, phospho-p38, p38, Mcl-1, and cyclin D1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MRP1, clAP-2, Survivin, Jak1, phospho-Jak1, Jak2, and phospho-Jak2 were obtained from Abcam (Cambridge, MA, USA). The antibody against β -Actin was purchased from Abgent (San Diego, CA, USA). Antibodies against P-gp, Caspase-8 and PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Shp2, Shp1, PTP1B was purchased from Epitomics (Burlingame, CA, USA). The antibody against c-Myc was from R&D Systems (Minneapolis, MN, USA). Rhodamine 123 (Rh123) and 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1) were from Beyotime Institute of Biotechnology (Haimen, China). The Annexin V-FITC apoptosis detection kit was purchased from KeyGen Biotech (Nanjing, China).

Western blotting analyses. Cell lysates were analyzed using standard western blotting to examine the levels of the indicated proteins.^{7,17,52}

Reverse transcript polymerase chain reaction (RT-PCR). Cells were treated with tanshinone-1 at the indicated concentrations for 12 h. The total cell RNA was extracted with the TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and reverse transcribed into cDNA. The cDNA was then subjected to PCR. The primers were as follows: 5'-GGATGCAGAGAGATCACTG-3' (forward), 5'-CGATCCACACGGAGTACTTG-3' (reverse) for β -actin;⁵³ 5'-GTATTC AAC TATCCACCC-3' (forward), 5'-GCTTTATTTCTTTGCCATC-3' (reverse) for *mdr-1*; 5'-CTGACAAGCTAGACCATGAATGT-3' (forward), 5'-TTACACCAAGCCGGCC TCTTT-3' (reverse) for *MRP1*.

Flow cytometry. Cell apoptosis, Rh123 efflux, and the loss of mitochondria membrane potential (MMP) were detected with a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA). Cells were treated with tanshinone-1 and collected for apoptosis detection as instructed by the Annexin V-FITC apoptosis detection kit. For the Rh123 efflux assay, cells were treated with 5 μ M verapamil or 40 μ M tanshinone-1 for 1.5 h before adding Rh123 (1 μ g/ml) for 30-min treatments. All the cells were collected and suspended in PBS for 1.5 h. To examine the loss of MMP, cells were treated with tanshinone-1 at the indicated concentrations for 18 h and collected for JC-1 staining (1 μ M). Samples were analyzed by flow cytometry, and 10 000 events were counted each time.

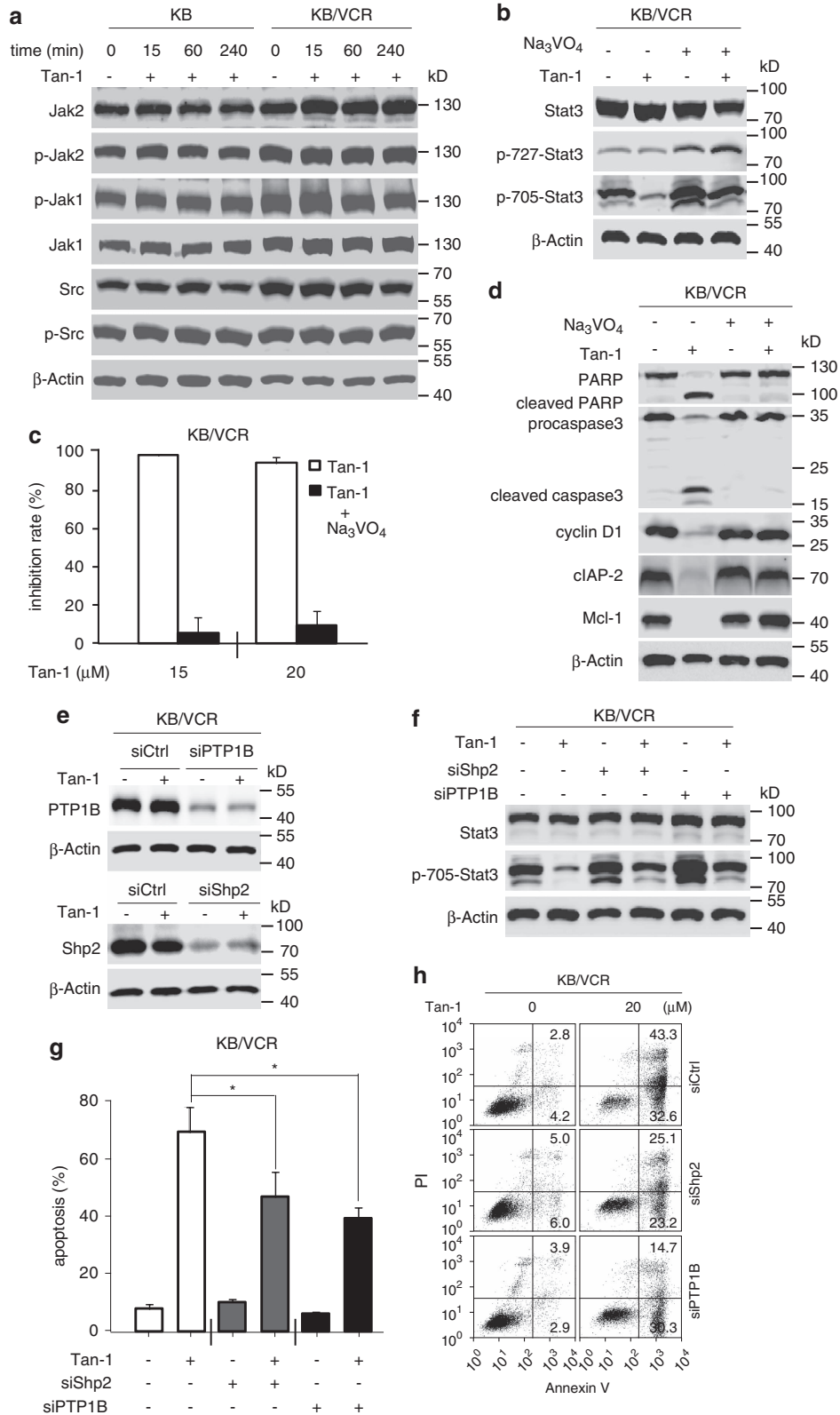
Gene knockdown using small interfering RNA (siRNA) technology.

