Cooperative Enhancement of Radiosensitivity After Combined Treatment of 17-(Allylamino)-17-Demethoxygeldanamycin and Celecoxib in Human Lung and Colon Cancer Cell Lines

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We investigated whether the combined treatment of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), an inhibitor of heat-shock protein 90 (hsp90), and celecoxib, an inhibitor of cyclooxygenase-2, can cooperatively enhance the radiosensitivity of various human cancer cells. Combined treatment with 17-AAG and celecoxib, at clinically relevant concentrations, cooperatively induced radiosensitization in all tested cancer cells, but not in normal cells. Cooperative radiosensitization by the drug combination was also shown in a human tumor xenograft system. We found that ataxia-telangiectasia and rad3-related (ATR) and ataxia-telangiectasia mutated (ATM) are novel client proteins of hsp90. Combined treatment with 17-AAG and celecoxib cooperatively induced downregulation of ATR and ATM. In conclusion, combined treatment with 17-AAG and celecoxib at clinically relevant concentrations may significantly enhance the therapeutic efficacy of ionizing radiation.

Introduction

RADIODTHERAPY IS ONE OF the three major cancer treatment modalities, the others being surgery and chemotherapy. Enhancing the radiosensitivity of cancer cells while preserving the health of normal cells is one of the most important tasks in clinical radiobiology, but no single drug able to accomplish this task has been introduced into routine clinical practice despite the development of a number of radiosensitizing agents. In chemotherapy, single chemotherapeutic agents alone are rarely administered to treat cancer patients, but are rather typically used in combination chemotherapy with two or more drugs. Combination chemotherapy enhances the anticancer effects of the individual treatments and decreases their toxicities by requiring lower concentrations of each drug, compared to those when either drug is administered alone. Similarly, single agents may not be able to effectively radiosensitize all the types of diverse-origin human cancers because different types of cancer frequently show very different radiosensitivities, and because tumors use complicated and diverse mechanisms to induce radioresistance (Chistiakov *et al.*, 2008).

Based on the limitations when using a single agent to radiosensitize cancer cells, we hypothesized that simultaneous administration of two or more radiosensitivity-targeted agents may be more effective and efficient in the enhancement of ionizing radiation (IR) effect compared with using a single agent. We previously showed that the com-

bined administration of celecoxib and gefitinib can induce synergistic cytotoxicity and radiosensitization in several cancer cell lines (Park *et al.*, 2006). Therefore, simultaneous administration of several targeted agents may be a useful strategy to develop widely effective and practical radiosensitization tools.

Various exogenously or endogenously derived stresses cause denaturation, aberrant expression, aggregation, or breakdown of cellular proteins. Accumulation of aberrant proteins is associated with a number of human diseases, including cancer (McClellan *et al.*, 2007; Powers *et al.*, 2008). Stress-exposed cells activate several protective modalities to maintain cellular homeostasis or genomic integrity (Stravopodis *et al.*, 2007). The molecular chaperone system, which regulates protein folding, stability, and activity, is a representative of these protective modalities. Heat-shock protein (hsp) 90 is a central component of this complex chaperone system (Powers *et al.*, 2008). Hsp90 is an evolutionarily conserved protein that is expressed in most cells. It is also an abundant protein, making up about 1%–2% of total proteins in cells (Tse *et al.*, 2009). Hsp90 does not act to fold non-native proteins, in contrast to hsp70, but binds to near-native proteins, and regulates cell cycle, meiosis, and cytokinesis when cells are facing environmental stresses (Zhao *et al.*, 2005; Powers *et al.*, 2007).

Recent studies have shown that hsp90 is frequently overexpressed in cancer cells, and that it interacts with many oncogenic client proteins (Tsutsumi *et al.*, 2008; Yano *et al.*, 2008). These client proteins are involved in various signal

transduction pathways, cell cycle regulation, growth control, angiogenesis, metastasis, and apoptosis. Hsp90 inhibition can simultaneously block these functions of multiple client proteins. Therefore, hsp90 is an important target for cancer therapy (Powers *et al.*, 2006; Neckers, 2007).

17-(Allylamino)-17-demethoxygeldanamycin, a derivative of geldanamycin (17-AAG), has entered phase II clinical trials as a hsp90 inhibitor. 17-AAG shows more than a 100-fold higher affinity for hsp90 in cancer cells than in normal cells (Powers *et al.*, 2007; Sauvageot *et al.*, 2009). Several studies also showed that 17-AAG induces synergistic anticancer effects when combined with several chemotherapeutic agents or IR (Enmon *et al.*, 2003; Kobayashi *et al.*, 2005).

Cyclooxygenase-2 (COX-2) is an enzyme that mediates biosynthesis of various prostanoids, including prostaglandins from arachidonic acid. COX-2 is rapidly induced by stimuli such as inflammation, cytokines, and growth factors in contrast to cyclooxygenase-1 (COX-1), which is constitutively expressed in cells. COX-2 is frequently overexpressed in malignant tumors in a variety of tissues (Park *et al.*, 2006). COX-2 is also known to be involved in carcinogenic processes through various pathways (Kim *et al.*, 2009). Therefore, COX-2 is also regarded as an important target molecule for cancer treatment as we previously reviewed (Jun *et al.*, 2009; Kim *et al.*, 2009).

COX-2 inhibitors are well known for their anticancer effects. Celecoxib is a COX-2-specific inhibitor that is currently used in clinical settings. Celecoxib is known to exert anticancer and chemo- or radiosensitizing effects by inhibiting various signaling pathways in both COX-2-dependent and -independent manners (Park *et al.*, 2006; Dittmann *et al.*, 2008). In addition, celecoxib interacts synergistically with other targeted agents (Shin *et al.*, 2005; Dittmann *et al.*, 2008).

Interaction between COX-2 and hsp90 has been rarely researched. A few studies showed that hsp90 inhibitors induce hsp70 expression, whereas chronic COX-2 inhibition reduces hsp70 expression (Neuhofer *et al.*, 2004; Powers *et al.*, 2008). These results suggest that hsp90 and COX-2 may be related to each other via hsp70. In addition, since hsp90 has many client proteins, many of which are related to tumor growth, and COX-2 also interacts with many critical tumor-related proteins in cells, these two molecules may share one or more critical proteins as their target. Taken together with the observations that both celecoxib and 17-AAG have radiosensitizing effects, these findings suggest the possibility that, when administered concurrently, 17-AAG and celecoxib may cooperatively interact to show synergistic anticancer effects or radiosensitization. In the present study, we investigated whether concurrent treatment of 17-AAG and celecoxib induces cooperative radiosensitizing effects in human cancer cells.

