Combination effects of SC144 and cytotoxic anticancer agents

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Previously, we synthesized a series of hydrazide class of compounds and examined their cytotoxicity in a number of cancer cell lines. Among these analogues, SC144 exhibited potent cytotoxicity against a panel of drug-sensitive and drug-resistant cancer cell lines. To further explore its therapeutic potentials in the combination settings, we evaluated the synergy between SC144 and selected conventional chemotherapeutic agents in in-vitro cancer cell models. SC144 showed synergism with both 5-fluorouracil and oxaliplatin when cotreated in colorectal cancer HT29 cells. Pretreatment with SC144 in oxaliplatin-resistant HTOXAR3 cells was more effective than oxaliplatin pretreatment. In addition, the combination of SC144 and paclitaxel exhibited synergism in MDA-MB-435 cells with a schedule-dependent block in cell cycle. In an MDA-MB-435 mouse xenograft model, coadministration of SC144 and paclitaxel delayed tumor growth in an SC144 dose-dependent manner. Evaluation of the pharmacokinetics of SC144 revealed that intraperitoneal administration of SC144 showed a two-compartmental pharmacokinetics elimination profile that was not observed in the oral dosing. In summary, these studies further validate SC144 as a novel anticancer agent and provide insights for developing combination therapies for both drug-sensitive and drug-resistant cancers. *Anti-Cancer Drugs* 20:312–320 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Development of drug resistance remains a major impediment for successful cancer treatment. It is well established that the mechanisms of drug resistance to most anticancer drugs are multifactorial [1]. These include genetic and epigenetic alterations in cells that are implicated in determining the response to treatment [2]. Various cellular mechanisms, including changes in drug targets, increasing repair of drug-induced DNA damage, and altered expression of drug influx and efflux transporters, are all responsible for drug resistance [3–7]. Among these factors, a group of genes encoding ATP-dependent transporters are of particular importance in drug resistance [8]. Drugs that are affected by these transporters include doxorubicin, vinblastine, paclitaxel, and cisplatin [9,10]. Unlike normal cells, cancer cells exhibit increased genetic instability as well as substantial heterogeneity between individual cells [11]. As a result, each cancer cell responds differently to anticancer drugs and therefore, may show cross-resistance to drugs of different class, a phenomenon known as multidrug resistance [12]. Understanding the resistance mechanisms of each drug may open up possibilities for designing new treatment regimes, such as using combination therapy to treat cancers refractory to current therapies [13].

Combination therapy is most effective when using drugs with different mechanisms of action, at their optimal doses, to prevent the emergence of resistance. In most cases, combination chemotherapy is more effective than monotherapy [14,15]. Herein, we explored the anticancer activity of a novel hydrazide, SC144, in combination with several conventional cytotoxic agents using cytotoxicity assay and cell cycle analysis [16,17]. The synergy exhibited by the combination of SC144 and paclitaxel in breast cancer MDA-MB-435 cells prompted us to further examine their in-vivo efficacy in combination in a mouse xenograft model using the same cancer cell line. In addition to its potential in combination therapy, we also evaluated the activity of SC144 in oxaliplatin-resistant HT29 cells. Collectively, our results unveil the multifaceted chemotherapeutic potential of SC144 in combination with selected conventional anticancer agents in relevant cell settings, providing a promising direction for further development into a clinically effective anticancer drug.

Methods

Cell culture

HT29 and MDA-MB-435 cells were purchased from the American Type Cell Culture (Manassas, Virginia, USA). The HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were

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kindly provided by Dr Bert Vogelstein (Johns Hopkins Medical Institutions, Baltimore, Maryland, USA). Oxaliplatin-resistant HTOXAR3 cells were established from HT29 as described earlier [5]. Cells were maintained as monolayer cultures in the appropriate media: RPMI 1640 (HT29 and HTOXAR3) or DMEM (MDA-MB-435) supplemented with 10% fetal bovine serum (Gemini-Bioproducts, Woodland, California, USA) and 2 mmol/l L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. To remove adherent cells from the flask for subculture and counting, cells were washed with PBS without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, Missouri, USA) for 5–10 min, resuspended with culture medium and centrifuged. All experiments were performed using cells in exponential growth. Cells were routinely checked for Mycoplasma contamination by using PlasmoTest (InvivoGen, San Diego, California, USA).

Cytotoxicity assays

Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier [18]. Cells were seeded in 96-well microtiter plates, allowed to attach overnight and subsequently treated with a continuous exposure to the corresponding drugs for 72 h. After the drug treatments, an aliquot of MTT solution (at a final concentration of 0.5 mg/ml) was added to each well and cells were incubated for an additional 4 h at 37°C. After removal of the supernatant, dimethyl sulfoxide (DMSO) was added and the absorbance was read at 570 nm. All assays were performed in triplicate. Inhibitory concentration 50% (IC_{50}) values were determined for each drug from a plot of log (drug concentration) versus percentage of cell kill. Standard deviation was calculated based on the IC_{50} values obtained from at least three independent experiments.