Cells were seeded in 12-well plates at 1.5×10^6 cells/ml. The specific siRNA, mixed with Lipofectamine RNAi MAX, was added into the culture medium without antibiotics. Cells were incubated for 48 h before treatment with the indicated drugs. The sequences of the siRNA target genes were: 5'-GAGUUGAAUUUACAG CUUATT-3' for *stat3* (1); 5'-GUUUGGAAUUAUGGUGAATT-3' for *stat3* (2);¹⁷ 5'-GAATCCTATGGTGAAACA-3' for *Shp2* (1);⁵⁴ 5'-AAGAATATGGCGTC ATGCGTG-3' for *Shp2* (2);⁵⁵ 5'-UAGGUACAGAGACGUCAGU-3' for *PTP1B* (1); 5'-UGACCAUAGUCGGAUAAA-3' for *PTP1B* (2), and 5'-AAAUCAACG GAAGAAGGUGUCU-3' for *PTP1B* (3).⁵⁶

Drug accumulation assays. KB and KB/VCR cells (1×10^7 cells) were seeded in 10-cm dishes, and were treated with tanshinone-1 for the indicated time. The cells were then scraped and washed twice with PBS. The samples were prepared as previously described.⁵⁷ The 10 μ l of final sample was injected into the

high performance liquid chromatography with a ZORBAX SB-C18 Column (4.6 × 150 mm, 5 μm, Agilent, Santa Clara, CA, USA) using 77:23(v/v) methanol/water as the mobile phase at a flow rate of 1.0 ml/min. Concentrations of tanshinone-1 in each sample eluted from the column were detected at 254 nm.

In vitro enzymatic activity assays. The activity of phosphatases was detected by BPS Bioscience Inc. (San Diego, CA, USA). All assays were conducted at room temperature for 15 min. The enzymatic reactions for phosphatases were conducted in duplicate in a transparent 96-well plate.



