Synergy of the Protein Farnesyltransferase Inhibitor SCH66336 and Cisplatin in Human Cancer Cell Lines

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ABSTRACT

The enzyme protein farnesyltransferase, which catalyzes the first step in the posttranslational modification of ras and a number of other polypeptides, has emerged as an important target for the development of anticancer agents. SCH66336 is one of the first farnesyltransferase inhibitors to undergo clinical testing. In the present study, we examined the effect of combining SCH66336 with several classes of antineoplastic drugs in various human tumor cell lines. Flow cytometry indicated that SCH66336 had no effect on the cell cycle distribution of treated cells. Nonetheless, colony-forming assays revealed that the antiproliferative effects of SCH66336 and 5-fluorouracil were less than additive. In contrast, the effects of SCH66336 and melphalan were additive. Moreover, the combination of SCH66336 + cisplatin produced antiproliferative effects that were additive or synergistic over a broad range of clinically achievable concentrations in A549 non-small cell lung cancer cells and T98G human glioblastoma cells, but less than additive in MCF-7 breast, HCT116 colon, or BxPC-3 pancreatic adenocarcinoma cells. Examination of the effect of drug sequencing in A549 cells revealed synergism when cells were exposed to SCH66336 and then cisplatin and antagonism when drugs were administered in the opposite order. The additive and synergistic effects resulted in enhanced apoptosis with the SCH66336 + cisplatin combination. Additional studies failed to show any effect of SCH66336 on the formation or removal of platinum-DNA adducts, raising the possibility that SCH66336 is affecting survival of cisplatin-treated cells downstream of the DNA lesions. These observations suggest that SCH66336 exhibits additive or synergistic effects when combined with cisplatin in a sequence- and cell line-dependent fashion. Additional preclinical and clinical study of this combination appears warranted.

INTRODUCTION

FT4 catalyzes the first step in the posttranslational modification of a number of cellular polypeptides, including ras (1), the nuclear intermediate filament proteins lamin B (2) and prelamin A (3), the centromere proteins CENP-E and CENP-F (4), protein tyrosine phosphatases PRL-1, -2 and -3 (5), cyclic GMP phosphodiesterase (6), rhodopsin kinase (7), the peroxisomal protein PxF (8), the cytoplasmic chaperone HDJ-2 (9), and the γ-subunit of the retinal protein transducin (10). FT has attracted attention because of its role in the processing of ras proteins, which function as molecular switches linking receptor and non-receptor tyrosine kinase activation to downstream cytoplasmic and nuclear events. Activating mutations in these ras proteins result in constitutive signaling, thereby stimulating cell proliferation and inhibiting apoptosis (11). Oncogenic ras mutations have been identified in ~30% of human cancers (12). Because the maturation of ras proteins was originally reported to be dependent on farnesylation (13), FT inhibition was envisioned as a strategy for interfering with ras-mediated cell transformation (14–16), although it is now clear that there might be additional targets as well (17, 18). In particular, the M, 21,000 small G protein rhoB has been hypothesized to be the target of FTIs. Inhibition of rhoB farnesylation is thought to lead to accumulation of geranylgeranylated rhoB (19), which inhibits cell proliferation (20, 21). Other data implicate a farnesylated protein associated with the PI3-kinase/Akt2-mediated cell survival pathway as the target of FTIs (22, 23).

Recently, four FTIs have been introduced into clinical trials. At least two of these, including SCH66336, are currently undergoing Phase II testing (16, 24). A clinical response was documented in a patient with NSCLC in a Phase I trial of this agent (25). In addition, ~40% of NSCLC cells carry a ras mutation (12). Because of these observations, there is considerable interest in testing FTIs in NSCLC as well as other solid tumors.