Compound dilutions

Stock solutions of 10 mmol/l of all compounds were prepared in DMSO and stored at −20°C. Further dilutions were made fresh in cell-culture media.

Drug combination studies

To define the best combination of SC144 and paclitaxel, three different treatment schedules were used (i) SC144 (24 h) → paclitaxel (24 h), (ii) paclitaxel (24 h) → SC144 (24 h) and (iii) SC144 + paclitaxel (48 h). Cell viability for each combination was assessed by MTT assay and data were analyzed using the Chou–Talalay method [19]. Briefly, the analysis of combined drug effects was performed in each experiment with serial dilutions of both drugs added at doses that typically correspond to 1/4, 1/2, 1, 1.5, and three times the individual IC_{50} values. Fractional survival was then calculated by dividing the number of cells in drug-treated plates by the number of cells in control plates. By using this method, it is possible to calculate the doses of the individual drugs and the combination required to produce varying levels of cytotoxicity. For each level of cytotoxicity, a parameter called the combination index (CI) was calculated according to the following equation: $CI_f = D_1/(D_f)_1 + D_2/(D_f)_2 + \lambda D_1 D_2/(D_f)_1(D_f)_2$, where D_1 and D_2 are the concentrations of the combination required to produce survival f. $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce f. The value for λ was set to one or zero depending on whether the drugs are assumed to be mutually nonexclusive or mutually exclusive, respectively, in their action. According to this method, synergism is indicated by a $CI < 1$, antagonism by a $CI > 1$, and additive by $CI = 1$.

Cell cycle analysis

Cell cycle perturbations were analyzed by propidium iodide DNA staining. Briefly, exponentially growing cells were treated with IC_{80} doses of each drug in a sequential combination schedule for 48 and 72 h. At the end of the treatment, cells were collected and washed with PBS after a gentle centrifugation at 1200 rpm for 5 min. Cells were thoroughly resuspended in 0.5 ml of PBS and fixed in 70% ethanol at −20°C overnight. Ethanol-fixed cells were then centrifuged at 1200 rpm for 5 min and washed twice in PBS to remove residual ethanol. For cell cycle analysis, the pellets were resuspended in 1 ml of PBS containing 0.02 mg/ml of propidium iodide and 0.5 mg/ml of DNase-free RNase A and incubated at 37°C for 30 min. Cell cycle profiles were obtained by using BD LSRII flow cytometer (BD Biosciences, San Jose, California, USA) and data were analyzed with BD FACSDiva software (BD Biosciences) and the ModFit LT software package (Verity Software House, Topsham, Maine, USA).

Pharmacokinetics study

Preliminary pharmacokinetics of SC144 was performed using eight mice, each time point consisting of two mice. The mice were stratified to various doses given as an intraperitoneal (i.p.) injection of SC144 (25 mg/kg), which was compared with the oral (25 mg/kg) administration. At the specified time points (1, 4, 8, and 24 h) after drug administration, blood was collected with heparinized syringes. The samples were allowed to settle in 500 µl Eppendorf vials, and then centrifuged at 1500 rpm for 10 min. The plasma was removed and transferred into a new eppendorf tube and frozen at −80°C until analysis.

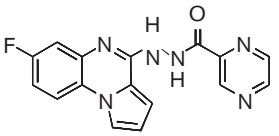
Liquid chromatography–mass spectrometry methodology

The concentration of SC144 was quantified using a validated liquid chromatography–mass spectrometry method in which the lower level of quantification was

0.1 ng/ml. Plasma levels were determined by adding 50 µl of SC144 (100 ng/ml) to 50 µl plasma and the entire sample was precipitated by adding 500 µl of methanol. Samples were then centrifuged at 15 000 rpm for 5 min at 4°C. The supernatant was evaporated completely, and the residue was reconstituted with 150 µl of mobile phase, and 10 µl reconstitute was injected into an Agilent 1100 HPLC system (Agilent Technologies Inc., Santa Clara, California, USA) linked to a API 3000 mass spectrometer (Applied Biosystems, Carlsbad, California, USA).

