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Sunitinib Malate Synergistically Potentiates Anti-Tumor Effect of Gemcitabine in Human Bladder Cancer Cells

Cheol Yong Yoon, Jung Sun Lee, Bo Sun Kim, Seong Jin Jeong, Sung Kyu Hong, Seok Soo Byun, Sang Eun Lee

Department of Urology, Bundang Hospital, Seoul National University College of Medicine, Seongnam, Korea

Purpose: Sunitinib malate (Sutent; Pfizer, New York, NY, USA) is a highly selective multi-targeted agent and has been reported to have potent anti-tumor effects against various tumors, including renal cell carcinoma and gastrointestinal stromal tumors. In this study, we explored *in vitro* the anti-tumor effect and related molecular mechanisms of sunitinib malate against human bladder cancer cell lines. We also determined the synergistic anti-tumor effect between sunitinib and conventional cytotoxic drugs, cisplatin and gemcitabine, in bladder cancer cells.

Materials and Methods: Six human cancer cell lines (HTB5, HTB9, T24, UMUC14, SW1710, and J82) were exposed to an escalating dose of sunitinib alone or in combination with cisplatin/gemcitabine, and the cytotoxic effect of the drugs was examined by CCK-8 assay. The synergistic effect between sunitinib and cisplatin/gemcitabine was determined by the combination index (CI) and clonogenic assay. Alterations in cell cycle (cyclin D, B1), survival (p-Akt, t-Akt), and apoptosis (Bax, Bad) regulator expression were analyzed by Western blotting.

Results: Like cisplatin and gemcitabine, sunitinib exerted a dose- and time-dependent anti-tumor effect in bladder cancer cells. However, sunitinib exhibited entirely different sensitivity profiles from cisplatin and gemcitabine. Sunitinib suppressed the expression of cyclin B1, p-Akt, and t-Akt while augmenting the expression of cyclin D and pro-apoptotic Bax and Bad in HTB5 cells. Analysis of the drug combination by the isobolic method and clonogenic assay revealed that sunitinib acts in synergy with gemcitabine in HTB5 cells.

Conclusions: These results indicate that sunitinib malate has a potent anti-tumor effect and may synergistically enhance the anti-tumor effect of gemcitabine in human bladder cancer cells.

Key Words: *Carcinoma; Cisplatin; Gemcitabine; Sunitinib; Urinary bladder*

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Corresponding Author:
Sang Eun Lee
Department of Urology, Bundang Hospital, Seoul National University College of Medicine, 300, Gumi-dong, Bundang-gu, Seongnam 463-707, Korea
TEL: +82-31-787-7341
FAX: +82-31-787-4057
E-mail: selee@snubh.org

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INTRODUCTION

Although most bladder cancer patients present with superficial disease, approximately 70% of cases experience disease recurrence and 10% to 20% experience progression to invasive disease. Even though bladder transitional cell carcinoma is relatively chemotherapy-sensitive, the responses are usually transient and most responding diseases recur within the first year, with median survival ranging from 12 to 14 months. Thus, during the past few decades, numerous trials have been conducted to develop new treatment regimens for advanced bladder cancer [1-4].

Sunitinib malate (Sutent; Pfizer, New York, NY, USA) is a multi-targeted receptor tyrosine kinase inhibitor that acts on vascular endothelial growth factor (VEGF) receptor 1, 2, and 3; platelet-derived growth factor (PDGF) receptor; stem cell receptor (KIT); and FMS-like tyrosine kinase-3 receptor (FLT3), and its anti-tumor activity has been dem-

onstrated in various tumors, including renal cell carcinoma (RCC), gastrointestinal stromal tumor, non-small-cell lung cancer, and colorectal cancer [5-9].

The anti-tumor effect of sunitinib malate in RCC is tightly related to the VEGF/PDGF signaling axis, distal effectors of the von Hippel-Lindau tumor suppressor. Sunitinib malate exerts its anti-angiogenic and pro-apoptotic effect in RCC through the suppression of this axis signaling. According to this knowledge, in the present study we examined *in vitro* the anti-tumor effect and related mechanisms of sunitinib malate in human bladder cancer cells, one of the hypervascular tumors with high VEGF expression [10-14]. We also explored the synergistic anti-tumor effect between sunitinib malate and conventional cytotoxic chemotherapy agents, gemcitabine and cisplatin, in bladder cancer cells.

MATERIALS AND METHODS

1. Cell lines and chemicals

Bladder cancer cell lines (HTB5, HTB9, T24, UMUC14, SW1710, and J82) were purchased from the ATCC (Manassas, VA, USA) and were maintained in RPMI (T24), DMEM (SW1710, UMUC14, SW1710, J82), and MEM (HTB5) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA, USA) and 100 U/ml penicillin and 100 mg/l streptomycin (Gibco BRL, Grand Island, NY, USA). Sunitinib malate and cisplatin were obtained from Pfizer (Pfizer Korea LTD., Seoul, Korea) and gemcitabine was obtained from Lilly (Lilly Korea LTD., Seoul, Korea).

