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Synergistic induction of cancer cell death and reduction of clonogenic resistance by cisplatin and FK228

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ABSTRACT

Human urinary bladder cancer is the fifth most common cancer in the United States, and the long-term disease-free survival in patients is still suboptimal with current chemotherapeutic regimens. Development of effective chemotherapeutic regimens is crucial to decrease the morbidity and mortality of this cancer. The goal of this study was to investigate the effectiveness of FK228 in increasing cisplatin's ability to induce bladder cancer cell death and reduce drug resistance. Our study revealed that FK228 combined with cisplatin synergistically induced cell death and reduced clonogenic survival of human urinary bladder cancer cells. The Erk-Nox pathway played an important role in mediating signals highly increased by this combined treatment to induce significantly-elevated levels of reactive oxygen species, leading to substantially-induced caspase activation and synergistically-increased death in cancer cells. Cisplatin was able to enhance the ability of FK228 to significantly reduce glutathione, indicating a novel activity of combined FK228 and cisplatin in reducing drug resistance. The ability of combined FK228 and cisplatin to synergistically induce cell death and reduce clonogenic survival was also applicable to colon cancer cells. Hence, combined use of FK228 with cisplatin should be considered in development of therapeutic strategies to control urinary bladder cancer and other cancer development and recurrence.

1. Introduction

Urinary bladder cancer is the fifth most common human cancer in the United States; in 2013, it is expected that more than 72,000 people will live with urinary bladder cancer, and more than 15,000 deaths are estimated [1]. The high recurrence rate of 60–70% and the risk of progression to invasive disease necessitates life-long surveillance, thereby making bladder cancer the most expensive cancer to manage [2–4]. Current chemotherapeutic regimens, which have shown to be beneficial to survival of patients with invasive cancer, are the cisplatin-based regimens, such as MVAC (methotrexate, vinblastine, adriamycin, and cisplatin) and cisplatin combined with gemcitabine [4,5]. However, despite the initial high response rates with these regimens, the overall 5-year survival rate is still suboptimal at less than 20% [4]. Hence, development of more effective regimens is crucial to decrease the morbidity and mortality of bladder cancer.

Cisplatin is a DNA-damaging agent for treating various human cancers, including brain, ovary, lung, and testis cancers in addition to bladder cancer [5,6]. Cisplatin also has potent *in vitro* cytotoxic activity against human colon cancer cells [7,8]. Though DNA damage is a major mechanism for cisplatin-induced cytotoxicity [6], cisplatin's cytotoxicity may also be mediated by reactive oxygen species (ROS) [4,9,10]. However, the efficacy of cisplatin is often dampened by cancer cells' drug resistance, mostly associated with glutathione (GSH)-based detoxification [6,11,12]. GSH, a tripeptide with a reactive sulfhydryl group, plays an important role in cellular detoxification of anticancer agents [4]. Although GSH depletors, such as buthionine sulfoximine and diethylmaleate, have been used to augment cisplatin's activity to induce cancer cell death [9], they are still ineffective in reducing cell resistance to cisplatin [13]. Apparently, additional studies are required to identify agents that will increase the efficacy of cisplatin to induce cancer cell death and reduce cell resistance.

FK228 (FR901228, romidepsin), is a depsipeptide and a histone deacetylase inhibitor [14,15]. FK228 was approved by the U.S. Food and Drug Administration for treatment of T cell lymphoma. However, the therapeutic value of FK228 for solid tumors, including lung, pancreatic, and esophageal cancer, is still under clinical trials [16,17]. Although FK228 is effective in inducing apoptosis of human urothelial carcinoma T24 cells *in vitro* and regression of T24 xenograft tumors *in vivo* [18], its therapeutic value in treatment of bladder cancer is yet to be determined. Our previous studies revealed that expression of oncogenic H-Ras in human bladder tumor J82 cells results in increased susceptibility and reduced clonogenic

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resistance to FK228 for inducing apoptosis [19–22]. Expression of oncogenic H-Ras promotes J82 cells to acquire tumorigenicity, mimicking an acquisition of H-*ras* gene activation that is seen in more than 35% of human bladder cancers [19,23]. FK228-induced ROS plays a key role in reduction of GSH and clonogenic survival [21,24]. The clonogenic survival is used an index for cell resistance to anticancer agents [25]. Whether FK228-induced ROS elevation and GSH depletion may improve the efficacy of cisplatin for bladder cancer control needs to be studied.