SC144 was separated using a C18 column. The analytes were eluted using a gradient of a mobile phase system containing two different components. Component A consisted of acetonitrile, whereas component B was 0.1% formic acid. The gradient program was 10% of acetonitrile from 0 to 2.5 min, after which time the acetonitrile increased from 10 to 95% for the next 4.5 min (or 7 min after injection). The level of acetonitrile was then switched back to 10% acetonitrile for the next 7 min. The flow rate

Table 1 **Cytotoxicity of SC144 in four colon cancer cell lines**



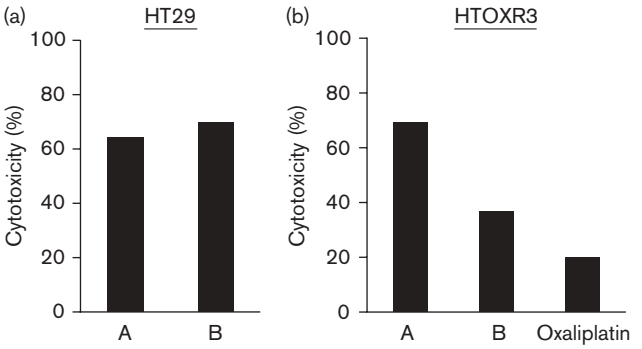
SC144

Cell line	Characteristics	IC_{50} (µmol/l)
HT29	p53 mutant	0.9 ± 0.06
HCT116 $p53^{+/+}$	p53 wild-type	0.6 ± 0.07
HCT116 $p53^{-/-}$	p53 null	1.0 ± 0.04
HTOXAR3	Oxaliplatin resistant HT29	0.8 ± 0.07

Table 2 **Cytotoxicity of six drugs in HT29 colon cancer cells**

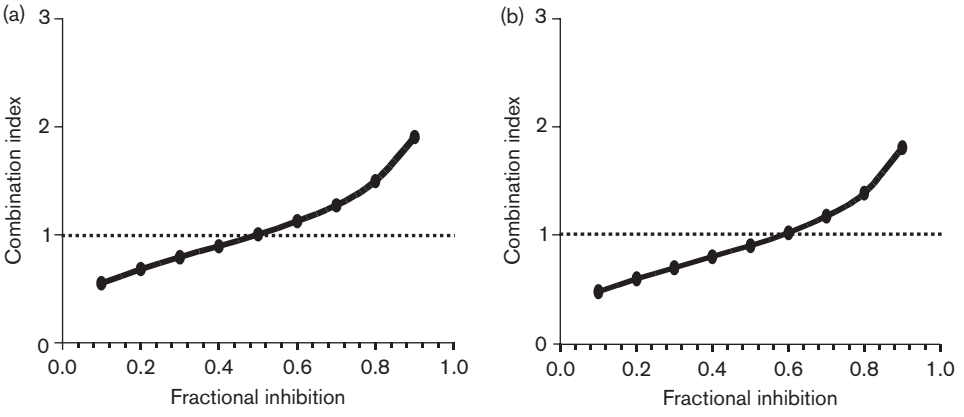
Drug	IC_{25} (µmol/l)	IC_{50} (µmol/l)
SC144	0.5	0.9
5-FU	1.9	5.5
CDDP	1.2	7.0
Oxaliplatin	0.22	0.55
CPT-11	0.009	0.045
Paclitaxel	0.13	0.4
Etoposide	0.4	8.0

Fig. 2



SC144 significantly enhanced the cytotoxicity of oxaliplatin in oxaliplatin-resistant HTOXAR3 cells. (a) Combination of SC144 and oxaliplatin exhibited similar cytotoxicity with both sequential treatment schedules in HT29 cells. (b) Pretreatment of oxaliplatin-resistant HTOXAR3 cells with SC144 markedly increased the activity of oxaliplatin in treatment A when compared with treatment B schedule. Treatment A: SC144 (24 h) → oxaliplatin (24 h). Treatment B: oxaliplatin (24 h) → SC144 (24 h).