As SCH66336 and other FTIs progress through clinical trials, there is increasing interest in examining the effects of these agents in combination with other anticancer drugs. Only three published studies have examined the effect of combining FTIs with other chemotherapeutic agents. Moasser et al. (26) evaluated the effect of combining the FTI L744832 with pacli-
tairol or epothilone B in cultured MCF-7 and MDA-MB-468 cells. Results of this analysis revealed synergy between L744832 and the two spindle poisons. Only a small number of cell lines were studied, however; and the mechanistic basis for the synergy was not identified. More recently, Liu et al. (27) studied the effect of combining SCH66336 with vincristine, cyclophosphamide, or 5-FU in transgenic mice; and Sun et al. (28) evaluated FTI2148 in combination with paclitaxel, gemcitabine, or cisplatin in nude mice bearing human tumor xenografts. The latter two studies demonstrated enhanced antitumor effects with certain combinations. By their very nature, however, these latter two studies were unable to distinguish between effects that were additive versus those that were synergistic in a strict mathematical sense. Moreover, the mechanistic basis for the enhanced antitumor effects was not determined.

Recent experiments (25, 29, 30) have raised the possibility that some biochemical alterations observed in neoplastic cells might be drug-specific rather than reflecting effects common to all FTIs. These observations highlight the potential importance of studying drug combinations using the drugs that will actually be tested clinically. Of the three previous studies examining FTIs in combination with other antineoplastic agents, only one (28) was performed with an FTI that is being tested in the clinic. Accordingly, we have begun to study the effect of combining SCH66336 with other agents in preclinical models. For these studies, we used colony-forming assays to examine the effect of treating cells with cisplatin, melphan, or 5-FU in a number of cell lines, including the A549 NSCLC line as well as T98G glioblastoma, MCF-7 breast, BxPC3 pancreatic, and HCT-116 colon carcinoma cell lines. Our study reveals that the combination of SCH66336 and cisplatin exhibits synergy that is dependent on sequence of administration and on the model system used.

MATERIALS AND METHODS

Materials. SCH66336 was supplied by Schering-Plough Research Institute (Piscataway, NJ). Cisplatin, 5-FU, and melphanal were from Sigma Chemical Co. (St. Louis, MO). A stock solution of SCH66336 (10 mM) in DMSO was prepared and stored in small, single-use aliquots at −20°C. Concentrated (1000-fold) solutions of cisplatin and melphanal in DMSO or 5-FU in ice-cold water were prepared immediately before use. All other agents were obtained as described previously (31).

Colony-forming Assays. Cell lines from American Type Culture Collection (Manassas, VA) were grown in the following media containing 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine: (a) A549 in RPMI 1640–5% (v/v) FBS (medium A); (b) BxPC3 and HCT-116 in RPMI 1640–10% (v/v) FBS; (c) T98G in minimal essential medium containing Earle’s salts, 10% (v/v) FBS, nonessential amino acids, and 1 mM sodium pyruvate (medium B); and (d) MCF-7 cells in medium B containing 10 μg/ml insulin.

After subconfluent monolayers were trypsinized, aliquots containing 500 A549 cells were plated in multiple 35-mm dishes containing 2 ml of medium A and incubated for 18–24 h at 37°C to allow cells to attach. Graded concentrations of drugs or equivalent volumes of diluents were then added to triplicate plates, which were incubated for 7 days. Resulting colonies were stained with Coomassie Blue and counted manually. Control plates typically contained 150–200 colonies. HCT-116 cells were treated identically. With T98G, MCF-7, and BxPC3 cells, 750, 1000, or 1000 cells, respectively, were plated in the media indicated above and incubated with drugs for 7 days (T98G), 10 days (BxPC3), or 14 days (MCF-7).

To examine the effect of sequential exposure, cells that had been allowed to adhere for 18–24 h were exposed to graded concentrations of cisplatin for 24 h. Graded concentrations of SCH66336 were then added to the medium, and cells were incubated for a total of 7–8 days to allow colonies to form. Exposure to the reverse sequence was performed in a similar fashion (SCH66336 exposure for 24 h before the addition of cisplatin and additional incubation for 7–8 days). In each experiment, additional plates were exposed to each drug alone at the same time that plates were exposed to drugs in sequence.