In this communication, we investigated the effectiveness of FK228 combined with cisplatin in inducing cancer cell death and reducing clonogenic survival. We also investigated the synergy and mechanism of FK228 combined with cisplatin in induction of cell death and reduction of clonogenic resistance in bladder and colon cancer cells to reveal their potential value in therapeutic control of cancer cell viability and recurrence.

2. Materials and methods

2.1. Cell cultures and reagents

J82, HT29 (American Type Culture Collection [ATCC], Rockville, MD), and oncogenic H-Ras(V12)-expressing, J82-Ras and HT29-Ras cells [19,26] were maintained in DMEM supplemented with 10% fetal bovine serum. Cultures were maintained in medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C and routinely subcultured every 2 to 3 d. Stock aqueous solutions of FK228 (National Cancer Institute, Chemistry and Synthesis Branch, collaboration with Dr. K.K. Chan, The Ohio State University) and chloromethyl–dichlorodihydrofluorescein–diacetate (CM–H_2DCF–DA) (Invitrogen, Carlsbad, CA) were prepared in DMSO and diluted in culture medium for assays. Stock aqueous solutions of *N*-acetyl-L-cysteine (NAC) (Alexis, San Diego, CA) and cisplatin (LKT, St. Paul, MN) were prepared in distilled water and diluted in culture media for assays.

2.2. Cell viability

A Methyl Thiazolyl Tetrazolium (MTT) assay kit (ATCC) was used to measure cell viability. Five $\times 10^3$ cells was seeded into each well of 96-well culture plates. After indicated treatments, cells were incubated with MTT reagent for 4 h, followed by incubation with detergent reagent for 24 h. Reduced MTT reagent in cultures was quantified with an ELISA reader (Bio-Tek, Winooski, VT) [19].

2.3. Clonogenic assay

Triplicates of 5 $\times 10^3$ cells were seeded in 60-mm culture dishes. After treatment, cultures were replaced with fresh medium and maintained for 7 or 14 d. Growing colonies (>30 cells) were counted under an anatomical microscope [19].

2.4. ROS measurement

Cells were incubated with 5 μM CM–H_2DCF–DA for 1 h to detect ROS level by flow cytometry; the mean DCF fluorescence intensity of 2 $\times 10^4$ cells was quantified using Multicycle software (Phoenix, San Diego, CA), as performed previously [20–22].

2.5. Measurement of GSH

Intracellular GSH levels were measured with a GSH assay kit (Cayman, Ann Arbor, MI), as performed previously [21,24]. In brief, 50 μl of deproteinated cell lysates were incubated with 150 μl reaction buffer at 37 °C for 30 min. GSH was determined by absorbance at 405 nm using GSH disulfide as a standard; the levels were expressed as GSH per mg cell lysate protein.

2.6. Caspase activity assay

Caspase-3/7 activity was measured using a Caspase-Glo assay kit (Promega, Madison, WI), as performed previously [20,21]. In brief, 30 μg of cell lysates was incubated with a caspase-3/7-specific proluminescent substrate at ambient temperature for 1 h. The released luminescence was measured in a luminometer plate reader (Bio-Tek).

2.7. Immunoblotting

Equal amounts of cellular proteins were resolved by electrophoresis in either 10% or 14% SDS–polyacrylamide gels and transferred to nitrocellulose filters for immunoblotting [19,26], using specific antibodies to detect phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, Nox-1, and β-actin (Santa Cruz, Santa Cruz, CA). Antigen–antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce, Rockford, IL).

2.8. Statistical analysis

The Student *t* test was used to analyze statistical significance, indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; a *P* value of $\leqslant 0.05$ was considered significant. Combination indices analysis was performed using the method of Chou and Talay [27] via the CalcuSyn software suite (version 2.1, Biosoft, Cambridge UK). Combination indices less than, equal to, and greater than 1 indicate synergistic, additive, and antagonistic effects, respectively.