Materials and Methods

Materials

Celecoxib was provided from Pfizer, Inc. 17-AAG and geldanamycin (GA) were purchased from Stressgen. Cycloheximide was purchased from Sigma.

Cell culture

A549, NCI-H460 lung cancer cell lines, BEAS-2B, an immortalized bronchial epithelial cell line, and HCT-116 colon

cancer cells were purchased from American Type Culture Collection (ATCC). MOR-P lung cancer cells were kindly provided by Dr. Y.M. Zhu (The University of Sheffield, UK).

in vitro clonogenic assay

To measure cytotoxicity, the cells were exposed to a vehicle (0.1% DMSO) or to graded doses of 17-AAG, celecoxib, or IR for 72 h at 37°C. To determine radiosensitizing effects, the cells were preincubated with 17-AAG or celecoxib at indicated concentrations for 4 h, exposed to graded doses of IR, and then further incubated for 68 h at 37°C. Thereafter, processes were followed as described previously (Park *et al.*, 2006).

Radiation enhancement ratio and synergy analysis

Radiation enhancement ratio (RER) for 17-AAG, celecoxib, or the combination of both drugs was determined at surviving fraction (SF)_{0.1} and SF_{0.25} from clonogenic survival assays as described previously (Park *et al.*, 2006). Synergisms for three agent combinations were analyzed using two different methods, median-drug effect analysis (Chou, 2006) and drug-independent action model analysis (Berenbaum, 1978; Park *et al.*, 2006). These are representative two models for synergy analysis and can also substitute for isobolographic analysis when used concurrently because these two models are basis of mode I and II lines of envelope of additivity in isobologram (Steel and Peckham, 1979). In addition, the isobolographic analysis becomes complicated to express when more than two agents are concurrently treated. Combination index (CI) for median drug effect analysis was evaluated as described previously (Berenbaum, 1978; Chou, 2006). CI is indicated that CI < 1 is synergistic, CI = 1 is additive, and CI > 1 is antagonistic. In independent action model analysis, observed SF (OSF) was obtained from the results of clonogenic assays after treatment with 17-AAG and celecoxib with IR, and expected SF (ESF) was calculated using independent action model, and then OSF and ESF were statistically compared as previously described (Park *et al.*, 2006).

Western blot analysis

Western blot was performed as described previously (Kim *et al.*, 2009). The antibodies used for western blot were as follows: ataxia telangiectasia and rad3-related (ATR; Santa Cruz Biotechnology), ataxia-telangiectasia mutated (ATM; Novus), β -actin (Sigma), and phospho-Tyr¹⁵-Cdk1 and Cdk1 (Cell Signaling Technology). All experiments were performed at least in triplicate. The levels of ATR, ATM, and phospho-Tyr¹⁵-Cdk1 proteins were quantified using Multi Gauge V3.0 program (FUJIFILM) and normalized by β -actin.

Reverse transcription–polymerase chain reaction

Total cellular RNA extraction and reverse transcription were as described previously (Kim *et al.*, 2009). We conducted polymerase chain reaction (PCR) using primers for ATR (forward primer: 5' AAAGTACTCTCAGCCAACCTC 3' and reverse primer: 5' GCATACTCATCAACTGCAAAGG 3') and ATM (forward primer: 5' GAGGTGCAAAAAA AGTCTTTTG 3' and reverse primer: 5' CTGAGATTTCGTTT GCATTCT 3') in a PCR machine (GeneAmp PCR System

FIG. 1. Lung cancer cells are more susceptible to 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and celecoxib than a normal cell line. **(A)** Basal heat-shock protein 90 (hsp90) and cyclooxygenase-2 (COX-2) expression levels were compared between a normal bronchial cell line (BEAS-2B) and lung cancer cells (NCI-H460, A549, and MOR-P) by western blot analysis. **(B, C)** Cells were treated with 17-AAG or celecoxib at indicated concentrations for 72 h, and then cell survival was monitored by clonogenic assay. **(D)** Inhibitory concentrations (IC) of each drug were determined from the data with clonogenic assays. Each IC₅₀ value of 17-AAG and celecoxib was statistically compared between normal and cancer cells. 17-AAG: * $p=0.010$; † $p=0.031$; # $p=0.006$. Celecoxib: * $p=0.049$; † $p=0.027$; # $p=0.045$. All experiments were performed at least in triplicate and error bars represent $\pm$ SE.

		17-AAG (nM)		Celecoxib (μM)		
Cell type	Cell line	IC ₅₀	IC ₂₅	IC ₅₀	IC ₂₅	IC ₁₀
lung normal	BEAS-2B	25.00 $\pm$ 0.037	12.50 $\pm$ 0.028	50.00 $\pm$ 0.037	> 40.00	> 40.00
lung cancer	NCI-H460	2.00 $\pm$ 0.015	1.25 $\pm$ 0.021	40.00 $\pm$ 0.056	20.00 $\pm$ 0.012	5.00 $\pm$ 0.033
lung cancer	MOR-P	2.50 $\pm$ 0.164	1.25 $\pm$ 0.016	15.00 $\pm$ 0.027	7.50 $\pm$ 0.148	3.75 $\pm$ 0.203
lung cancer	A549	2.50 $\pm$ 0.019	1.25 $\pm$ 0.010	20.00 $\pm$ 0.038	10.00 $\pm$ 0.033	5.00 $\pm$ 0.011

9700; Applied Biosystems). The mRNA levels of *ATR* and *ATM* were also quantified using Multi Gauge V3.0 program and normalized by *GAPDH*.

Establishment of human tumor xenograft and tumor growth delay assay in vivo

All animal studies were carried out following protocols approved by Laboratory Animal Research Center in Samsung Medical Center. The nude mice (BALBC nu/nu) aged 7–8 weeks (Orient) were housed in sterile filter-capped cages, fed, and watered. All handling and implantation procedures before IR were conducted in a laminar-flow biosafety hood.