Fig. 1



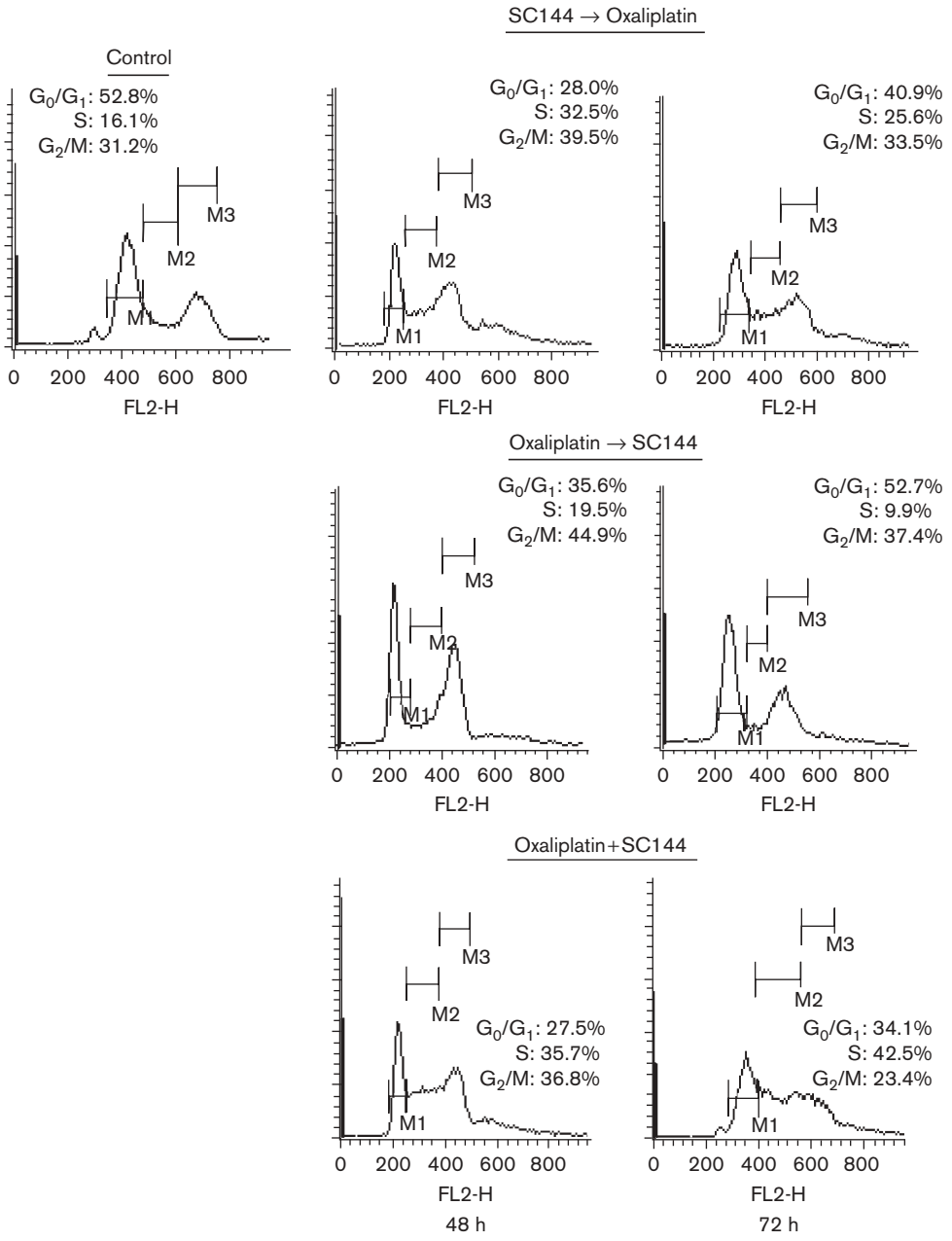
SC144 is synergistic with 5-fluorouracil (5-FU) and oxaliplatin in HT29 cells. (a) SC144 synergistically enhanced the cytotoxicity of 5-FU at lower fractions of cell kill according to the Chou–Talalay method. (b) Synergism was also seen between SC144 and oxaliplatin when the fractional inhibition was below 60%.

was set at 350 µl/min. The concentration of SC144 was monitored using selective reaction monitoring for M + → transition ion of 323.1 → 305.4.

In-vivo mouse xenograft studies

Human breast cancer MDA-MB-435 cells in logarithmic growth from in-vitro cell culture were inoculated subcutaneously in the flank of athymic nude mice (2×10^6 cells/mouse) under aseptic conditions. Tumor growth was assessed by bi-weekly measurement of tumor diameters with a Vernier caliper (length × width). Tumor weight was calculated according to the following formula: TW (mg) = tumor volume (mm^3) = $d^2 \times D/2$, where d and D are the shortest and longest diameters, respectively. Tumors were allowed to grow to an average volume of 100 mm^3. Animals were then randomly assigned to control

Fig. 3



Cell cycle arrest by combination of SC144 and oxaliplatin in HT29 cells. Pretreatment of HT29 cells with SC144 or simultaneous treatment halted cells at S-phase.

and treatment groups, and received vehicle (DMSO in sesame oil) or a combination of paclitaxel (10 mg/kg) and SC144 (25, 50, 75, and 100 mg/kg, dissolved in DMSO and diluted in sesame oil) through i.p. injections once daily for 15 days. Treatment of each animal was based on individual body weight. After 15 days of treatment, the tumor volumes in each group were measured once a week until the control tumor reached 1800 mm^3. Treated animals were checked daily for treatment toxicity/mortality.