3. Results and discussion

3.1. Synergistic cell death induced by FK228 and cisplatin in bladder cancer cells

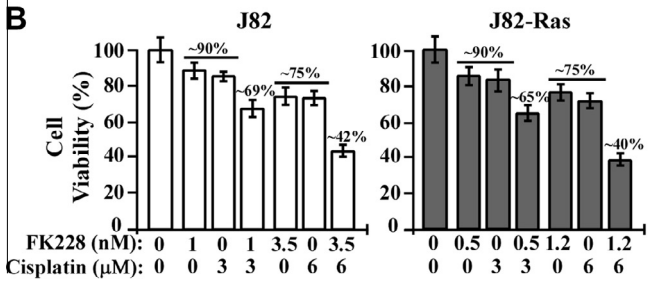
To investigate the effectiveness of FK228 and cisplatin in combination versus individually in induction of cancer cell death, we measured inhibitory concentrations of FK228 and cisplatin for J82 and oncogenic H-Ras-expressing, J82-Ras cells. As shown in Fig. 1A, FK228 IC_{10} and IC_{25} values for J82 cells were determined to be 1 and 3.5 nM, respectively, and for J82-Ras cells, 0.5 and 1.2 nM, respectively. Cisplatin IC_{10} and IC_{25} values were determined to be 3 and 6 μM, respectively, for both J82 and J82-Ras cells. Accordingly, expression of oncogenic H-Ras resulted in increased susceptibility of J82 cell to FK228, but not to cisplatin, for inducing cell death.

In determining whether FK228 and cisplatin were able to cooperatively induce cell death, we detected that treatment of J82 and J82-Ras cells with FK228 at IC_{10} values combined with cisplatin at IC_{10} values resulted in reduced cell viability to approximately 69% and 65%, respectively (Fig. 1B). Similarly, treatment of J82 and J82-Ras cells with FK228 at IC_{25} values combined with cisplatin at IC_{25} values resulted in reduced cell viability to approximately 42% and 40%, respectively. Using the Chou–Talalay method [27], we determined combination indices to be less than 1 when using combined FK228 and cisplatin at either their IC_{10} or IC_{25} values (Fig. 1C), indicating that combined FK228 and cisplatin treatments synergistically increased cell death in both J82 and J82-Ras cells.

We showed that the Erk pathway plays an essential role in mediating signals for Nox-1 elevation, ROS production, and caspase activation in FK228-induced apoptosis of J82 and J82-Ras cells [21]. Activation of the Erk pathway and elevation

A

Anticancer agents	IC Values	J82	J82-Ras
FK228 (nM)	IC_{10}	1	0.5
	IC_{25}	3.5	1.2
Cisplatin (μM)	IC_{10}	3	3
	IC_{25}	6	6