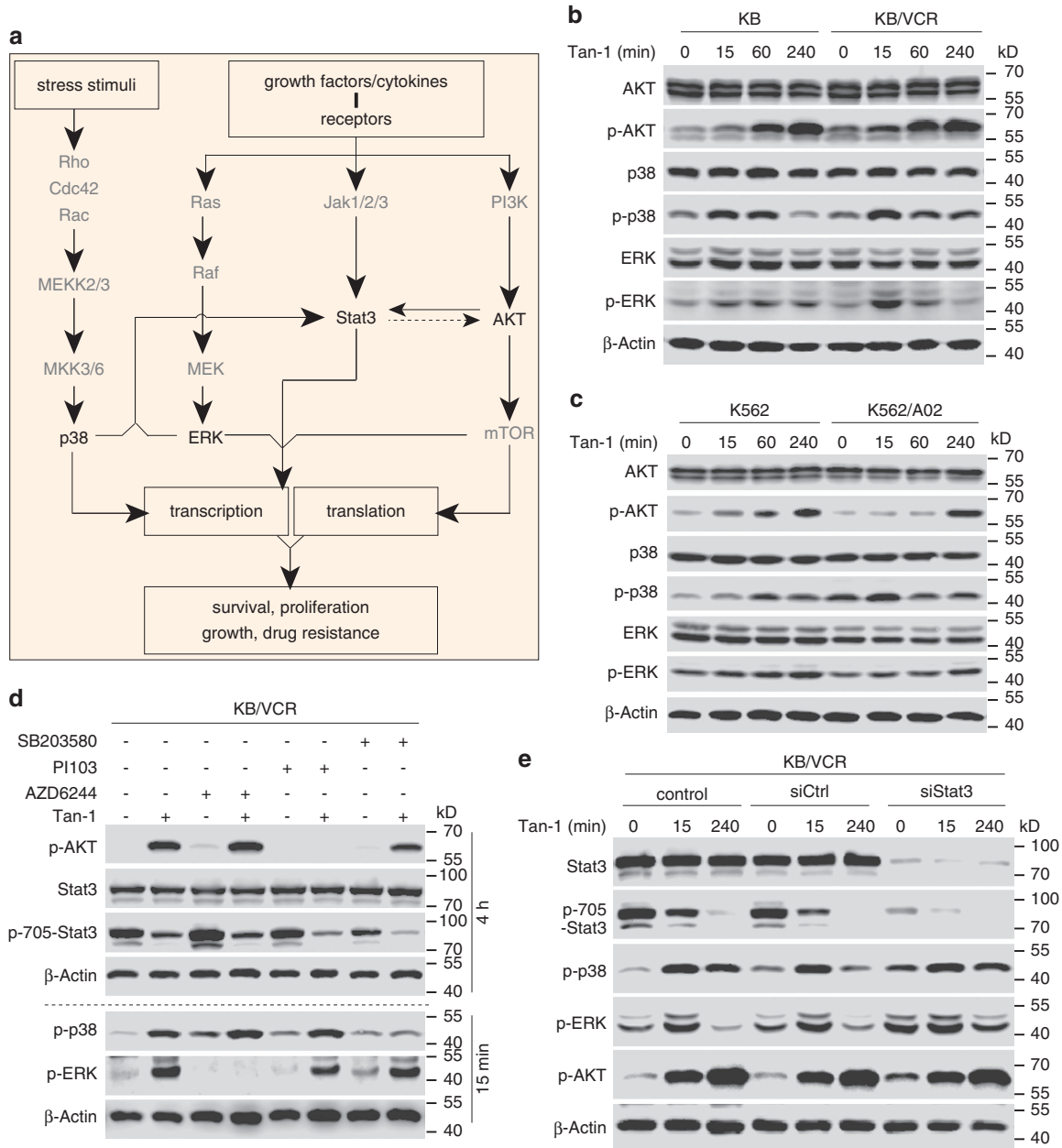


Figure 4 Tanshinone-1 activated p38, AKT, and ERK signaling. (a) Signaling pathways consisting of Stat3, p38, AKT, and ERK, may crosstalk and influence their respective biological effects. (b and c) Phosphorylation was analyzed by western blotting. Tanshinone-1 (40 μ M, Tan-1) enhanced phosphorylation of p38, AKT, and ERK in MDR KB/VCR and parental KB cells (b) and in MDR K562/A02 and parental K562 cells (c). (d) The impact of p38, AKT, and ERK inhibitors on Tan-1-mediated enhancement of cellular p38, AKT, and ERK phosphorylation. KB/VCR cells were pretreated with PI103 (10 μ M, AKT) or AZD6244 (5 μ M, ERK) for 30 min or SB203580 (50 μ M, p38) for 10 min. The cells were then exposed to 20 μ M of Tan-1 for 15 min (p-p38 and p-ERK) or 4 h (p-AKT and p-705-Stat3) followed by western blotting analyses. (e) The impact of siStat3-induced Stat3 reduction on the Tan-1-mediated phosphorylation of p38, AKT, and ERK. KB/VCR cells were pretreated with siCtrl or siStat3 for 48 h. The cells were then exposed to 40 μ M of Tan-1 for the indicated time and were analyzed by western blotting