The mice were divided into eight groups with similar average weight (five mice per group) and 4×10^6 NCI-H460 cells suspended in 50 μ L of RPMI were injected into the subcutaneous tissue of right hind leg of each mouse. Ten

days after implantation, tumor-bearing mice were intraperitoneally injected with vehicle (equal volume DMSO), celecoxib (15 mg/kg body weight), 17-AAG (40 mg/kg body weight), or drug combination (15 mg/kg celecoxib + 40 mg/kg 17-AAG) for 7 consecutive days. From 2nd day of drug treatment, mice in IR-combined groups were irradiated with 2 Gy on the tumor-bearing right-hind leg for 5 consecutive days (total dose was 10 Gy). Changes in tumor size and body weight were measured every other day. Tumor volume was determined by the formula $V = LW^2/2$, where V is volume of tumor, L is longest length of tumor, and W is width (perpendicular length to the L) of the tumor. Enhancement factor (EF) for 17-AAG, celecoxib, or the combination of both drugs was determined as previously described (Pyo *et al.*, 2001): $EF = (GD_{drug + xIR} - GD_{drug}) / GD_{xIR}$. In this formula, “drug + xIR” represents combined treatment of 17-AAG + IR, celecoxib + IR, or 17-AAG + celecoxib + IR; “drug” represents 17-

FIG. 2. The combined treatment of 17-AAG and celecoxib increases cytotoxicity cooperatively in cancer cells but not in a normal cell line. **(A–E)** Cells were treated with 17-AAG at IC_{50} for cancer cells (A549, MOR-P, NCI-H460, or HCT-116) and at 2.5 nM (the highest concentration among IC_{50} of tested cancer cell lines) for the normal BEAS-2B cell line, and celecoxib at indicated concentrations for 72 h and then surviving fraction (SF) was determined by clonogenic assay. The SFs of combined treatment groups with 17-AAG and celecoxib were normalized by that of 17-AAG alone. **(F)** Cooperatively cytotoxic effects for 17-AAG and celecoxib combination were analyzed by comparing clonogenic death fractions by combined treatment **(C)** and sum of death fractions by each drug treatment **(A + B)**. All experiments were performed at least in triplicate and error bars represent $\pm$ SE. “#” and “†,” indicate statistically significant ($p < 0.05$) cooperative effects by combined treatment of 17-AAG and celecoxib when compared to each 17-AAG and celecoxib alone, respectively. ns, nonspecific effect.

F

Cell lines	C	A + B	p value (C vs A+B)
A549	0.84 $\pm$ 0.003	0.51 $\pm$ 0.070	0.049
MOR-P	0.76 $\pm$ 0.020	0.64 $\pm$ 0.004	0.048
H460	0.74 $\pm$ 0.128	0.57 $\pm$ 0.165	0.047
HCT116	0.88 $\pm$ 0.006	0.61 $\pm$ 0.043	0.040
BEAS-2B	0.23 $\pm$ 0.083	0.38 $\pm$ 0.076	0.208

*C; Death fraction by combined treatment of 17-AAG and celecoxib
A and B; Death fractions by each drug alone (17-AAG and celecoxib)

FIG. 2. (Continued).

AAG or celecoxib alone treatment, or combination of both drugs; and "xIR" represents radiation plus vehicle treatment. Growth delay (GD) was calculated as the time for treated tumors to reach an average volume of 0.6 or 0.8 cm³ minus the time for control tumors to reach 0.6 or 0.8 cm³.

Statistical analysis

Data from the clonogenic assay were also calculated as $\pm$ SD or SE via the pooling of the results of three independent experiments. The data were analyzed by Student's *t*-test to compare the two groups. Data from animal experiments were expressed as mean $\pm$ SE.

Results

Lung cancer cells are more susceptible to 17-AAG and celecoxib than is a normal cell line

We compared expression levels of hsp90 and COX-2 in a normal cell line and cancer cells. Hsp90 overexpression was observed in all lung cancer cells (NCI-H460, A549, and MOR-P) in contrast to a normal cell line (BEAS-2B). COX-2 overexpression was found in A549 and MOR-P cells but not in NCI-H460 and BEAS-2B cells (Fig. 1A).

To determine the effect of each inhibitor in tested normal and cancer cells, cells were treated with 17-AAG or celecoxib at various concentrations for 72 h, and SFs were obtained by

clonogenic assay. 17-AAG and celecoxib showed greater cytotoxic effects in lung cancer cells compared to a normal cell line (Fig. 1B, C).

Based on the data from clonogenic survival analyses, we determined the inhibitory concentration (IC) for each drug (Fig. 1D). IC₅₀ values of 17-AAG *in vitro* were below plasma concentration ($\leq 10 \mu\text{M}$) (Modi *et al.*, 2007) in all tested normal or cancer cells. IC₅₀ values of 17-AAG in lung cancer cells were 10–12.5 times lower than values for the drug in a normal cell line (2.00–2.50 nM vs. 25.00 nM). In contrast, IC₅₀ values of celecoxib *in vitro* were determined to be above plasma concentration ($\geq 10.00 \mu\text{M}$) (Davies *et al.*, 2000) in all tested cells. IC₁₀ values of celecoxib were below plasma concentration in all cancer cells, but not in BEAS-2B cells (3.75–5.00 μM vs. $>40.00 \mu\text{M}$). These results suggest that lung cancer cells may be more susceptible to 17-AAG and celecoxib compared to normal bronchial epithelial cells.

Combined treatment of 17-AAG and celecoxib increases cytotoxicity cooperatively in cancer cells but not in a normal cell line

We measured the cytotoxic effect of combination treatment with 17-AAG and celecoxib. Cells were treated with either 17-AAG at IC₅₀ or celecoxib at the indicated concentrations, or with combinations of both drugs for 72 h, and then SFs were determined by clonogenic assay. The SFs of

TABLE 1. SYNERGISM ANALYSES FOR TRIPLE COMBINED TREATMENT WITH 17-AAG AND CELECOXIB WITH RADIATION ACCORDING TO THE INDEPENDENT ACTION MODEL AND MEDIAN-DRUG EFFECT METHOD IN FOUR CANCER CELL LINES AND ONE NORMAL CELL LINE