Analysis of Combined Drug Effects. Dose-response curves were initially generated for each agent to estimate its IC50 for the cell line under study. In subsequent experiments, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously or sequentially at a fixed ratio of doses that corresponded to one-half, five-eighths, three-quarters, seven-eighths, 1, and 1.5 times the individual IC50s. Cells were exposed continuously to the candidate drugs. Fractional survival (f) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Data were analyzed by the method of Chou and Talalay (32). In brief, \( \log(1/f) - 1 \) was plotted against \( \log(\text{drug dose}) \). From the resulting median effect curves, the X intercept (log IC50) and slope m were calculated for each drug and for the combination by the method of least squares. These parameters were then used to calculate doses of the individual drugs and the combination required to produce varying levels of cytotoxicity \( f = 0.95, 0.90, 0.85, \ldots , 0.05 \) according to the equation:

\[
Dose_f = Dose_{ICS}[\frac{1 - f}{f}]^m
\]

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival \( f \) could be divided into the component doses \((D_1)\) and \((D_2)\) of drugs 1 and 2, respectively. For each level of cytotoxicity, a parameter called the CI was calculated according to the equation:

\[
CI = \frac{(D_1)/(D_1)_{50} + (D_2)/(D_2)_50 + \alpha (D_1)/(D_1)_50(D_2)/(D_2)_50}{(D_1)/(D_1)_{50} + (D_2)/(D_2)_50}
\]

where \((D_1)_{50}\) and \((D_2)_{50}\) are concentrations of the combination required to produce survival \( f \). \((D_1)_{50}\) and \((D_2)_{50}\) are the concentrations of the individual drugs required to produce \( f \) and \( \alpha = 1 \) or 0 depending on whether the drugs are assumed to be mutually nonexclusive or mutually exclusive, respectively. In this method, synergy is indicated by CI < 1, additivity by CI = 1, and antagonism by CI > 1 (32).

Platinum Measurement. Platinum-DNA adducts in T98G cells were assayed as described previously (31, 33). Briefly, five subconfluent 100-mm tissue culture plates containing \( \sim 10^7 \) T98G cells/plate were incubated with 4 μM SCH66336 in medium B for 24 h. Cisplatin (40 μM) was then added to the medium for 2 h. Cells were then washed twice with serum-free medium and incubated at 37°C in medium B with or without
SCH66336 for 0–24 h as indicated. At the completion of the incubation, plates were washed once in ice-cold PBS. Cells were released by brief trypsinization, collected by centrifugation at 200 g for 10 min, washed three times in ice-cold PBS, and lysed in 5 ml of TEN buffer [10 mM Tris-HCl (pH 7.4 at 20°C), 10 mM EDTA, and 150 mM NaCl] supplemented with 0.4% SDS and 1 mg/ml proteinase K. DNA purification and assay for elemental platinum by mass spectroscopy were performed as described previously (31, 33).

Cell Cycle Analysis. Logarithmically proliferating A549 cells were incubated with 0–10 μM SCH66336 for 48 h, washed with drug-free RPMI 1640, released by trypsinization, and sedimented at 200 g for 10 min. After a wash with ice-cold PBS, cells were fixed at 4°C in 50% (v/v) ethanol, digested with RNase A, stained with propidium iodide, and subjected to flow microfluorimetry as described previously (34). Data were analyzed using ModFit software (Verity Software, Topsham, ME).

Assays for Apoptosis. Assays for apoptosis were performed as described previously (35). Briefly, T98G cells were plated in 10 100-mm tissue culture plates and incubated at 37°C for 12–16 h to allow cells to adhere. The cells were then treated with 1, 5, 10, or 20 μM cisplatin alone or in combination with 400 nM SCH66336 for 120 h. The nonadherent cells were harvested and washed twice in ice-cold PBS. The adherent cells were collected after trypsinization and washed twice in ice-cold PBS. Each cell population was counted and then the two populations were combined. The cells were fixed in 3:1 (v/v) methanol:acetic acid, stored at 4°C overnight, dropped onto glass slides, and mounted in 50% glycerol-100 mM Tris-HCl (pH 7.4) containing 1 μg/ml Hoechst 33258. Cells were then observed at ×400 and counted on a Zeiss Axioplan microscope equipped with an epi-illuminator and appropriate filters. Samples were photographed at ×1000 using a Zeiss Axiocam.