C

Combination indices

FK228/ Cisplatin	J82	J82-Ras
IC_{10}	0.61	0.62
IC_{25}	0.60	0.63

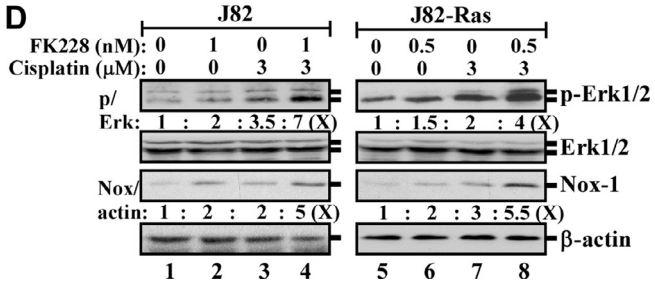


Fig. 1. Synergistic cell death induced by FK228 and cisplatin in bladder cancer cells. (A) J82 and J82-Ras cells were treated with FK228 at a range of concentrations from 0.1 to 5000 nM and cisplatin at a range of concentrations from 0.1 to 5000 μM for 48 h, cell viability determined, and IC_{10} and IC_{25} values determined for FK228 and cisplatin for J82 and J82-Ras cells. (B) J82 (white *columns*) and J82-Ras (dark *columns*) cells were treated with FK228 at IC_{10} and IC_{25} value doses in the presence and absence of cisplatin at IC_{10} and IC_{25} value doses for 48 h. Cell viability was measured, and relative cell viability was normalized by the value determined in untreated counterpart J82 and J82-Ras cells, set as 100%. *Columns*, mean of tetraplicates; *bars*, SD. (C) Combined effects (B) were evaluated. (D) J82 and J82-Ras cells were treated with FK228 at 0, 0.5, or 1 nM in the presence and absence of 3 μM cisplatin for 24 h. Cell lysates were analyzed to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-actin as a control, and these levels were quantified by densitometry. The levels of Nox-1 (Nox/actin) were calculated by normalizing with the level of β-actin and the level set in untreated control cells as 1 (X, arbitrary unit). The level of specific phosphorylation of Erk1/2 (p/Erk) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in control cells as 1 (X, arbitrary unit). All results are representative of three independent experiments.

of Nox-mediated ROS also reportedly play important roles in cisplatin-induced cell death [28–30]. In studying whether the Erk-Nox pathway was involved in FK228- and cisplatin-induced, synergistic cell death, we detected that treatment with combined FK228 and cisplatin at their IC_{10}.values increased Erk1/2 phosphorylation and Nox-1 level (Fig. 1D, lanes 4 & 8) to higher levels than their counterparts induced by either FK228 or cisplatin alone in J82 (lanes 2 & 3) and J82-Ras (lanes 6 & 7) cells. These results indicate that activation of the Erk-Nox pathway was involved in the synergistic induction of cell death by combined FK228 and cisplatin; a highly-induced Erk-Nox pathway appeared to result in high-level ROS, leading to significant cell death.

3.2. ROS in FK228- and cisplatin-induced synergistic cell death

In determining if intracellular ROS level was highly increased in conjunction with FK228- and cisplatin-induced synergistic cell death, we detected that treatment of J82 and J82-Ras cells with FK228 or cisplatin at their IC_{10} values resulted in mild increases of ROS (Fig. 2A) and caspase activity (2B) in correlation with mild degrees of reduced cell viability (2C). Treatment with combined FK228 and cisplatin resulted in significant increases of ROS level (2A) and caspase activity (2B) in correlation with high degrees of reduction of cell viability (2C). Using the general antioxidant NAC [31], we detected that blockage of ROS by NAC (2A) resulted in an effective inhibition of FK228- and cisplatin-induced caspase activation (2B) and cell death (2C). These results indicate that the level of ROS was elevated, resulting in significant caspase activation for synergistically-induced cell death by combined FK228 and cisplatin treatment. Activation of the Erk-Nox pathway conceivably played an important role in mediating signals synergistically induced by combined agents to induce ROS and caspase activation, leading to cancer cell death.

3.3. Synergistic reduction of clonogenic survival by FK228 and cisplatin

In studying whether treatment with combined FK228 and cisplatin may result in significant reduction of clonogenic survival