Treatment group	Independent action model					Median-drug effect method	
	ESF	OSF	Ratio (ESF/OSF)	p-Value	Synergism	CI	Synergism
BEAS-2B cells							
2.5 nM 17-AAG + 20 μM Celx	0.809 $\pm$ 0.037	0.769 $\pm$ 0.083	1.05	0.426	No	0.57 $\pm$ 0.108	Yes
2.5 nM 17-AAG + 5 μM Celx + 2 Gy IR	0.455 $\pm$ 0.062	0.515 $\pm$ 0.026	0.88	0.340	No	1.43 $\pm$ 0.119	Antagonism
2.5 nM 17-AAG + 20 μM Celx + 2 Gy IR	0.276 $\pm$ 0.016	0.266 $\pm$ 0.012	1.04	0.240	No	1.00 $\pm$ 0.011	Additive
A549 cells							
2.5 nM 17-AAG + 5 μM Celx	0.468 $\pm$ 0.007	0.115 $\pm$ 0.008	4.07	0.001	Yes	0.62 $\pm$ 0.031	Yes
2.5 nM 17-AAG + 1 μM Celx + 2 Gy IR	0.250 $\pm$ 0.015	0.095 $\pm$ 0.010	2.62	0.100	Marginally yes	0.97 $\pm$ 0.011	Yes
2.5 nM 17-AAG + 2.5 μM Celx + 2 Gy IR	0.245 $\pm$ 0.004	0.048 $\pm$ 0.007	5.12	0.010		0.91 $\pm$ 0.009	Yes
2.5 nM 17-AAG + 20 μM Celx + 2 Gy IR	0.166 $\pm$ 0.001	0.008 $\pm$ 0.003	21.96	0.010	Yes	0.93 $\pm$ 0.014	Yes
MOR-P cells							
2.5 nM 17-AAG + 7.5 μM Celx	0.419 $\pm$ 0.025	0.242 $\pm$ 0.021	1.73	0.050	Yes	0.64 $\pm$ 0.005	Yes
2.5 nM 17-AAG + 3.75 μM Celx + 2 Gy IR	0.198 $\pm$ 0.011	0.059 $\pm$ 0.004	3.35	0.030	Yes	0.71 $\pm$ 0.005	Yes
2.5 nM 17-AAG + 7.5 μM Celx + 2 Gy IR	0.160 $\pm$ 0.003	0.025 $\pm$ 0.0003	6.28	0.010	Yes	0.70 $\pm$ 0.026	Yes
2.5 nM 17-AAG + 20 μM Celx + 2 Gy IR	0.073 $\pm$ 0.001	0.007 $\pm$ 0.003	10.33	0.010	Yes	0.78 $\pm$ 0.009	Yes
NCI-H460 cells							
2.0 nM 17-AAG + 20 μM Celx	0.530 $\pm$ 0.001	0.348 $\pm$ 0.035	1.52	0.019	Yes	1.11 $\pm$ 0.043	Antagonism
2.0 nM 17-AAG + 5 μM Celx + 2 Gy IR	0.241 $\pm$ 0.002	0.056 $\pm$ 0.006	4.31	0.010	Yes	0.85 $\pm$ 0.001	Yes
2.0 nM 17-AAG + 20 μM Celx + 2 Gy IR	0.206 $\pm$ 0.009	0.020 $\pm$ 0.001	10.31	0.030	Yes	0.81 $\pm$ 0.017	Yes
HCT-116 cells							
20.0 nM 17-AAG + 5 μM Celx	0.447 $\pm$ 0.030	0.146 $\pm$ 0.006	3.07	0.027	Yes	0.56 $\pm$ 0.005	Yes
20.0 nM 17-AAG + 5 μM Celx + 2 Gy IR	0.205 $\pm$ 0.002	0.020 $\pm$ 0.001	10.48	0.003	Yes	0.81 $\pm$ 0.003	Yes
20.0 nM 17-AAG + 20 μM Celx + 2 Gy IR	0.183 $\pm$ 0.003	0.008 $\pm$ 0.003	21.87	0.002	Yes	0.93 $\pm$ 0.043	Yes

Mean $\pm$ SE, 17-AAG; IC₅₀, 10 μM $\leq$ celecoxib; within plasma concentration, 20 μM celecoxib; over plasma concentration. The *p*-value was statistically compared between ESF and OSF.

17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; CI, combination index; IR, ionizing radiation; ESF, expected surviving fraction; OSF, observed surviving fraction.

the combined treatment groups were normalized by the SF from cells treated with 17-AAG alone to show the degree of cooperative cytotoxic effect between the drugs (Fig. 2A–E). The combined treatments of 17-AAG and celecoxib enhanced cell death in all tested cancer cells probably in a more than

additive manner, but only in an additive manner in a normal cell line (Fig. 2).

To quantitatively assess the cooperative cytotoxic effect of two drugs, we further analyzed clonogenic assay data using three different analytic methods: (1) analysis using an

FIG. 3. The combined treatment of 17-AAG and celecoxib shows synergistic radiosensitizing effects in cancer cells but not in a normal cell line. Cells were pre-incubated with 17-AAG at IC_{50} or celecoxib at indicated concentrations, or the combination of both drugs for 4 h, exposed to graded doses of γ -radiation, and then SF was determined by clonogenic assay. The SFs of combined groups were normalized by that of drug alone or drug combination. Error bars represent $\pm$ SE, which was calculated after the pooling of the results of three independent experiments. (A) A549, (B) MOR-P, (C) NCI-H460, (D) HCT116, (E) BEAS-2B.

independent action model, (2) analysis using the median-drug effect method (CI value), and (3) statistical comparison of SFs from combined drug treatment group with sum of SFs from either drug alone treatment. The results of analysis by independent action model and statistical comparison between $SF_{(\text{drug combination})}$ and sum of $SF_{(\text{either agent alone})}$ s showed that the combined treatments of 17-AAG and celecoxib could cooperatively induce cell death ($ESF/OSF > 1$ in Table 1 and $C > A + B$ in Fig. 2F) in all cancer cell lines tested (A549, MOR-P, H460, and HCT-116), but not in a normal cell line (BEAS-2B). The analysis results by median-drug effect method also showed that 17-AAG+celecoxib synergistically enhances cell death in three cancer cell lines (CIs were < 1 in A549, MOR-P, and HCT-116) (Table 1). However, CI values for NCI-H460 cells were > 1 (antagonistic), and for BEAS-2B cells were 0.57 (synergistic) after combined drug treatment (Table 1). In summary, three out of four cancer cell lines showed synergistic cell death by 17-AAG+celecoxib combination treatment according to all three analytical methods. At the same time, a normal cell line showed additive cell death by the same drug combination according to two analytical methods, but the median-drug effect method showed synergistic cell death in these cells.

A mathematical model that can be universally applied to all biological cases has not been developed to determine synergy between agents. Synergy (or additivity) is a rather conceptual term and is hard to determine in complicated biological systems (Greco *et al.*, 1995; Peterson and Novick, 2007). Not surprisingly, different calculation models frequently result in conflicting outcomes. Our data also showed slight discrepancies in results of drug-drug cooperation calculated from the two models, and we suggest that the independent action model seemed to fit our data better according to the shape of the graphical data (Fig. 2C, E). Synergistic-looking data may be additive in reality. However, additive-looking data (Fig. 2E) do not seem to be synergistic.