Statistical analysis

Eight mice were assigned to each group and the results were expressed as the mean ± SEM. Statistical analysis and *P* value determination were performed by using two-tailed paired *t*-test with a confidence interval of 95% for the determination of the differences between groups. A *P* value of less than 0.05 was considered to be statistically significant. Analysis of variance was used to test for significance among groups. The SAS statistical software package (SAS Institute, Cary, North Carolina, USA) was used for statistical analysis.

Results and discussion

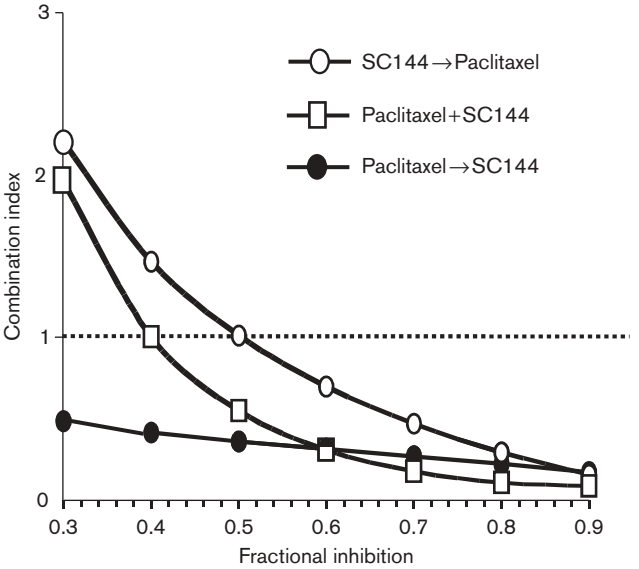
Growth inhibition of drug combinations

The cytotoxicity of SC144 was examined in four human colon cancer cell lines, HT29, HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, and the oxaliplatin-resistant cell line, HTOXAR3. In all the cell lines tested, SC144 showed cytotoxicity in the submicromolar range (Table 1). Earlier, we used HTOXAR3 cell line established from HT29 cells and showed altered expression of several genes involved in drug uptake, DNA repair, and detoxification mechanisms in this resistant cell line [5]. The potent growth inhibitory activity of SC144 in HTOXAR3 suggests that SC144 utilizes different cytotoxicity mechanisms in comparison with conventional chemotherapeutic agents and therefore, may be a good candidate for combination therapy to delay resistance. To compare the toxicity of SC144 with that of the conventional chemotherapeutic agents, we selected six anticancer drugs and evaluated their activity in HT29 cells. All agents showed potent activity (Table 2). Of the six agents tested, CPT-11 was the most potent agent with an IC_{50} of 45 nmol/l. Paclitaxel and oxaliplatin were also active with IC_{50} values of approximately 0.5 µmol/l. The IC_{50} of SC144 was comparable with both paclitaxel and oxaliplatin in HT29 cells. In comparison, etoposide and cisplatin had IC_{50} values of 8 µmol/l and were found to be the least active in this panel. To understand the growth inhibitory effect of SC144 in combination with current standard chemotherapeutic agents for colon cancer, we examined the cell fractions affected by SC144 and 5-fluorouracil in HT29 cells by using the Chou–Talalay method. A constant ratio of 1 : 0.24 between 5-fluorouracil and SC144 was used in this study. As shown in Fig. 1a, a synergistic effect was seen at the low proportions of cell death (e.g. fractional inhibition $< 50\%$). At higher fractional inhibition, additive or antagonistic effects were observed. Similar anticancer properties were also seen when SC144 and oxaliplatin were used in HT29 cells (Fig. 1b). These observations suggest that the interactions between SC144 and these two anticancer agents result in a synergistic effect only at the lower fractions of cell inhibition.

Pretreatment of SC144 significantly enhances the activity of oxaliplatin in HTOXAR3 cells