Figure 3 Tanshinone-1 (Tan-1) activated cellular phosphatases. (a) Tan-1 did not inhibit upstream kinases of Stat3. Cells were treated with 40 μ M Tan-1 and analyzed by western blotting. (b) Na_3VO_4 prevented the decrease of phospho-705-Stat3. Cells were pretreated with 20 μ M Na_3VO_4 for 30 min, followed by treatment with 40 μ M Tan-1 for 4 h, and were then analyzed by western blotting. (c) Na_3VO_4 completely prevented the Tan-1-mediated proliferation inhibition. Cells were pretreated with 20 μ M Na_3VO_4 for 30 min, followed by the treatment with Tan-1 for 72 h, and were then analyzed by SRB assays and expressed as mean \pm S.D. (d) Na_3VO_4 decreased cleavage of procaspase 3 and PARP and the reduction of cyclin D1, Mcl-1, and cIAP-2. Cells were pretreated with 20 μ M Na_3VO_4 for 30 min, exposed to 20 μ M Tan-1 for 24 h, and then analyzed by western blotting. (e and f) Knockdown of Shp2 or PTP1B reversed the reduction of phospho-705-Stat3. Cells were transfected with siRNA against Shp2 and PTP1B for 48 h (e). The cells were then treated with 20 μ M Tan-1 for 4 h (f) and analyzed by western blotting. (g and h) Knockdown of Shp2 and PTP1B reduced the number of Annexin V/PI-positive cells. KB/VCR cells were transfected with siRNA against Shp2 and PTP1B for 48 h, followed by treatment with 20 μ M Tan-1 for 18 h, stained with Annexin V/PI, and then analyzed by flow cytometry. The apoptosis rates from three independent experiments were expressed as mean \pm S.D.; * $P < 0.05$ (g). Representative histograms are shown in h