The combined treatment of 17-AAG and celecoxib shows cooperative radiosensitizing effects in cancer cells

We examined whether combined treatments with 17-AAG and celecoxib at clinically achievable concentrations induce cooperative radiosensitizing effects. Cells were incubated with celecoxib at levels below plasma concentration (< 5.00 – $10.00 \mu\text{M}$) and/or 17-AAG at IC_{50} (2.00 – 2.50 nM) for 4 h, exposed to graded doses of γ -radiation, and then used in a clonogenic assay to determine SF. The SFs of 17-AAG+celecoxib+IR treatment groups were normalized by the SF of the 17-AAG+celecoxib treatment group.

Treatment with either drug alone did not show radiosensitizing effects at these low concentrations for all tested cell lines. However, combined treatments with 17-AAG and celecoxib showed significant radiosensitizing effects in all cancer cells tested (Fig. 3A–D). In contrast, the combined drug treatment did not radiosensitize the normal cell line even when celecoxib was used at an above-plasma concentration ($20.00 \mu\text{M}$) (Fig. 3E). These results suggest that the combined treatment with 17-AAG and celecoxib can induce cooperative radiosensitization in various types of cancer cells but not in normal cells at below clinically available concentrations,

whereas treatment with either drug alone at these low concentrations does not exert a radiosensitizing effect.

Next, to further evaluate the cooperative radiosensitizing effect of the two-drug combination treatment, RERs were measured for 17-AAG, celecoxib, or the combination of both drugs at $SF_{0.1}$ or $SF_{0.25}$ determined from the clonogenic assays shown in Figure 3. The RERs for the combined treatment of 17-AAG and celecoxib were between 1.3 and 1.6 at $SF_{0.1}$ and between 1.5 and 1.9 at $SF_{0.25}$ in the tested cancer cell lines, whereas treatment with either drug alone showed RERs between 0.97 and 1.1 (Table 2). In contrast, RERs for the combined drug treatment in the normal cell line were between 1.06 and 1.15 (Table 2). These results show that the combined treatment of 17-AAG and celecoxib can induce more than moderate-degrees of radiosensitization in the tested cancer cells at very low concentrations of each drug.

Potential of tumor cell killing effect by triple combined treatment with 17-AAG, celecoxib, and IR

To investigate how much the triple-combination treatment (17-AAG+celecoxib+IR) enhances the cytotoxic effect compared to the additive summation of the effects of each treatment alone, we compared the OSF with the ESF after the triple combination treatment using independent action model (Table 1). In addition, we calculated the CI values using median-drug effect analysis (Table 1). The triple-combination treatment in a normal cell line showed no cooperative effects, which the OSF was almost the same as the ESF, with the ESF/OSF ratio in the range of 0.88–1.04 and CIs also were ≥ 1 . In contrast, cooperative cytotoxic effects were shown in all tested cancer cells, and the highest ESF/OSF ratios at physiologically achievable concentrations in each cancer cell line were between 4.31 and 10.48, and were between 10.31 and 21.96 with higher concentrations of celecoxib. In addition, CIs of all tested cancer cell lines were < 1 after triple-combination (Table 1). Independent action model and median-drug effect method showed concordant results after triple-combination treatment. These results suggest that the triple-combined treatment with 17-AAG, celecoxib, and IR may induce cooperatively enhanced cell death in cancer cells but not in normal cells.

TABLE 2. RADIATION ENHANCEMENT RATIO FOR 17-AAG, CELECOXIB, OR COMBINED DRUG TREATMENT AT $SF_{0.1}$ AND $SF_{0.25}$ IN CANCER CELLS AND NORMAL BRONCHIAL EPITHELIAL BEAS-2B CELLS

	Cell line	17-AAG (IC_{50})	Celecoxib ($\leq IC_{10}$)	Combined
$SF_{0.1}$	A549	1.05 ± 0.04	1.02 ± 0.08	1.44 ± 0.04
	MOR-P	1.00 ± 0.01	1.00 ± 0.00^a	1.42 ± 0.04
	NCI-H460	1.06 ± 0.11	1.02 ± 0.01	1.59 ± 0.06
	HCT-116	1.07 ± 0.03^a	1.00 ± 0.00	1.32 ± 0.01
	BEAS-2B	1.04 ± 0.02	1.01 ± 0.01	1.06 ± 0.02
$SF_{0.25}$	A549	1.11 ± 0.06	1.03 ± 0.10	1.72 ± 0.08
	MOR-P	1.00 ± 0.00	0.97 ± 0.02^a	1.68 ± 0.01
	NCI-H460	1.11 ± 0.13	1.03 ± 0.02	1.88 ± 0.04
	HCT-116	1.02 ± 0.01^a	1.00 ± 0.00	1.53 ± 0.02
	BEAS-2B	1.07 ± 0.01	1.00 ± 0.00	1.15 ± 0.03

^a IC_{25} , normal BEAS-2B cells were treated with 2.5 nM 17-AAG or $20 \mu\text{M}$ celecoxib, the highest concentrations among IC_{50} or IC_{25} values of each drug for cancer cells.

Taken together, combined treatment with 17-AAG and celecoxib showed cooperative radiosensitization and enhanced cell death with IR up to 4 to 10 times greater than expected additive values at clinically achievable, very low concentrations of each drug.

ATR and ATM are novel client proteins of hsp90

We recently showed that celecoxib downregulated ATR and that this may be an underlying mechanism for radiosensitization by this drug (Jun *et al.*, 2009). In addition,

FIG. 4. The combined treatment 17-AAG and celecoxib cooperatively downregulates protein levels of ataxia-telangiectasia and rad3-related (ATR) and ataxia-telangiectasia mutated (ATM) but not of mRNA. Four cancer cells **a**, **e**, and **k**, A549, **b** and **f**, MOR-P, **c**, **g**, **i**, and **j**, NCI-H460, **d** and **h**, HCT116, **l**, BEAS-2B were incubated with 50 nM geldanamycin (GA) or 17-AAG for indicated times (0, 4, or 8 h) at 37°C. The protein or mRNA levels of ATR or ATM were measured by western blot (**A**) and by reverse transcription-polymerase chain reaction (RT-PCR) (**B**), respectively. (**C**) NCI-H460 cells were incubated with 30 µg/mL cycloheximide with or without 50 nM 17-AAG for 0, 0.5, 1, 2, 4, and 6 h. (**D**) A549 and BEAS-2B cells were incubated with 2.5 nM 17-AAG, 5 µM celecoxib, or 17-AAG + celecoxib for 24 h at 37°C. The protein levels of ATR, ATM, or β-actin were detected by western blot and quantified using Multi Gauge V3.0 program. All experiments and measurements were done at least in triplicate; 0 h; 0.1% DMSO alone treated.