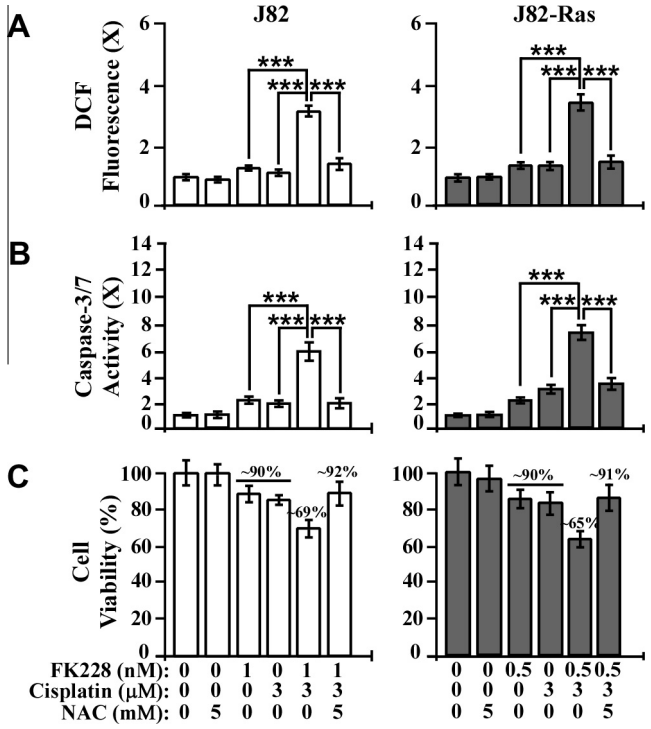


Fig. 2. ROS in FK228- and cisplatin-induced synergistic cell death. J82 (white *columns*) and J82-Ras (dark *columns*) cells were treated with FK228 at 0, 0.5, or 1 nM in the presence and absence of 3 μM cisplatin and/or 5 mM NAC for 24 h (A and B) or 48 h (C). (A) Cultures were then labeled with CM-H_2DCF-DA for flow cytometric analysis of ROS levels. Relative fluorescence intensity, as fold induction (X, arbitrary unit), was normalized by the fluorescence intensity determined in untreated, counterpart J82 and J82-Ras cells, set as 1. (B) Caspase-3/7 activity. Relative caspase activity, as fold induction (X, arbitrary unit), was normalized by the values determined in the untreated counterpart J82 and J82-Ras cells, set as 1. (C) Cell viability. Relative cell viability was normalized by the value determined in untreated counterpart J82 and J82-Ras cells, set as 100%. *Columns*, mean of tetraplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by $^{***}P < 0.001$. All results are representative of three independent experiments.

and GSH content in cancer cells, we detected that treatment of J82 and J82-Ras cells with FK228 or cisplatin at their IC_{10} values resulted in reduction of clonogenic survival to ranges between 75% and 72% (Fig. 3A). Treatment with combined FK228 and cisplatin at IC_{10} values significantly reduced clonogenic survival to approximately 13% in J82 and to 7% in J82-Ras cells. Treatment with cisplatin at IC_{25} values reduced clonogenic survival to ranges between 65% and 61%, and co-treatment with FK228 at IC_{10} values resulted in a complete elimination of clonogenic survival to 0%. The combination indices were less than 1 in using combined FK228 and cisplatin at IC_{10} values in reduction of clonogenic survival (Fig. 3B), indicating that FK228 combined with cisplatin synergistically reduced clonogenic survival of J82 and J82-Ras cells. Studying GSH content revealed that treatment with FK228 at its IC_{10} value reduced GSH in both J82 and J82-Ras cells, but treatment with cisplatin at its IC_{10} value did not induce any significant reduction of GSH; interestingly, treatment with combined FK228 and cisplatin at their IC_{10} values reduced GSH more than did FK228 alone (Fig. 3C). The GSH level reduced by combined FK228 and cisplatin was closely correlated with degrees of clonogenic survival synergistically reduced by combined FK228 and cisplatin. The ability of FK228 to reduce GSH appeared to be enhanced by co-treatment with cisplatin in reducing clonogenic survival, though cisplatin by itself was unable to induce significant reduction of GSH.

Our previous studies indicate that FK228-induced ROS plays an important role in depletion of GSH, contributing to the reduction of clonogenic resistance of J82 and J82-Ras cells to FK228 [21]. In contrast, cisplatin was able to induce ROS elevation but failed to induce GSH reduction. Whether cisplatin-induced ROS may participate in FK228-induced ROS to enhance the ability of FK228 to