2. Analysis of *in vitro* anti-tumor effect

The CCK-8 assay was used to determine the anti-tumor effect of each drug. Briefly, cells in 96-well plates were treated with escalating doses of sunitinib malate (0.313-20 μM), cisplatin (0.039-40.0 μg/ml), or gemcitabine (0.313-10.0 μM) for 24, 48, and 72 hours and then 10 μl of CCK-8 solution (Cell counting kit-8, Dojindo Molecular Technologies, Inc. Gaithersburg, MD, USA) was added to 100 μl of media in each well and absorbance was determined at 450 nm after 4 hours of incubation.

3. Determination of synergistic anti-tumor effect between sunitinib malate and cisplatin/gemcitabine

HTB5 cells were exposed to sunitinib malate alone or simultaneously with cisplatin or gemcitabine and the synergistic effect between the two drugs was determined by the combination index (CI) by use of the Chou and Talalay method as described previously [15]. The CI indicates a synergistic effect when <1.0, an antagonistic effect when >1.0, and an additive effect when equal to 1.0.

4. Colony-forming assay

For the clonogenic assay, $2x10^3$ HTB5 cells were plated in 6 cm culture discs and treated with increasing doses of sunitinib malate (5 μM, 10 μM, and 20 μM) for 48 hours. The drug was then washed out with PBS and the cells were maintained for another 10 days before visualization of colonies formed with 0.4% crystal violet staining. Only colonies larger than 0.2 mm in diameter were counted for quantitative analysis.

5. Analysis of cell cycle- and survival-related protein expression

HTB5 cells exposed to a graded concentration of sunitinib malate (5 μM, 10 μM, and 20 μM) for 48 hours were harvested and incubated for 15 to 20 min in 200 μl of RIPA lysis buffer containing IGEPAL CA630, sodium lauryl sulphate, sodium chloride, Tris-EDTA, deoxycholic acid, and protease inhibitor cocktail (Thermos Scientific, Rockford, IL, USA). Thirty micrograms of protein was fractionated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with TBST containing 5% milk for 1 hour, the membranes were incubated overnight at 4ºC with the respective primary antibody [cyclin D, cyclin B1, phospho-Akt (p-Akt), and total-Akt (t-Akt) from Cell Signaling Technology, Danvers, MA, USA, and Bax and Bad from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA]. The membranes were then incubated with secondary antibodies and protein expression was detected with an enhanced chemiluminescence Western blot substrate kit (Pierce, Rockford, IL, USA).

6. Statistical analysis

Unless specified, the results were expressed as the Mean±SD of the data collected from at least three independent experiments. Statistical significance was determined by the two-sample t-test and p-values <0.05 and 0.01 were considered as significant.

RESULTS

1. Anti-tumor effect of sunitinib malate on human bladder cancer cells

Sunitinib malate showed a dose- and time-dependent anti-tumor effect in all cell lines tested except J82, in which it exerted dose-dependent but not time-dependent activity (Fig. 1). In the concentration range of 10 μM or higher, a 72-hour exposure to sunitinib malate suppressed the proliferation of HTB5 (82.8±9.4%), HTB9 (74.9±28.2%), UMU14 (72.3±4.5%), and SW1710 (80.5±6.8%) cells up to 70% or higher, whereas T24 (32.9±7.6%) and J82 (28.7±0.7%) showed only partial responses to sunitinib treatment (Fig. 1). All cell lines were also exposed to graded doses of cisplatin (0.039-40.0 μg/ml) or gemcitabine (0.313-10.0 μM) for 48 to 72 hours to determine the sensitivity to each drug, and the results are summarized in Table 1. Compared with moderately differentiated HTB9 cells (grade 2), for which the IC_{50} values for 48-hour and 72-hour exposure were 5.23±1.0 μM and 2.31±0.95 μM, respectively, all other poorly differentiated cell lines (grades 3, 4) demonstrated relatively higher resistance to sunitinib (IC_{50} 5.34±0.67 μM to 11.89±1.34 μM) depending on the exposure time. HTB9