B By RT-PCR

C NCI-H460

FIG. 4. (Continued).

FIG. 4. (Continued).

hsp90 has been shown to have checkpoint kinase 1 (chk1) as a client protein. Chk1 is an important downstream molecule of ATR in the G_2 checkpoint to regulate G_2 arrest in IR-exposed cells. Recent studies have shown that inhibition or abrogation of the G_2 /M checkpoint sensitizes cancer cells to chemotherapy or IR (Nomura *et al.*, 2005; Tse *et al.*, 2007). We were curious whether ATR or ATM might be client proteins for hsp90. If so, then it could be hypothesized that cooperative downregulation of ATR and/or ATM by 17-AAG and celecoxib might be able to induce cooperative radiosensitization, and that this may be an underlying mechanism for the synergistic radiosensitization by both drugs in combination.

Therefore, we examined whether ATR, ATM, or both could be client proteins of hsp90 using the methods that were previously described (Nomura *et al.*, 2005). Four cancer cell lines were treated with 50 nM GA or 17-AAG for the indicated times (4 or 8 h), and then protein and mRNA levels of ATR and ATM were analyzed by western blot (Fig. 4A) and semiquantitative reverse transcription-PCR (Fig. 4B). The protein levels of ATR and ATM were both significantly decreased by GA and 17-AAG in all cancer cells (Fig. 4A), but mRNA levels were not changed (Fig. 4B), indicating that these molecules could be client proteins of hsp90 (Nomura *et al.*, 2005).

To further verify whether ATR and ATM are novel client proteins of hsp90, NCI-H460 cells were treated with cycloheximide with or without 17-AAG. The protein levels of ATR and ATM were decreased more rapidly in cells treated with 17-AAG and cycloheximide than in cycloheximide alone-

treated cells (Fig. 4C). HCT-116 and MOR-P cells also showed similar patterns to NCI-H460 cells when they were treated with cycloheximide alone or cycloheximide + 17-AAG (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertonline.com/dna). These results indicate that ATR and ATM may be novel client proteins of hsp90.

Cooperative downregulation of ATR and ATM by 17-AAG and celecoxib

To investigate whether ATR and ATM were cooperatively downregulated by 17-AAG and celecoxib, A549 and BEAS-2B cells were incubated with 2.5 nM 17-AAG, 5 μ M celecoxib, or a combination of both drugs for 24 h, and then the levels of ATR and ATM were analyzed by western blot (Fig. 4D). Treatment with either drug alone at these low concentrations did not affect the levels of ATR and ATM proteins on both A549 and BEAS-2B cells, but the combined treatment of both drugs downregulated protein levels of ATR and ATM on A549 cells (Fig. 4D-k) but not BEAS-2B cells (Fig. 4D-l). We also compared endogenous protein levels of ATR and ATM in four cancer cell lines that originated from different organs (HCT-116, MOR-P, H460, and MCF-7) and in a normal cell line (BEAS-2B) (Supplementary Fig. S2). ATR was highly expressed in four cancer cell lines compared to normal cells, but ATM levels were not higher than normal cells in all cancer cells. In addition, it has been shown that 17-AAG has more than a 100-fold higher affinity for hsp90 in cancer cells than in normal cells (Powers *et al.*,

2007; Sauvageot *et al.*, 2009). These suggest that the synergistic radiosensitizing effects may be induced by cooperative downregulation of ATR and ATM proteins in cancer cells but not in normal cells after the combined treatment of 17-AAG and celecoxib.

The combined treatment of 17-AAG and celecoxib cooperatively decreases level of pTyr¹⁵-Cdk1 after IR treatment

We further investigated whether combined treatment of 17-AAG and celecoxib induces cytotoxicity or radiosensitization via changing cell cycle after downregulating ATR and ATM. The level of pTyr¹⁵-Cdk1, a marker for G₂ phase, at 6 h after IR exposure was decreased by combined treatment of both drugs, whereas either drug alone-treatment with IR did not change the level of pTyr¹⁵-Cdk1, when compared to IR alone (Fig. 5). This result suggests that the combined treatment of 17-AAG and celecoxib may attenuate the IR-induced G₂ checkpoint activation, and thus supports our observations of cooperatively downregulating ATR and ATM by combined drug treatment.

The combined treatment of 17-AAG and celecoxib cooperatively enhances in vivo tumor GD after IR exposure

To investigate whether the cooperative radiosensitizing effects by 17-AAG plus celecoxib can be shown *in vivo* system, we performed tumor GD assay using human tumor xenograft in BALB/C nude mice. NCI-H460 human lung cancer cells were injected into the subcutaneous tissue of right hind leg and the tumors were grown *in vivo* for 10 days. The tumor-bearing mice were treated with celecoxib (15 mg/kg), 17-AAG (40 mg/kg), or combination of both drugs for 7 consecutive days, with or without IR exposure (2 Gy × 5 times).

EF was calculated as described in Materials and Methods. Combined administration of both drugs exerted cooperative enhancement of tumor GD after irradiation compared with either drug treatment alone plus radiation (Fig. 6A). The EFs were >2.0 after combination drug treatment (Fig. 6B).

We monitored common toxicities such as weight loss, change in skin texture, nausea or vomiting, diarrhea, fatigue, poor feeding, and shortness of breath (Fracasso *et al.*, 2011) until mice were sacrificed. However, we could not observe any toxicities described above, including weight loss (Supplementary Fig. S3), in mice in all groups.

These results indicate that 17-AAG and celecoxib also cooperatively enhance radiation effect without increasing side effects *in vivo*, and good EFs (>2.0) could be acquired after combination of mild- to moderate-degree radiosensitizers.

Discussion

In this study, we investigated whether the combined treatment of 17-AAG and celecoxib can enhance the radiosensitivity of various human lung and colon cancer cells. We found that a combined treatment with 17-AAG and celecoxib could result in cooperative enhancement of cytotoxic and radiosensitizing effects in the tested cancer cell lines, but not in a normal cell line (Fig. 3). These effects were achieved at very low (~2.00–2.50 nM) and clinically relevant concentra-

FIG. 5. The combined treatment of 17-AAG and celecoxib cooperatively decreased level of pTyr¹⁵-Cdk1 after ionizing radiation (IR) treatment. NCI-H460 lung cancer cells were pretreated with 2 nM 17-AAG, 5 μM celecoxib, or combination of both drugs for 4 h and then exposed or not to 6 Gy IR. The cells were recovered for 6 h and levels of pTyr¹⁵-Cdk1 were determined by western blot analysis (A). Densitometric analysis of pTyr¹⁵-Cdk1 level (B). Con, 0.1% DMSO control; A, 2 nM 17-AAG, C, 5 μM celecoxib; A + C, 2 nM 17-AAG + 5 μM celecoxib.

tions (~10 μM) of 17-AAG and celecoxib, respectively. At these low concentrations, combined treatment with 17-AAG and celecoxib showed at least a moderate degree of radiosensitizing effects (RERs of 1.5–1.9 at SF_{0.25}) in all tested cancer cells, whereas neither drug alone caused any radiosensitization at these concentrations (Table 2).