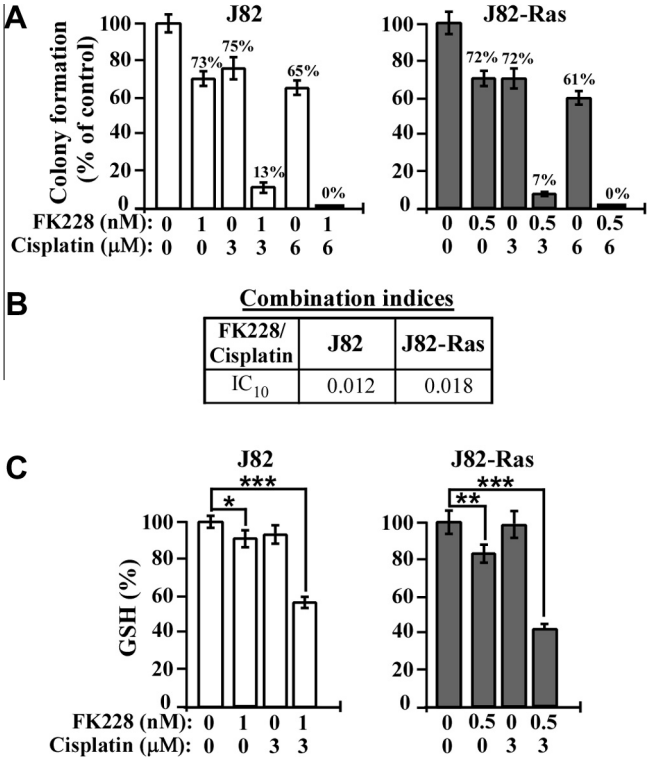
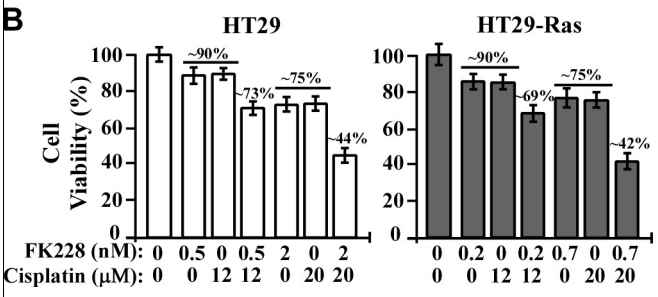


Fig. 3. Synergistic reduction of clonogenic survival by FK228 and cisplatin. (A) J82 (white *columns*) and J82-Ras (dark *columns*) cells were treated with FK228 at 0, 0.5, or 1 nM in the presence and absence of 3 or 6 μM cisplatin for 48 h. Cultures were then replaced with fresh medium. On day 7, untreated control cultures and by day 14, FK228- and cisplatin-treated cultures were stained with crystal violet (0.5% w/v). Cell colonies were counted, and relative colony formation was normalized by the value determined in untreated counterpart J82 and J82-Ras cells, set as 100%. (B) Combined effects (A) were evaluated. (C) J82 and J82-Ras cells were treated with FK228 at 0, 0.5, or 1 nM in the presence and absence of 3 μM cisplatin for 48 h. GSH content was determined, and relative GSH level was normalized by the value determined in untreated counterpart J82 and J82-Ras cells, set as 100%. *Columns*, mean of triplicates (A) or triplicates (C); *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All results are representative of three independent experiments.

A

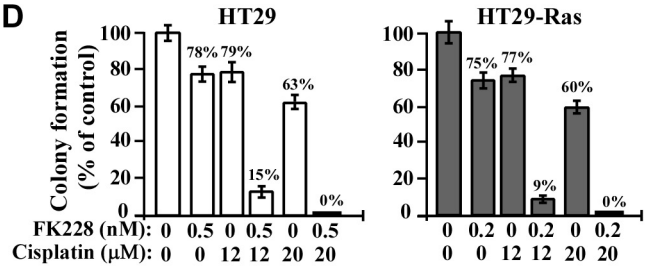
Anticancer agents	IC Values	HT29	HT29-Ras
FK228 (nM)	IC_{10}	0.5	0.2
	IC_{25}	2	0.7
Cisplatin (μM)	IC_{10}	12	12
	IC_{25}	20	20