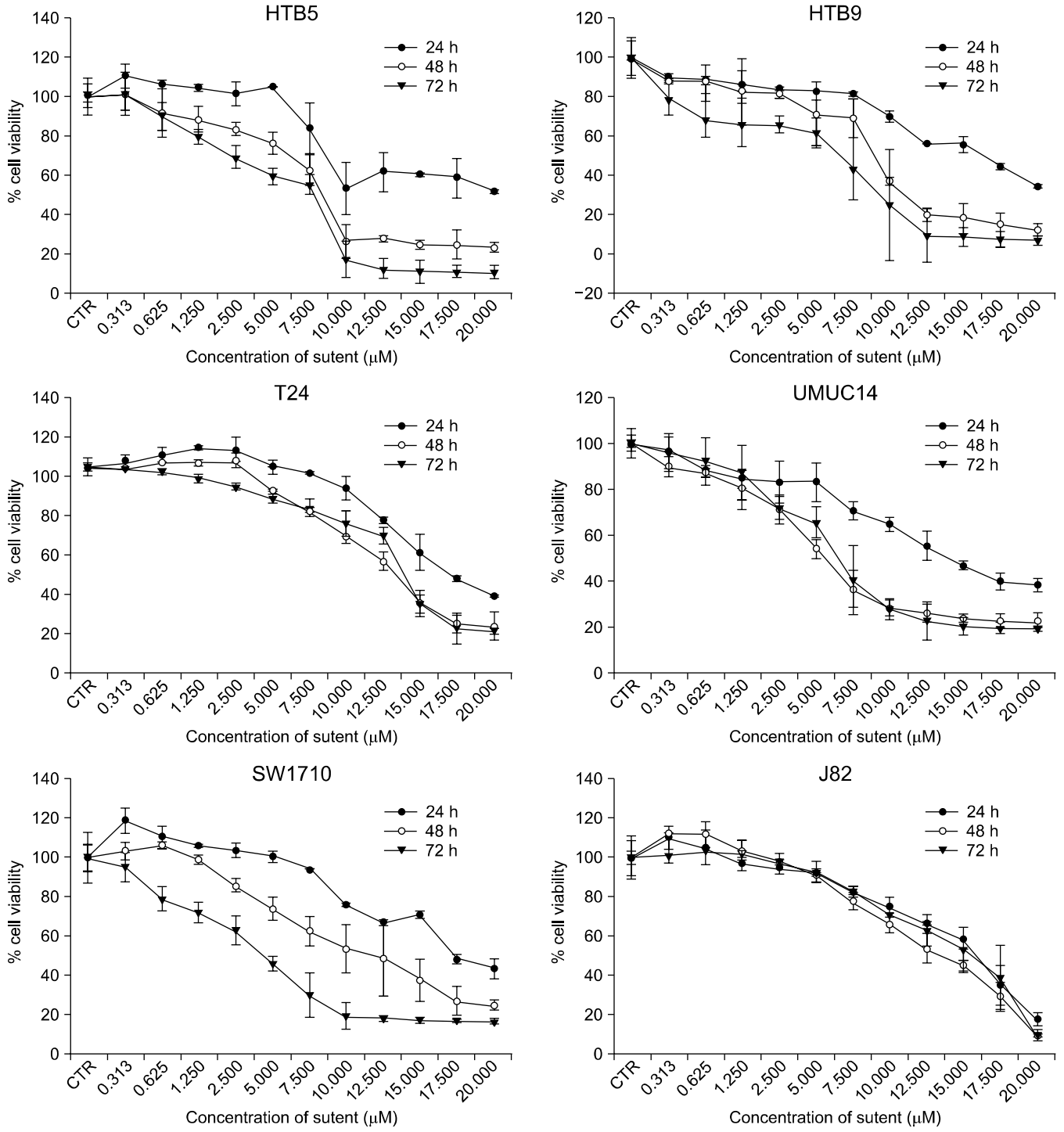

FIG. 1. Dose- and time-dependent anti-tumor effect of sunitinib malate in human bladder cancer cell lines. Six human bladder transitional cell carcinoma cell lines of various differentiation (HTB5 - grader 4; HTB9 - grade 2; T24 - grade 3; UMUC14 - grade 4; SW1710 - grade 3; and J82 - grade 3) were exposed to escalating doses of sunitinib malate (0.313-20 μM) for 24, 48, and 72 hours and the anti-tumor effect in each cell line was determined by CCK-8 assay. Each data point represents the Mean±SD of at least three independent experiments.

cells showed moderate sensitivity to either cisplatin or gemcitabine (IC_{50} values for cisplatin and gemcitabine at 72 hours were 0.71±0.13 μg/ml and 1.1±0.22 μM, respectively). Poorly differentiated HTB5 (grade 4) and T24 (grade 3) cells showed the highest sensitivity to cisplatin (IC_{50} 0.41±0.6 to 1.33±0.7 μg/ml), but exhibited relatively lower sensitivity to either sunitinib (IC_{50} 4.05±0.91 to 9.86±1.75) or gemcitabine (IC_{50} 9.59±8.7 to 142.49± 112.02 μM).

TABLE 1. IC_{50} and relative resistance of bladder cancer cell lines

Cell line	48 h		72 h	
	IC_{50} (SD)	Relative resistance	IC_{50} (SD)	Relative resistance
Sunitinib (μM)				
HTB9	5.23 (1.00)	1	2.31 (0.95)	1
HTB5	7.13 (1.59)	1.3	4.05 (0.91)	1.6
T24	9.93 (0.97)	1.9	9.86 (1.75)	4.2
UMUC14	5.34 (0.67)	1.0	4.69 (0.59)	2.0
SW1710	10.57 (1.79)	2.0	3.51 (0.55)	1.5
J82	14.29 (3.31)	2.7	11.89 (1.34)	5.1
Cisplatin (μg/ml)				
HTB9	1.69 (0.81)	1	0.71 (0.13)	1
HTB5	0.82 (0.19)	0.4	0.41 (0.18)	0.6
T24	1.33 (0.47)	0.7	0.46 (0.11)	0.6
UMUC14	1.83 (0.17)	1.1	0.89 (0.21)	1.3
SW1710	2.19 (0.56)	1.3	1.28 (0.57)	1.8
J82	4.98 (2.01)	2.9	0.77 (0.31)	1.1
Gemcitabine (μM)				
HTB9	1.3 (0.25)	1	1.1 (0.22)	1
HTB5	142.49 (112.02)	109.6	111.89 (88.54)	101.7
T24	12.55 (9.17)	9.7	9.59 (5.11)	8.7
UMUC14	0.3 (0.02)	0.2	0.1 (0.03)	0.1
SW1710	0.41 (0.13)	0.3	0.27 (0.09)	0.2
J82	8.29 (3.18)	6.4	6.59 (1.45)	5.9