RESULTS

Lack of Effect of SCH66336 on Cell Cycle Distribution. Previous studies have demonstrated that SCH66336 inhibits protein farnesylation in a variety of cell lines at low micromolar concentrations (4, 28, 30). In particular, we have shown that 0.25 μM SCH66336 inhibits prenylation of a variety of polypeptides, including prelamin A and HDJ-2 (30). To build on these earlier results, we subsequently examined the effect of SCH66336 on cell cycle distribution. Flow cytometry indicated that treatment with increasing doses of SCH66336 for up to 48 h failed to cause any significant effect on the cell cycle distribution of A549 cells (results not shown). Similar effects were observed in T98G cells.5

Effect of Combining SCH66336 with 5-FU. In view of the lack of effect of SCH66336 on cell cycle distribution, we examined the effect of combining SCH66336 with the S-phase specific agent 5-FU. Because SCH66336 is being tested clinically using prolonged oral administration schedules (25, 36), and because 5-FU is also administered by prolonged infusion in some regimens (37), cells were exposed to one or both agents continuously for 7 days until colonies formed. Results obtained when A549 cells were treated with SCH66336 and 5-FU are shown in Fig. 1. Each of these agents by itself inhibited colony formation (Fig. 1, A and B, ○). When cells were exposed to a fixed (4:1) ratio of SCH66336 and 5-FU, colony formation was inhibited more than when cells were exposed to SCH66336 alone (Fig. 1A, ●). On the other hand, the addition of SCH66336 seemed to add little to the antiproliferative effects of 5-FU in this cell line (Fig. 1B, ●).

5 J. N. Davis and A. A. Adjei, unpublished observations.
To determine whether the effects were additive, less than additive, or greater than additive, the data were analyzed by median effect method (32). Of the various mathematical methods available to address this question (reviewed in Refs. 32 and 38), this approach was chosen because it requires data sets that are within the limits of experimental feasibility (50–100 tissue culture plates) in colony-forming assays. This method is mathematically equivalent to the isobologram method if two drugs are assumed to be mutually exclusive (32, 38). Because the formula for calculating the CI of mutually nonexclusive drug interactions (32) has recently been questioned (38), we included results in each panel are representative of three or more experiments. Bars, ± 1 SD.

Synergistic Cytotoxicity of SCH66336 and Cisplatin.

When A549 cells were simultaneously exposed to cisplatin and SCH66336 for 7 days, cisplatin markedly increased the antiproliferative effects of SCH66336 (Fig. 2). The CI of this combination was <1 over much of the range examined (Fig. 2D), with mean CI values of 0.8 ± 0.3 at the IC50 and 0.6 ± 0.2 at the IC90 (n = 3). The effect of combining SCH66336 and cisplatin was also examined in other cell lines. The CI consistently dropped below 1 at concentrations of the combination between the IC50 and the IC90 in T98G glioblastoma cells (CI = 0.8 ± 0.2 at the IC50, and 0.9 ± 0.1 at the IC90; n = 3; Fig. 2E). In contrast, the effects of the combination were less than additive in MCF-7 (CI = 1.3 ± 0.2 at the IC50), BxPC3 (CI = 1.3 ± 0.2 at the IC50), and HCT116 cells [CI = 1.4 ± 0.4 at the IC50 (data not shown)].

Enhancement of Cytotoxicity by SCH66336 Combined with Melphalan.

When SCH66336 was combined with the DNA cross-linking agent melphalan, the effects were strictly additive. The CI (1.06 ± 0.05 at the IC50; n = 3), was indistinguishable from 1 at all levels of cytotoxicity (Fig. 1E). The CI of this combination was likewise ~1 at all levels of cytotoxicity in T98G cells.

Lack of Effect of SCH66336 on Retention of Platinum-DNA Adducts.

The cytotoxicity of cisplatin is related to the intracellular retention of platinum-DNA adducts (39). In previous studies we showed that the dual topoisomerase I/topoisomerase II inhibitor pyrazoloacridine (40) sensitized cells to cisplatin by inhibiting removal of platinum-DNA adducts (31). To determine whether a similar phenomenon might account for

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To explore the potential effect of drug sequencing, A549 cells were exposed to cisplatin for 24 h before SCH66336 was added. In the reverse sequence, SCH66336 was incubated with A549 cells for 24 h before cisplatin was added. When cisplatin exposure preceded SCH66336 treatment, cytotoxicity was less than additive (CI ≥ 1; Fig. 3C). With the opposite sequence, however, the CI was <1 over most of the range of cytotoxicity (Fig. 3F), indicating that the synergistic effects of the combination were dependent on the cells being exposed to SCH66336 before or during cisplatin exposure.