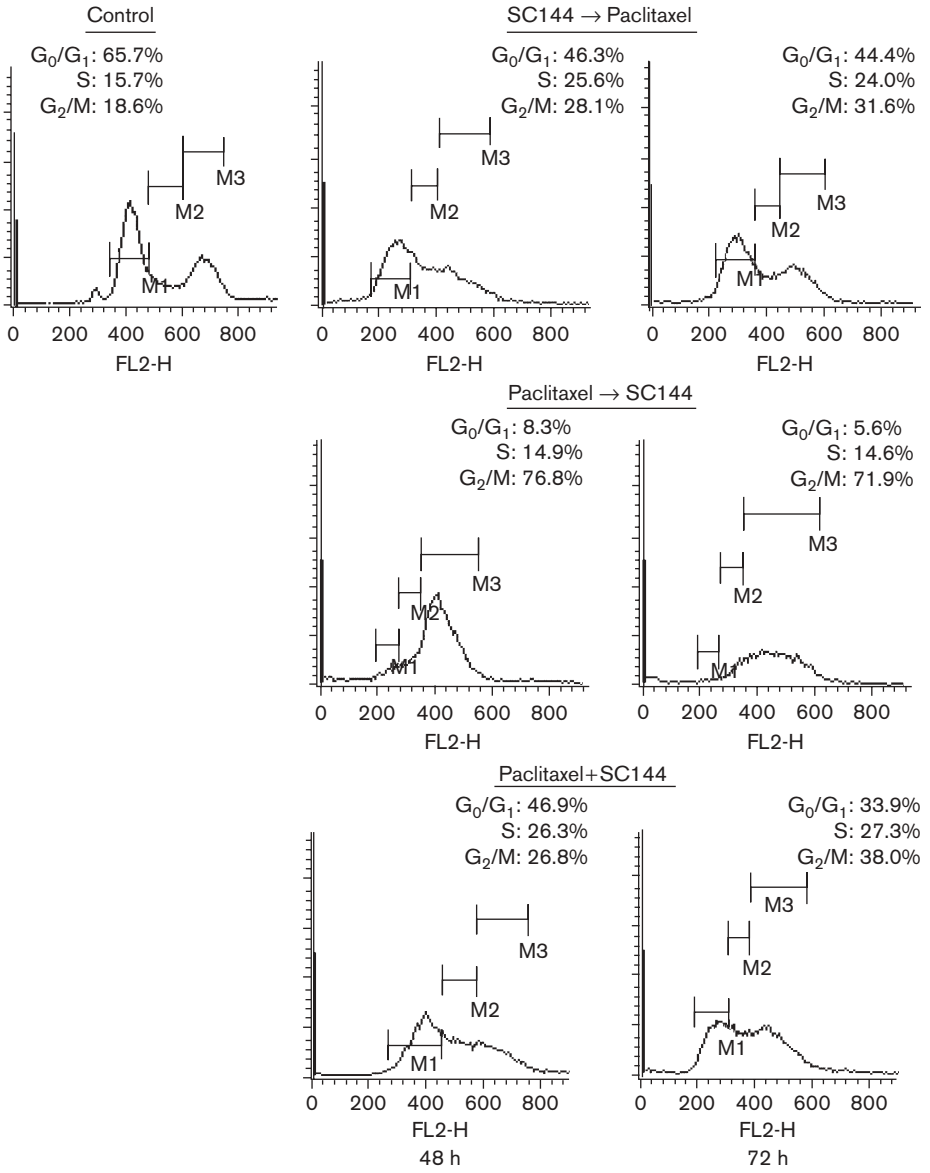
Oxaliplatin has now been widely used in combination therapies for the treatment of metastatic colorectal cancer [20]. However, resistance to oxaliplatin-based therapies often occurs in a similar kinetic pattern to other platinum agents [5,21]. To explore the therapeutic potential of SC144 in oxaliplatin-resistant colorectal cancers, we examined the cell growth inhibition with different treatment schedules of SC144 and oxaliplatin in combination, in HT29 and HTOXAR3 cells [5]. In HT29 cells, the cytotoxicity elicited by the combination of SC144 and oxaliplatin was independent of the sequential treatment (Fig. 2a). In HTOXAR3 cells, preexposure to SC144 seems to sensitize resistant cells towards oxaliplatin treatment. In contrast, when oxaliplatin was initially added, there was only 20% increase in cell growth inhibition as compared with oxaliplatin alone (Fig. 2b). This enhancing effect of SC144 on oxaliplatin cytotoxicity in the resistant cells warrants further examination to dissect the mechanisms of action [22].

Fig. 4



The synergism between SC144 and paclitaxel is dependent on the treatment schedule in MDA-MB-435 cells. Sequential treatment of paclitaxel-pretreated MDA-MB-435 cells with SC144 showed synergism. The other two treatment schedules showed synergism at higher proportions of cell kill.

Fig. 5



Cell cycle analysis in MDA-MB-435 cells after exposure to SC144 and paclitaxel with different treatment schedules. The synergistic treatment schedule between SC144 and paclitaxel disrupted cell cycle progression at the G_2/M phase.

SC144 and oxaliplatin affect cell cycle progression in a schedule-dependent manner

Oxaliplatin has been reported to cause G_2/M phase arrest in colorectal cancer HT29 cells [23]. To understand the effect of SC144 and oxaliplatin in combination, on cell cycle progression, we examined the cell cycle interruption in response to different treatment schedules. Single treatment of SC144 halted cells at S-phase as early as 24 h after exposure (data not shown). Sequential addition of oxaliplatin to SC144-treated cells or simultaneous administration of both agents caused the cells to arrest at S-phase, whereas sequential addition of SC144 to oxaliplatin-treated cells stopped the cell cycle progression at G_2/M phase (Fig. 3), suggesting that the mechanisms of action of SC144 and oxaliplatin in combination is schedule dependent.