IC_{50}: inhibitory concentration 50, SD: standard deviation

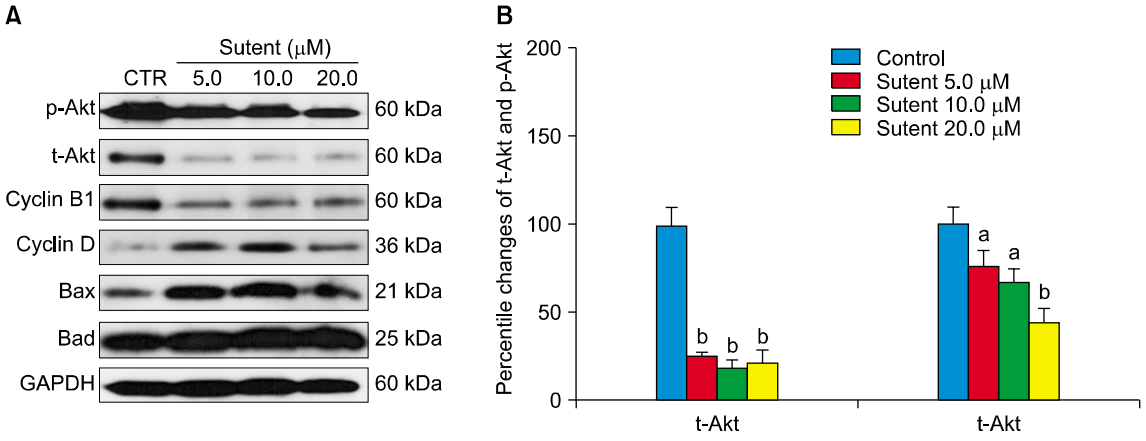

FIG. 2. Analysis of of cell cycle and survival regulatory protein expression in the HTB5 cell line. (A) HTB5 cells were exposed to increasing doses of sunitinib malate (5.0 μM, 10.0 μM, and 20.0 μM) for 72 hours and cyclin D, cyclin B1, p-Akt, t-Akt, Bax, and Bad expression was determined by Western blotting. (B) p-Akt and t-Akt expression was measured by densitometric method and percentile changes from untreated control were analyzed. Each data point represents the Mean±SD of two independent experiments. ([a] and [b]: $p<0.05$, respectively; control vs. each treatment).

2. Sunitinib-induced alterations of cell cycle and survival regulator expression

Exposure to sunitinib caused a decreased expression of cyclin B1 in HTB5 cells, whereas cyclin D expression increased (Fig. 2). Sunitinib treatment significantly suppressed both p-Akt ($p<0.05$) and t-Akt ($p<0.01$) expression in HTB5 cells and also caused an increase in the expression of the pro-apoptotic Bax and Bad (Fig. 2).

3. Synergistic anti-tumor effect between sunitinib and gemcitabine against bladder cancer cells

To test the synergistic anti-tumor effect, poorly differentiated HTB5 cells were exposed to sunitinib alone or in combination with gemcitabine. Over a wide range of concentrations, sunitinib exerted a dose-dependent anti-tumor effect and its combination with gemcitabine yielded a significantly greater anti-tumor effect than that of either agent alone (Fig. 3). These results were also confirmed by