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the synergism observed with SCH66336 and cisplatin, we compared the formation and removal of platinum-DNA adducts in the absence and in the presence of SCH66336. Results are shown for A549 cells, although identical results were also obtained in T98G cells.

As indicated in Fig. 4A, a 24-h pretreatment with 4 μM SCH66336 did not affect the number of platinum-DNA adducts formed during a 2-h treatment with cisplatin. In contrast, the niacin derivative 6-aminonicotinamide, which increases cisplatin accumulation in a variety of cell lines (41), increased the number of cisplatin-DNA adducts that formed in the same experiments.

In additional experiments, the effect of SCH66336 on removal of platinum-DNA adducts was examined. Cells were treated with SCH66336 or diluent for 24 h. Cisplatin was then added for 2 h. Cells were then washed and incubated for 4–24 h in the continued presence of diluent or SCH66336. As indicated in Fig. 4B, the presence of SCH66336 had no effect on the retention of platinum-DNA adducts.

Enhancement of Cisplatin-induced Apoptosis. Recent studies have questioned whether colony-forming assays provide an accurate reflection of cell killing (42, 43). To determine whether the greater-than-additive effects of cisplatin and SCH66336 reflected enhanced cell killing, T98G cells were treated with one or both agents for up to 120 h and then examined morphologically for evidence of apoptosis. Results of this analysis revealed that cisplatin induces apoptosis (Fig. 5). Moreover, treatment with SCH66336 increased the proportion of apoptotic cells despite the lack of effect on platinum-DNA adduct formation or retention.

DISCUSSION
In the present study, we have demonstrated that SCH66336, which exhibited antitumor activity in NSCLC in a Phase I clinical trial (25) and is currently undergoing Phase II testing in several solid tumors (16), produces sequence-dependent synergistic cytotoxicity when combined with cisplatin over a broad range of clinically achievable drug concentrations in A549 and T98G cells (Figs. 3 and 4). Additional experiments have demonstrated that similar results are obtained when another FTI in clinical trials, R115777, is used in place of SCH66336, suggesting that the cytotoxic synergy might be related to inhibition of FT. The mechanism responsible for the synergistic effects of SCH66336 and cisplatin requires additional investigation. Nonetheless, the present studies provide several important observations related to potential explanations for this synergy.

First, SCH66336 does not sensitize cells to all anticancer drugs. In particular, our studies revealed that SCH66336 does not potentiate the effects of 5-FU (Fig. 1, B and D), an observation that has potential implications for efforts to combine FTIs with fluoropyrimidines in the treatment of colon cancer. Moreover, our studies demonstrated that combinations of the FTI with melphalan (Fig. 2E) or gemcitabine (not shown) were strictly additive. The failure of SCH66336 to synergize with these agents suggests that SCH66336 is affecting sensitivity in a drug-specific manner rather than affecting the core cell death machinery. Consistent with this hypothesis, exposure of cells to SCH66336 fails to cause changes in levels of apoptotic regulators such as Bcl-2, BAD, Bax, and Bcl-XL.

Second, the synergism between cisplatin and SCH66336 was cell line-specific. Synergy was demonstrated in A549 and T98G cells but not in MCF-7, BxPC3, or HCT116 cells. Interestingly, the occurrence of synergism did not appear to correlate with the presence of wild-type ras (BxPC3 and MCF-7) as compared with mutant ras (A549 and HCT116). The presence of wild-type (A549, MCF-7, and HCT116) or mutant p53 (T98G)
also failed to correlate with the synergism. Moreover, there was no correlation between intrinsic sensitivity to SCH66336 and the occurrence of synergism, which was observed in T98G cells (SCH66336 IC\textsubscript{50}; 75 nM) and A549 cells (SCH66336 IC\textsubscript{50}; 2 mM) but not MCF-7 cells (IC\textsubscript{50}; 25 nM), HCT116 cells (IC\textsubscript{50}; 100 nM), or BxPC3 cells (IC\textsubscript{50}; 400 nM). These results not only imply that examining one cell line in vitro for evidence of synergy might be misleading, but also raise the possibility that SCH66336 might be affecting cisplatin sensitivity by altering the function of a polypeptide that acts in a cell type-specific manner.