One major issue for this kind of experiment is about synergism among agents (drug, radiation, etc.) for a targeted effect. Many mathematical models have developed to assess the closest additive value for certain agent combinations, because additive value is a conceptually difficult issue to determine. There have been two representative mathematical models to explain additivity and synergism. One is the Loewe additivity model (median-drug effect is based on this model) (Berenbaum, 1978; Chou, 2006) and the other one is the Bliss independence model (Park *et al.*, 2006). However, the status of synergy analysis methodology is that there is no perfect model to be universally applied to all biological cases (Greco *et al.*, 1995; Peterson and Novick, 2007).

In this study, we used these two different model-based calculation methods to quantitatively evaluate the cooperative cytotoxic and radiosensitizing effects after drug combination, but not to determine the real synergism itself between agents, which is rather a complicated and still unsolved

FIG. 6. The combined treatment of 17-AAG and celecoxib effectively delayed tumor growth in BALB/C nude mice via enhancing radiosensitivity. **(A)** NCI-H460 lung cancer cells (4×10^6 cells/50 μ L) were injected into the subcutaneous tissue of the right hind leg as described in Materials and Methods. Tumor-bearing mice were given i.p. with celecoxib (15 mg/kg), 17-AAG (40 mg/kg), or drug combination (celecoxib + 17-AAG) for 7 consecutive days after 10 days postimplantation, with or without irradiation on tumor (2 Gy $\times$ five times) starting from the next day after drug administration. The mice were monitored every 2–3 days for changes in tumor growth, body weight, and health status. Control groups were given i.p. with equal volume of DMSO. **(B)** The enhancement factor (EF) ratio was determined at tumor volume 0.6 and 0.8 cm³ as described in Materials and Methods. Error bars represent $\pm$ SE. The symbols are $p < 0.05$ and represented as follows: * and #—IR alone versus 17-AAG + IR and IR alone versus celecoxib + 17-AAG + IR at tumor volume 0.6 cm³; $\ddagger$ —IR alone versus celecoxib + 17-AAG + IR at tumor volume 0.8 cm³.

scientific issue. The results from the independent action model showed that the total antitumor effect could be enhanced 4 to 10 times that of the expected additive values after triple-combination (17-AAG + celecoxib + IR) treatments (Table 1). The results by the median-drug effect method were generally concordant with the ones from the independent action model. Both calculation methods were well matched in the triple-combination cases.

We also found that 17-AAG and celecoxib could cooperatively enhance the tumor GD by radiotherapy in an *in vivo* xenograft system (Fig. 6). Good EF (2.4) could be acquired after combination of mild- to moderate-degree radiosensitizers. Taken together, the data indicate that combined treatment of 17-AAG and celecoxib can radiosensitize cancer cells in a cooperative manner in *in vivo* as well as *in vitro* systems.

Interestingly, cooperative cytotoxic effects between 17-AAG and celecoxib were strong in all tested cancer cells, but not in a normal cell line (Fig. 2). Hsp90 has many client proteins, including various oncoproteins, and celecoxib is known to have many COX-2-dependent and -independent target molecules. Therefore, our observations may indicate that 17-AAG and celecoxib interact intimately in cells and may share one or more cancer-related target proteins, leading to cooperative cytotoxic effects. Research to identify these common target molecules will not be easy. However, we focused on molecules involved in the G₂ checkpoint, especially ATR and ATM, because we previously found that COX-2 profoundly prolongs IR-induced G₂ arrest and it is caused by upregulation of ATR by COX-2 (Shin *et al.*, 2005;

Kim *et al.*, 2009). In addition, hsp90 also has many client proteins involved in cell cycle regulation, including Chk1, which is directly downstream of ATR. Therefore, we examined whether ATR and ATM are client proteins of hsp90, and identified both ATR and ATM as client proteins of hsp90. Further, we found that the combined treatment with both drugs induced significant downregulation of ATR and ATM at the low concentrations, which were used in the clonogenic assays in the current study, whereas neither drug alone showed any significant change in ATR or ATM level at these concentrations (Fig. 4D–K).

Recently, On *et al.* (2011) reported that inhibition of ATR/ATM-Chk1/Chk2 pathway by anti-cancer agents can trigger premature mitotic entry and result in mitotic catastrophe that defined as cell death associated with inappropriate entry into mitosis. Prise *et al.* (2009) also described that inhibition of ATR and ATM increases radiosensitivity in directly irradiated cells. Celecoxib could also downregulate ATR expression at transcription level (Kim *et al.*, 2009). These suggest that, in combined treatment of 17-AAG and celecoxib, 17-AAG degrades ATR/ATM proteins and celecoxib concurrently inhibits transcription of ATR, and it results in cooperative downregulation of endogenous ATR/ATM levels.

We also found that the level of pTyr¹⁵-Cdk1, a marker for G₂ phase, at 6 h after IR exposure was decreased by combined treatment of both drugs, whereas either drug alone-treatment with IR did not change the level of pTyr¹⁵-Cdk1, when compared to IR alone (Fig. 5). This result indicates that the combined treatment of 17-AAG and celecoxib attenuated the IR-induced G₂ checkpoint activation and thus supports

our observations of synergistically downregulating ATR and ATM by combined drug treatment.

Base on these results, we suggest that synergistic downregulation of ATR and/or ATM by 17-AAG and celecoxib may be related to the underlying mechanism for the synergistic cytotoxic or radiosensitizing effects of these drug combinations.

In summary, we found that combined treatment with 17-AAG and celecoxib at clinically relevant concentrations significantly enhanced the therapeutic efficacy of IR in *in vitro*/*in vivo* systems. We also found that cooperative downregulation of ATR and ATM, principal G₂/M checkpoint molecules, may be related to the underlying mechanisms for this synergistic radiation-enhancing effect. Our results suggest that the combined-targeted radiosensitization strategy may be an effective and clinically applicable therapeutic tool.

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Disclosure Statement

No competing financial interests exist.