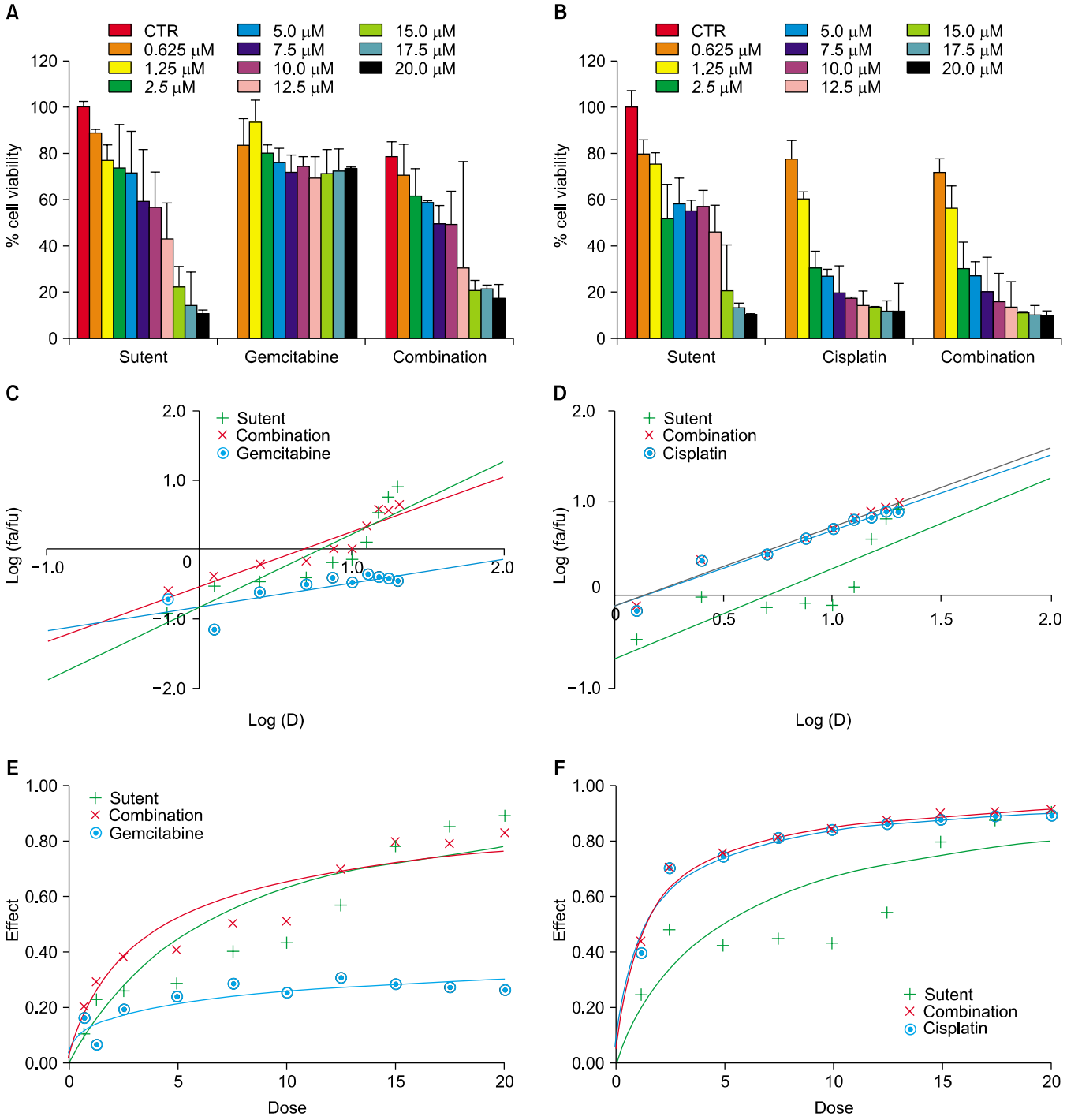

FIG. 3. Combination treatment of human bladder cancer cells with sunitinib malate. To check the synergistic anti-tumor effect between sunitinib malate and a conventional chemotherapy agent, the HTB5 cell line was exposed to increasing doses of sunitinib malate alone or in combination with gemcitabine (A) or cisplatin (B) at a fixed ratio (1:1) for 48 hours and the anti-tumor effect was analyzed by CCK-8 assay. The median-effect plot (C) and the dose-effect plot (E) of the sunitinib and gemcitabine combination showed a synergistic anti-tumor effect for the mid-range of dose combinations, whereas the sunitinib and cisplatin combination (D, F) showed no significant synergistic effect over the whole-dose combinations tested.

the clonogenic assay in which combination treatment of HTB5 cells with sunitinib and gemcitabine showed significantly higher suppression of colony formation (4.5±2.9% of untreated control) than single treatment with either 2.5 μM of sunitinib malate (85.3±1.31% of untreated control, p=0.005) or 0.625 μM of gemcitabine (88.1±13.6% of control, p=0.001) (Fig. 4). Because the sunitinib and gemcitabine combination indicated synergism, we performed a fixed ratio (1 to 1) combination treatment of HTB5 cells to determine the combination index. These results are