SC144 increases paclitaxel cytotoxicity in a schedule-dependent manner

To further explore the combination therapeutic potential of SC144 in different cancer models, we evaluated the effect of SC144 and paclitaxel on the growth inhibition of MDA-MB-435 cells with different treatment schedules using the Chou–Talalay method. We chose paclitaxel

because weekly paclitaxel treatment is highly effective as a first-line chemotherapy regime in advanced breast cancer patients [24]. Herein, low individual doses of both agents were used in this experiment. The interactions between SC144 and paclitaxel resulted in 90% of cell fractions being affected. Interestingly, the anticancer activity between SC144 and paclitaxel seemed to be sequence dependent, where SC144 administration after paclitaxel pretreatment revealed synergism at all the fractions affected (Fig. 4). In contrast, the effects of the other two schedules were found to be only additive and antagonistic at the lower fractions of cell death (fractional inhibition $< 40\%$ with simultaneous use of the two drugs, fractional inhibition $< 50\%$ when SC144 was followed by paclitaxel). At higher proportions of cell death, synergistic anticancer activity was observed with these two treatment schedules.

The schedule-dependent interaction between paclitaxel and SC144 led us to examine the effect of their combination on cell cycle progression. Similar to oxaliplatin, single treatment of paclitaxel halted cells at G_2/M phase 24 h after exposure (data not shown). Sequential addition of paclitaxel to SC144-treated cells or simultaneous treatment with both agents interrupted cell cycle at S and G_2/M phase, whereas sequential addition of SC144 to paclitaxel-primed cells arrested cells at G_2/M phase (Fig. 5), implying that the characteristic of the interaction between SC144 and paclitaxel is dependent on the treatment schedule.

Pharmacokinetics of SC144

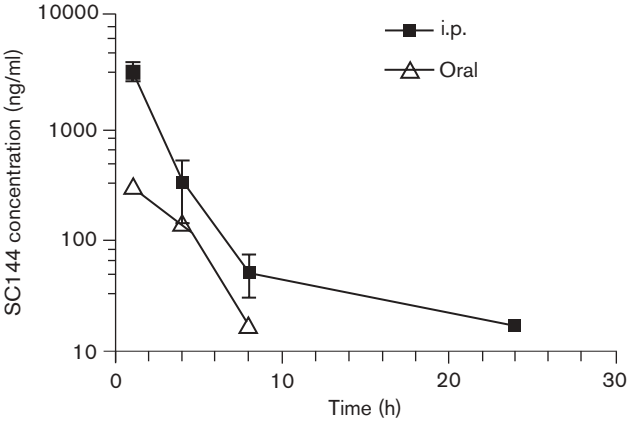
To understand the optimal dosing regime, a preliminary pharmacokinetics profile of SC144 was also evaluated (Table 3). The mice were treated either with i.p. or oral route administration of SC144 and serial blood draws were performed. The plasma concentration was evaluated by using liquid chromatography–mass spectrometry. One hour after the i.p. administration of SC144, the concentration in the blood was 3345 ng/ml. Sequential plasma analysis revealed an apparent two-compartmental pharmacokinetics elimination, which was not detected in the oral dosing (Fig. 6). Oral dosing of SC144 showed rapid absorption. The half-life between i.p. and oral dosages was similar, which was determined to be approximately 2 h when using a noncompartmental analysis.

Table 3 Representative pharmacokinetics parameters of SC144 in mouse

PK parameters	i.p.	Oral
C_{max} (ng/ml)	3345 ± 926	312
T_{max} (h)	1	1
Kel (h^{-1})	0.438 ± 0.342	0.422
$t_{1/2}$ (h)	2.275 ± 1.77	1.643
AUC (ng/ml × h)	6684 ± 169	999

AUC, area under the curve; i.p., intraperitoneal.