C

Combination indices

FK228/ Cisplatin	HT29	HT29-Ras
IC_{10}	0.76	0.70
IC_{25}	0.69	0.67



E

Combination indices

FK228/ Cisplatin	HT29	HT29-Ras
IC_{10}	0.03	0.01

Fig. 4. Synergistic cell death induced by FK228 and cisplatin in colon cancer cells. (A) HT29 and HT29-Ras cells were treated with FK228 at a range of concentrations from 0.1 to 5000 nM and cisplatin at a range of concentrations from 0.1 to 5000 μM for 48 h, cell viability determined, and IC_{10} and IC_{25} values of FK228 and cisplatin for HT29 and HT29-Ras cells determined. (B) HT29 (white *columns*) and HT29-Ras (dark *columns*) cells were treated with FK228 at IC_{10} and IC_{25} value doses in the presence and absence of cisplatin at IC_{10} and IC_{25} value doses for 48 h. Cell viability was measured, and relative cell viability was normalized by the value determined in untreated counterpart HT29 and HT29-Ras cells, set as 100%. (C) Combined effects (B) were evaluated. (D) HT29 and HT29-Ras cells were treated with FK228 at 0, 0.2, or 0.5 nM in the presence and absence of cisplatin at 12 or 20 μM for 48 h. Then, cultures were replaced with fresh medium. On day 7, untreated cultures and on day 14, FK228- and cisplatin-treated cultures were stained with crystal violet (0.5% w/v). Cell colonies were counted, and relative colony formation was normalized by the value determined in untreated counterpart HT29 and HT29-Ras cells, set as 100%. (E) Combined effects (D) were evaluated. *Columns*, mean of tetraplicates (B) or triplicates (D); *bars*, SD. All results are representative of three independent experiments.

reduce GSH for synergistic reduction of clonogenic survival of bladder cancer cells remains to be studied.

3.4. Induction of cell death and reduction of clonogenic survival in colon cancer cells

To investigate whether the synergistic induction of cell death and reduction of clonogenic survival by combined FK228 and cisplatin were limited to bladder cancer cells, we studied the effects of combined FK228 and cisplatin on human colon cancer HT29 and HT29-Ras cells. We determined the IC_{10} and IC_{25} values of FK228 and cisplatin for HT29 and HT29-Ras cells (Fig. 4A). Then, we determined that treatment of HT29 and HT29-Ras cells with combined FK228 and cisplatin at IC_{10} values resulted in reduced cell viability to approximately 73% and 69%, respectively; treatment of these cells with combined FK228 and cisplatin at IC_{25} values reduced cell viability to approximately 44% and 42%, respectively (Fig. 4B). The combination indices were less than 1 in using combined FK228 and cisplatin to reduce cell viability (Fig. 4C), indicating that the effectiveness of combined FK228 and cisplatin was synergistically increased in induction of HT29 and HT29-Ras cell death. The ability of combined FK228 and cisplatin to synergistically induce cell death was applicable to colon cancer cells in addition to urinary bladder cells. We also detected that treatment with FK228 or cisplatin at their IC_{10} values resulted in reduction of clonogenic survival to ranges between 79% and 75%, and treatment with combined FK228 and cisplatin at IC_{10} values resulted in reduction to approximately 15% in HT29 and to 9% in HT29-Ras cells (Fig. 4D). Treatment with cisplatin at its IC_{25} value resulted in reduction of clonogenic survival to approximately 63% in HT29 and to 60% in HT29-Ras cells, and co-treatment with combined FK228 at IC_{10} values resulted in a complete elimination of clonogenic survival to 0%. The combination indices were less than 1 in using combined FK228 and cisplatin to reduce clonogenic survival of HT29 and HT29-Ras cells (Fig. 4E). These results indicate that the ability of FK228 and cisplatin to synergistically reduce clonogenic survival was applicable to colon cancer cells in addition to urinary bladder cells.

Two key concerns in therapeutic development are how to increase the efficacy of anticancer regimens to induce cancer cell death and how to reduce cancer cell drug resistance. In this communication, we demonstrated that combined use of FK228 and cisplatin synergistically induced cell death and reduced clonogenic survival of human urinary bladder and colon cancer cells. The Erk-Nox pathway played an important role in mediating signals increased by FK228 combined with cisplatin to profoundly elevate ROS, leading to significant caspase activation and synergistic cancer cell death. Although cisplatin by itself failed to reduce GSH, cisplatin was able to enhance the ability of FK228 to significantly reduce GSH in synergistic reduction of clonogenic survival of cancer cells, indicating a novel activity of combined FK228 and cisplatin in reducing drug resistance. Our results, for the first time, indicate that FK228 should be considered in a new regimen with cisplatin in therapeutic control of development and recurrence of urinary bladder and other cancers. However, the mechanism associated with the novel ability of combined FK228 and cisplatin to synergistically reduce drug resistance of cancer cells remains to be addressed.

Acknowledgments

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