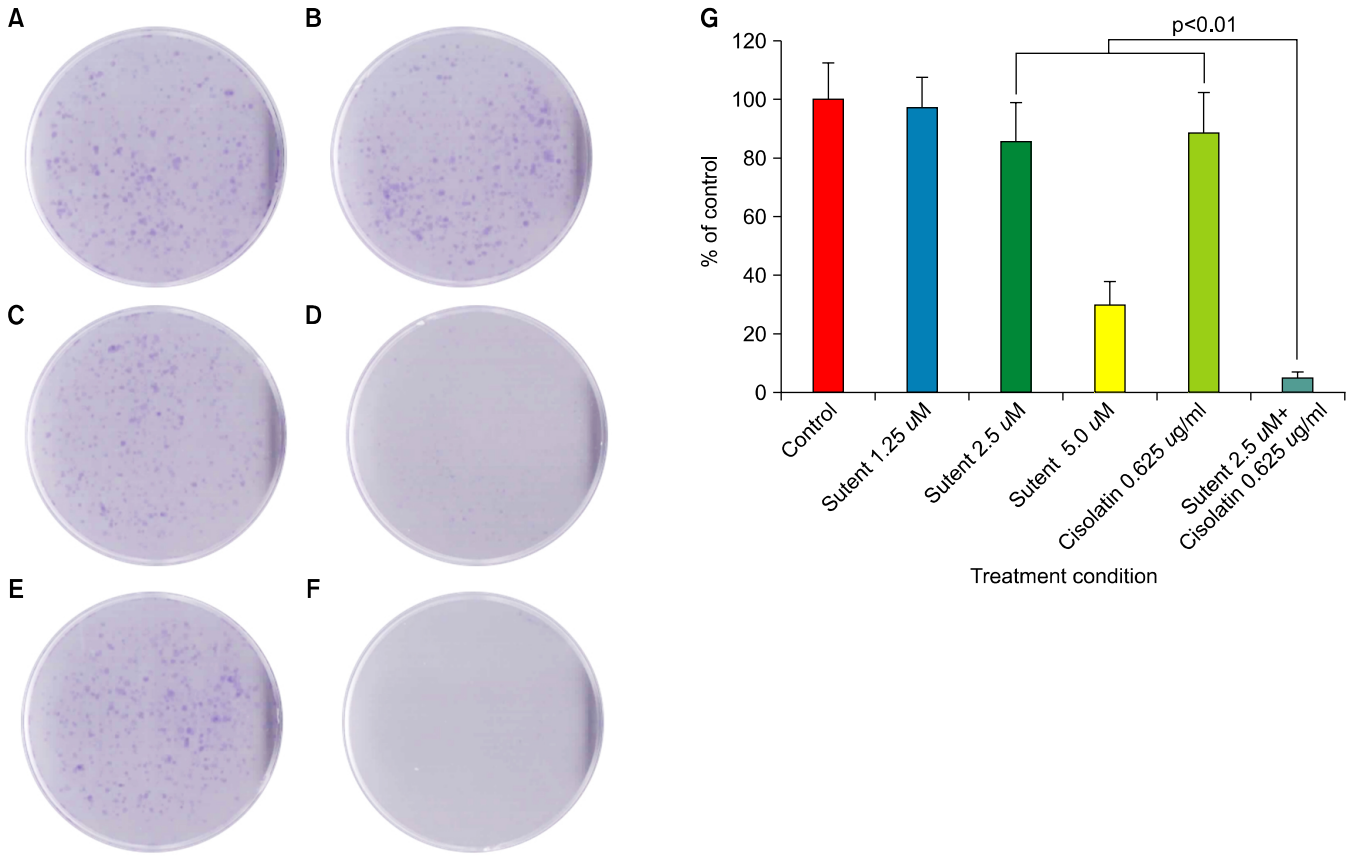

FIG. 4. Colony formation of sunitinib-treated HTB5 cells. Two thousand HTB5 cells were seeded in 6 cm culture plates and permitted to attach overnight. After 48 hours of single or combination treatment with sunitinib malate and cisplatin, cells were washed with PBS and cultured in drug-free media for an additional 7 days before crystal violet staining. Only the number of colonies having a diameter of 0.2 mm or larger were counted for analysis. Changes in colony formation in with sunitinib malate ((B) 1.25 μM, (C) 2.5 μM, (D) 5.0 μM) or cisplatin ((E) 0.625 μg/ml) single treatment or combination treatment ((F) 2.5 μM of sunitinib malate plus 0.625 μg/ml of cisplatin) from untreated control (A) are expressed as percentages. Each data point represents the Mean±SD of duplicated experiments.

TABLE 2. Parameters related with dose-effect responses of sunitinib malate and gemcitabine in HTB5 bladder cancer cells

Compound	m[a]	Dm (μM)[b]	r[c]
Sunitinib	1.05532	6.08988	0.89844
Gemcitabine	0.35456	198.63541	0.91838
Sunitinib+ Gemcitabine	0.79855	4.52277	0.93821

[a]: coefficient signifying the shape of the dose-effect curve, [b]: dose of drugs required to inhibit 50% of cell proliferation, [c]: correlation coefficient signifying conformity of the data to the mass-action law.

summarized in Table 2 and 3. The median effect doses (Dm=IC_{50}) for sunitinib, gemcitabine, and their combination were 6.089 μM, 198.635 μM, and 4.522 μM, respectively, indicating a more effective anti-tumor effect of the combination than of either agent alone. The median effect analysis showed CIs$<$1 between fa=0.15 and 0.7, which suggests a synergistic anti-tumor effect between the two drugs across the broad range of fraction affected (15-70% cell death) (Fig. 5). The dose-reduction index (DRI) also showed that the sunitinib and gemcitabine combination produced favorable dose reduction along the wide fa range (Table 3).

No detectible synergistic anti-tumor effect was shown when HTB5 cells were treated with sunitinib together with cisplatin.