Third, the present studies demonstrate that SCH66336 sensitizes cells to cisplatin in a manner that is different from that of recently described modulating agents. In particular, the action of SCH66336 appears to be distinct from that of 6-aminonicotinamide, which enhances the action of cisplatin by increasing drug accumulation and subsequent formation of platinum-DNA adducts (Ref. 41 and Fig. 4A). The amount of platinum covalently bound to DNA was compared with that found in cells lysed immediately after cisplatin exposure.

Fourth, the synergism between SCH66336 and cisplatin was also sequence-dependent. In particular, synergism was observed when cells were exposed to the two agents simultaneously or to SCH66336 and then to cisplatin, whereas antagonistic, which inhibits removal of platinum-DNA adducts (31). Because an SCH66336-induced alteration in the formation or removal of platinum-DNA adducts in bulk DNA was not detectable using the assay methodology used in these previous studies, the effects of SCH66336 on the action of cisplatin appear to be more subtle.

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**Fig. 4** Effect of SCH66336 on retention of platinum-DNA adducts. A, T98G cells were treated for 24 h with diluent, 400 nM SCH66336 or 62.5 μM 6-aminonicotinamide, then exposed for 2 h to 40 μM cisplatin. After extensive washing as described previously (31), highly purified cellular DNA was isolated so that platinum-DNA adducts could be quantified (31). In contrast to 6-aminonicotinamide (6AN), SCH66336 did not enhance platinum-DNA adducts. B, cells were incubated with diluent (□) or 400 nM SCH66336 (●) for 24 h. Cisplatin (40 μM) was then added for 2 h. After extensive washing, cells were incubated in cisplatin-free medium in the continued absence (□) or presence (●) of 400 nM SCH66336 for the indicated times and harvested for measurement of platinum-DNA adducts. The amount of platinum covalently bound to DNA was compared with that found in cells lysed immediately after cisplatin exposure.

**Fig. 5** Effect of SCH66336 on cisplatin-induced apoptosis. T98G cells were incubated with diluent or 400 nM SCH66336 and varying concentrations of cisplatin. Cells were harvested and examined morphologically for evidence of apoptosis. Data shown are an average of three experiments. SCH66336 alone induced apoptosis in <4% of cells. A, morphology of cells treated for 120 h with diluent, 2 μM cisplatin, 400 nM SCH66336, or both agents. B, percentage of cells with apoptotic morphology after treatment with varying concentrations of cisplatin in the presence of 400 nM SCH66336 (open bars) or diluent (closed bars) for the indicated length of time.
onism was observed when cisplatin preceded SCH66336. These results suggest that SCH66336 might be enhancing the effects of cisplatin by inhibiting the farnesylation of a polypeptide that ordinarily participates in cisplatin resistance. Although the function of the critical farnesylated polypeptide is currently unknown, several possibilities can be envisioned. One possibility is that SCH66336 is selectively affecting removal of a subset of the platinum-DNA adducts, e.g., by affecting strand-specific or transcription-coupled repair. Alternatively, it is possible that SCH66336 is affecting a component of the mismatch repair pathway, a pathway that has been implicated in sensitivity to cisplatin (44, 45). Finally, it is possible that SCH66336 is altering cisplatin sensitivity by affecting a polypeptide involved in the recognition of platinated DNA (reviewed in Ref. 46).

Although additional experiments are required to distinguish between these possibilities, the present observations have potentially important implications for future development of FTIs. SCH66336 and cisplatin not only display synergy in some cell lines in vitro, but these synergistic antiproliferative effects are observed at drug concentrations (0.05–4 μM SCH66336 and 2.5–20 μM cisplatin) that are readily achievable in vivo (25, 47). Although the effects of SCH66336 and cisplatin were only additive in other cell lines, it is important to stress that additivity implies that the antiproliferative effects of the combination were greater than either agent alone. Given the activity of cisplatin against a broad spectrum of human neoplasms (46), the additive and greater-than-additive effects observed in the present study suggest that additional preclinical and clinical studies of FTIs in combination with this cisplatin appear to be warranted.

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