Fig. 6

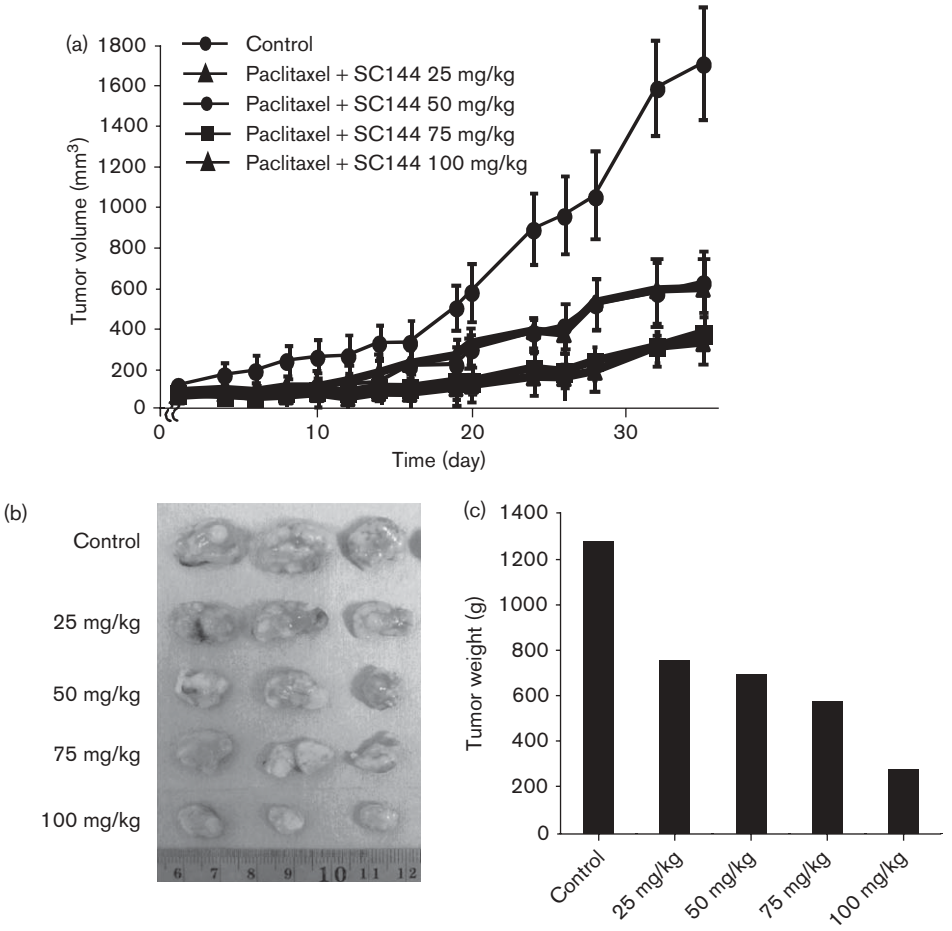


The pharmacokinetic distribution of SC144 in mice. Mice were treated with SC144 through intraperitoneal (i.p.) and oral route. Plasma concentrations of SC144 are plotted against treatment time.

Coadministration of SC144 and paclitaxel significantly inhibits tumor growth in a human breast cancer mouse xenograft model

The in-vivo efficacy of the combination of SC144 and paclitaxel was evaluated in a nude mouse xenograft model using human breast cancer MDA-MB-435 cells. On the basis of the pharmacokinetics profile of SC144, we treated the mice with daily i.p. injections of DMSO in sesame oil (controls) and 10 mg/kg paclitaxel in combination with indicated doses of SC144 (25, 50, 75, and 100 mg/kg, respectively). After 15 days of dosing, the compound treatments were discontinued and the animals were monitored bi-weekly for 5 weeks. Figure 7a shows the tumor volume (mean ± SEM) of different treatment groups over time. Combination treatment of SC144 and paclitaxel significantly reduced tumor burden and delayed tumor growth in a dose-dependent manner where higher doses of SC144 resulted in lower tumor volume ($P < 0.05$). Furthermore, the mean tumor volume between treatment groups showed a gradual decrease with increasing SC144 dose at the end of the experiment (Fig. 7b). Quantification of tumor size shown in Fig. 7b is presented in Fig. 7c. It is noteworthy that the trend of our in-vivo observation is consistent with our in-vitro cell culture combination studies in which simultaneous treatment of SC144 and paclitaxel elicited synergism only at higher doses. No organ toxicity was observed by histopathological examination of the organs derived from the mice (data not shown). Taken together, these results show that the combination of SC144 and paclitaxel inhibits the tumor growth and that higher doses of SC144 further decrease tumor size. Additional studies are in progress to better understand the mechanism of action and synergy of SC144 in different cancer models.

Fig. 7



Co-treatment of SC144 and paclitaxel delayed the tumor growth in a human breast cancer MDA-MB-435 mouse xenograft model. (a) Combination of SC144 and paclitaxel reduced the sizes of tumors in a dose-dependent manner. Values represent the median tumor weight for each group. (b) A representative comparison image of the tumors derived from the control and treated mice. (c) Quantification of the mean weight of the excised tumors in Fig. 7b.

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