DISCUSSION

Sunitinib malate is a small molecular multi-targeted tyrosine kinase inhibitor that directly inhibits VEGFR, PDGFR, KIT, and FLT3. The main anti-tumor mechanism of sunitinib malate is through its inhibition of angiogenesis and the induction of tumor apoptosis by inhibiting tyrosine kinases expressed by cancer. It is well reported that the VEGF level is highly correlated with the clinical output of well vascularized tumors such as RCC and GIST, and on the basis of the results of clinical trials, the use of sunitinib malate for patients with advanced RCC hRCCand gastrointestinal stromal tumor was approved by the Food and Drug Administration.

High serum VEGF levels are associated with high blad-

TABLE 3. Dose-effect relationships of sunitinib malateand gemcitabine in HTB5 cell lines at 72 hours of exposure

Sunitinib (μM)	Gemcitabine (μM)	Fraction affected (fa)	Combination index (CI)	Dose reduction index (DRI)
0.7593		0.1		2.630
1.6372		0.2		2.054
2.7285		0.3		1.743
4.1471		0.4		1.524
6.0899		0.5		1.346
8.9427		0.6		1.190
13.5924		0.7		1.040
22.6520		0.8		0.883
48.8458		0.9		0.689
	0.4043	0.1		1.400
	3.9812	0.2		4.995
	18.2061	0.3		11.631
	63.3009	0.4		23.255
	198.6354	0.5		43.919
	623.3094	0.6		82.944
	2,167.1890	0.7		165.839
	9,910.6937	0.8		386.152
	9.759e+004	0.9		1,377.291
Combination (sunitinib:gemcitabine=1:1)				
0.28869	0.28869	0.1	1.094	
0.79701	0.79701	0.2	0.687	
1.56530	1.56530	0.3	0.660	
2.72202	2.72202	0.4	0.699	
4.52277	4.52277	0.5	0.765	
7.51481	7.51481	0.6	0.852	
13.06803	13.06803	0.7	0.967	
25.66523	25.66523	0.8	1.136	
70.85528	70.85528	0.9	1.451	

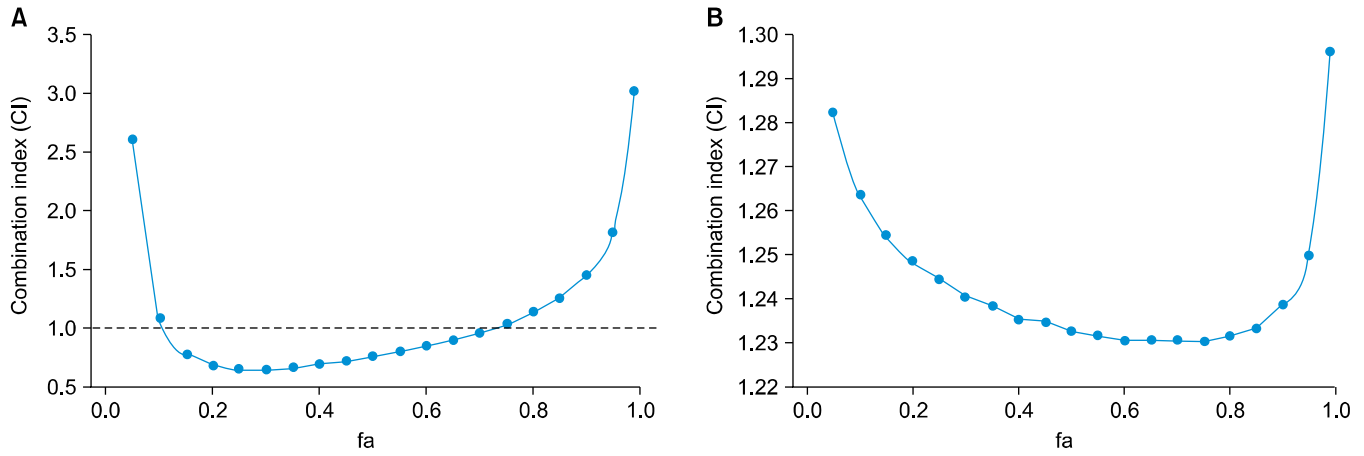

FIG. 5. fa-combination index (CI) plot of sunitinib malate combination treatment. HTB5 cells were treated with escalating concentrations of gemcitabine (A) or cisplatin (B) either alone or in combination with sunitinib at a 1:1 fixed ratio for 48 hours and the CI was calculated by using the Chou and Talalay equation as described previously. On the basis of the CI values at each fraction affected (fa), the fa-CI plot was generated in which CI < 1, CI=1, and CI > 1 denote synergism, additivity, and antagonism, respectively.

der cancer stage, grade, vascular invasion, and metastases. Also, high VEGF mRNA expression in superficial bladder cancer is related to early tumor recurrence and progression to a more invasive phenotype [10,16-19]. These findings suggest the possible anti-tumor and anti-angiogenic effect of sunitinib malate in advanced bladder cancer. However, only a few sunitinib malate clinical trials for bladder cancer have been performed recently, with mixed

initial results, and there have been only a few *in vitro* or *in vivo* studies of the anti-tumor effect and related mechanisms of sunitinib malate in bladder cancer [10,20,21].