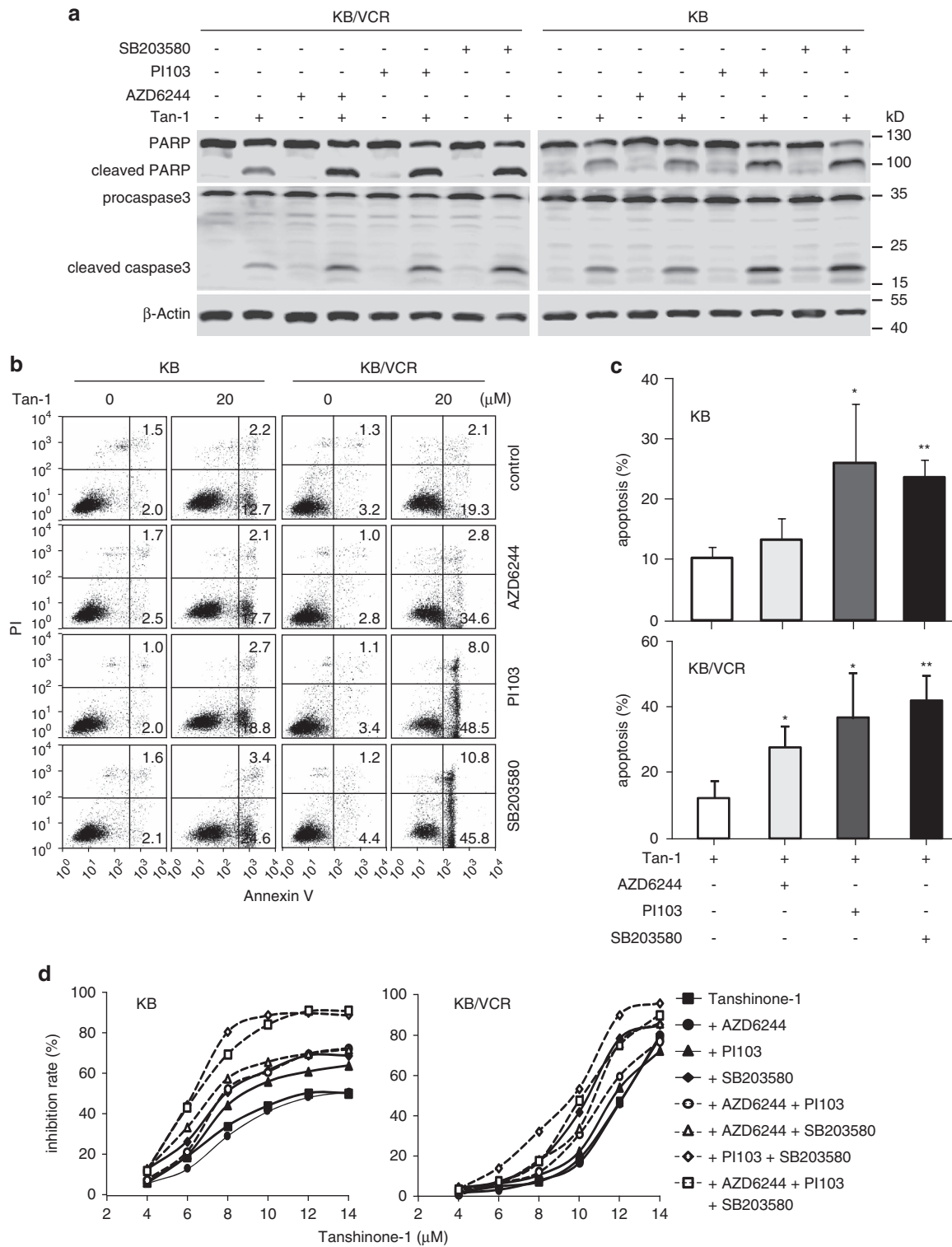


Figure 5 Cotreatments of p38, AKT and ERK inhibitors potentiated the tanshinone-1 (Tan-1)-induced apoptosis and cytotoxicity. (a–c) MDR KB/VCR and parental KB cells were pretreated with PI103 (10 μM, AKT) or AZD6244 (5 μM, ERK) for 30 min or SB203580 (50 μM, p38) for 10 min. The cells were then exposed to 20 μM of Tan-1 for 14 h. The cleavage of procaspase3 and PARP were detected by western blotting (a) and apoptotic induction was examined by flow cytometry (b). The apoptosis percentage from three independent experiments was expressed as mean ± S.D.; **P* < 0.05; ***P* < 0.01 (c). (d) Cells were pretreated with different combinations of PI103 (10 μM, 30 min), AZD6244 (5 μM, 30 min), or SB203580 (50 μM, 10 min), followed by exposure to gradient concentrations of Tan-1 for 14 h. The growth inhibition rates were analyzed by SRB assays. The data from three independent experiments were expressed as mean (S.D. was not shown for clearness and readability)

Each well contained 200 μ l of reaction buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 3 mM DTT), 1 mM pNPP, a phosphatase enzyme, and the test compound. The reaction was initiated by adding a phosphatase, and the reaction progress was monitored continuously for 15 min at an absorbance of 405 nm using a Tecan Infinite M1000 plate reader. The luminescence data were analyzed using the computer software, GraphPad Prism. In the absence of compounds, the slope (VT) of the data set was defined as 100% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity = (VC/VT) \times 100, where VC is the slope of the data set in the presence of the compound.

The tyrosine kinase activity assay was detected by enzyme-linked immunosorbent assays as reported previously.⁵¹ The inhibitory activity of tanshinone-1 against Jak1 was tested by Reaction Biology Corporation (Malvern, PA, USA); the impact of tanshinone-1 on Jak2 and Jak3 was examined by BPS Bioscience (San Diego, CA, USA).

Expression of constitutively activated Stat3. It is reported that residue substitutions of the mouse Stat3 at A661 and A663 with cysteine result in constitutive activation of Stat3.^{58,59} We amplified the human wild-type *stat3* expression plasmid from the HG10034-M-H clone (Sino Biological Inc., Beijing, China), and constructed an expression plasmid containing constitutively activated human Stat3 (pCMV-H3C) by site-directed mutagenesis as reported previously;^{58,59} the mouse constitutively activated Stat3 plasmids were gifts from Drs Hua Yu and Jie-Hui Deng (Beckman Research Institute, USA) and Dr. James Darnell (The Rockefeller University, USA). Cells were seeded in 12-well plates at 1.5×10^6 cells per ml and incubated overnight. And then the indicated plasmids were transfected into the KB cells for indicated time before the treatment with tanshinone-1.

Statistical analysis. Data were presented as mean \pm S.D., and statistical significance was assessed using a Student's *t*-test. Differences were considered significant at $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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