In this study, we explored *in vitro* the anti-tumor effect of sunitinib malate on human bladder cancer cell lines of various differentiation and analyzed the results compared with the conventional chemotherapeutic agents cisplatin and gemcitabine. All three drugs exhibited a dose- and time-dependent anti-tumor effect against bladder cancer cells. However, the sensitivity spectra of the drugs differed according to the bladder cancer cell lines tested. For example, UMUC14 cells (grade 4) showed relatively fair sensitivity to all three drugs (IC_{50} 4.69±0.59 μM for sunitinib malate, 0.89±0.21 μg/ml for cisplatin, and 0.1±0.03 μM for gemcitabine with 72 hours of exposure), whereas HTB5 (grade 4) exhibited high sensitivity only to cisplatin treatment (IC_{50} 0.41±0.6 μg/ml), exhibiting moderate and extremely low sensitivity to sunitinib malate (IC_{50} 4.05±1.6 μM with 72 hours of exposure) and gemcitabine (111.89± 88.54 μM with 72 hours of exposure), respectively. These results show that the sensitivities of bladder cancer cell lines to sunitinib might be regulated by factors different from those determining sensitivity to cisplatin or gemcitabine. In other words, it is possible that sunitinib malate could be used as a first- or second-line therapy in selected patients with advanced bladder cancer regardless of the response to cisplatin- or gemcitabine-based chemotherapy.

In our study, exposure of HTB5 cells to sunitinib malate resulted in a marked suppression of cyclin B1 expression accompanied by increased expression of cyclin D. Sunitinib malate also suppressed the expression of p-Akt and t-Akt. These findings indicate that the anti-tumor mechanism of sunitinib malate in bladder cancer is at least partly through G2 phase cell cycle arrest and suppression of the Akt survival pathway. The sunitinib-mediated augmentation of pro-apoptotic Bax and Bad expression suggests the possible involvement of the mitochondrial apoptotic pathway in the anti-tumor action of sunitinib malate against bladder cancer cells.

In tumors such as RCC, testicular germ cell tumors, breast cancer, and pancreatic cancer, sunitinib malate has been reported to have synergistic anti-tumor activity with various treatment modalities, including targeted agents, chemotherapy drugs, and even radiation therapy [22-28]. However, studies of the synergistic anti-tumor effect between sunitinib malate and conventional cytotoxic agents in bladder cancer are very rare. Because cisplatin and gemcitabine are the active drugs and mainstay of chemotherapy regimens for advanced bladder cancer, in this study we analyzed the synergistic anti-tumor effect between sunitinib malate and these two drugs. Concomitant treatment of HTB5 cells with sunitinib malate and gemcitabine significantly enhanced the anti-tumor effect of both drugs along the fa value between 0.15 to 07, indicating the optimal synergistic anti-tumor effect between the two drugs at the mid-range of combination doses. The clonogenic assay also demonstrated compatible results. Although sunitinib malate as a single agent caused dose-dependent suppression of colony formation of HTB5 cells, the overall decrease in clonogenicity was significantly greater when HTB5 cells were exposed to sunitinib malate and gemcitabine simultaneously. Although combination of high-dose- range sunitinib malate and cisplatin showed a mild additive anti-tumor effect (CI=1.23), over a wide range of dose combinations, no significant synergistic enhancement in anti-tumor effect between sunitinib malate and gemcitabine against HTB5 was shown. These results are compatible with those obtained by Sonpavde et al, in which sunitinib malate and cisplatin showed a synergistic anti-tumor effect against human bladder cancer cell lines only in high-dose combinations [29].

In summary, our findings suggest a synergistic anti-tumor effect between sunitinib malate and gemcitabine in bladder cancer cells, providing a rationale for the development of a sunitinib-based combination therapy regimen with a conventional chemotherapy agent such as gemcitabine for advanced bladder cancer.

CONCLUSIONS

In the present study, sunitinib malate showed dose- and time-dependent anti-tumor activity against human bladder cancer cell lines of various differentiations. The sensitivity profile of sunitinib malate differed from the profiles identified for cisplatin and gemcitabine, indicating that no cross-sensitivity or resistance exists between sunitinib malate and cisplatin/gemcitabine. Also, the combination treatment of sunitinib malate and gemcitabine was superior to either agent as a single treatment regarding anti-tumor potential. These findings suggest the possibility of clinical application of sunitinib-based single targeted therapy or combination treatment with conventional cytotoxic chemotherapy drugs for advanced bladder cancer. Considering the extremely poor prognosis of patients with advanced bladder cancers, the development of sunitinib-based novel treatment regimens is of utmost clinical importance. Because the IC_{50} of sunitinib malate *in vitro* is reported to be significantly higher than the low nanomolar free and active drug concentrations obtained *in vivo*, further studies are needed to confirm the synergistic anti-tumor effect of sunitinib-based combination treatment *in vivo* [29].

Conflicts of Interest

The authors have nothing to disclose.

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