References

- Berenbaum, M.C. (1978). A method for testing for synergy with any number of agents. *J Infect Dis* **137**, 122–130.
- Chistiakov, D.A., Voronova, N.V., and Chistiakov, P.A. (2008). Genetic variations in DNA repair genes, radiosensitivity to cancer and susceptibility to acute tissue reactions in radiotherapy-treated cancer patients. *Acta Oncol* **47**, 809–824.
- Chou, T.C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* **58**, 621–681.
- Davies, N.M., McLachlan, A.J., Day, R.O., and Williams, K.M. (2000). Clinical pharmacokinetics and pharmacodynamics of celecoxib: a selective cyclo-oxygenase-2 inhibitor. *Clin Pharmacokinet* **38**, 225–242.
- Dittmann, K.H., Mayer, C., Ohneseit, P.A., Raju, U., Andrantschke, N.H., Milas, L., and Rodemann, H.P. (2008). Celecoxib induced tumor cell radiosensitization by inhibiting radiation induced nuclear EGFR transport and DNA-repair: a COX-2 independent mechanism. *Int J Radiat Oncol Biol Phys* **70**, 203–212.
- Enmon, R., Yang, W.H., Ballangrud, A.M., Solit, D.B., Heller, G., Rosen, N., Scher, H.I., and Sgouros, G. (2003). Combination treatment with 17-N-allylamino-17-demethoxy geldanamycin and acute irradiation produces supra-additive growth suppression in human prostate carcinoma spheroids. *Cancer Res* **63**, 8393–8399.
- Fracasso, P.M., *et al.* (2011). A phase 1 study of UCN-01 in combination with irinotecan in patients with resistant solid tumor malignancies. *Cancer Chemother Pharmacol* **67**, 1225–1237.
- Greco, W.R., Bravo, G., and Parsons, J.C. (1995). The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev* **47**, 331–385.
- Jun, H.J., Kim, Y.M., Park, S.Y., Park, J.S., Lee, E.J., Choi, S.A., and Pyo, H. (2009). Modulation of ionizing radiation-induced G2 arrest by cyclooxygenase-2 and its inhibitor, Celecoxib. *Int J Radiat Oncol Biol Phys* **75**, 225–234.
- Kim, Y.M., Lee, E.J., Park, S.Y., Cho, K.H., Kim, J.Y., and Pyo, H. (2009). Cyclooxygenase-2 up-regulates ataxia telangiectasia and Rad3 related through extracellular signal-regulated kinase activation. *Mol Cancer Res* **7**, 1158–1168.
- Kobayashi, S., Nantz, R., Kitamura, T., Higashikubo, R., and Horikoshi, N. (2005). Combined inhibition of extracellular signal-regulated kinases and HSP90 sensitizes human colon carcinoma cells to ionizing radiation. *Oncogene* **24**, 3011–3019.
- McClellan, A.J., Xia, Y., Deutschbauer, A.M., Davis, R.W., Gerstein, M., and Frydman, J. (2007). Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. *Cell* **131**, 121–135.
- Modi, S., *et al.* (2007). Combination of trastuzumab and tane-spimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: a phase I dose-escalation study. *J Clin Oncol* **25**, 5410–5417.
- Neckers, L. (2007). Heat shock protein 90: the cancer chaperone. *J Biosci* **32**, 517–530.
- Neuhofer, W., Holzapfel, K., Fraek, M.L., Ouyang, N., Lutz, J., and Beck, F.X. (2004). Chronic COX-2 inhibition reduces medullary HSP70 expression and induces papillary apoptosis in dehydrated rats. *Kidney Int* **65**, 431–441.
- Nomura, M., Nomura, N., and Yamashita, J. (2005). Geldanamycin-induced degradation of Chk1 is mediated by proteasome. *Biochem Biophys Res Commun* **335**, 900–905.
- On, K.F., Chen, Y., Ma, H.T., Chow, J.P.H., and Poon, R.Y.C. (2011). Determinants of mitotic catastrophe upon abrogation of the G2 DNA damage checkpoint by UCN-01. *Mol Cancer Ther* **10**, 784–794.
- Park, J.S., Jun, H.J., Cho, M.J., Cho, K.H., Lee, J.S., Zo, J.I., and Pyo, H. (2006). Radiosensitivity enhancement by combined treatment of celecoxib and gefitinib on human lung cancer cells. *Clin Cancer Res* **12**, 4989–4999.
- Peterson, J.J., and Novick, S.J. (2007). Nonlinear blending: a useful general concept for the assessment of combination drug synergy. *J Recept Signal Transduct Res* **27**, 125–146.
- Powers, M.V., Clarke, P.A., and Workman, P. (2008). Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. *Cancer Cell* **14**, 250–262.
- Powers, M.V., and Workman, P. (2006). Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors. *Endocr Relat Cancer* **13 Suppl 1**, S125–S135.
- Powers, M.V., and Workman, P. (2007). Inhibitors of the heat shock response: biology and pharmacology. *FEBS Lett* **581**, 3758–3769.
- Prise, K.-M., and O'Sullivan, J.-M. (2009). Radiation-induced bystander signalling in cancer therapy. *Nat Rev Cancer* **9**, 351–360.
- Pyo, H., Choy, H., Amorino, G.P., Kim, J., Cao, Q., Hercules, S.K., and DuBois, R.N. (2001). A selective cyclooxygenase-2 inhibitor, NS-398, enhances the effect of radiation *in vitro* and *in vivo* preferentially on the cells that express cyclooxygenase-2. *Clin Cancer Res* **7**, 2998–3005.
- Sauvageot, C.M., *et al.* (2009). Efficacy of the HSP90 inhibitor 17-AAG in human glioma cell lines and tumorigenic glioma stem cells. *Neuro Oncol* **11**, 109–121.
- Shin, Y.K., Park, J.S., Kim, H.S., Jun, H.J., Kim, G.E., Suh, C.O., Yun, Y.S., and Pyo, H. (2005). Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer

- cells expressing differential COX-2 levels. *Cancer Res* **65**, 9501–9509.
- Stravopodis, D.J., Margaritis, L.H., and Voutsinas, G.E. (2007). Drug-mediated targeted disruption of multiple protein activities through functional inhibition of the Hsp90 chaperone complex. *Curr Med Chem* **14**, 3122–3138.
- Steel, G.G., and Peckham, M.J. (1979). Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. *Int J Radiat Oncol Biol Phys* **5**, 85–91.
- Tse, A.N., Carvajal, R., and Schwartz, G.K. (2007). Targeting checkpoint kinase 1 in cancer therapeutics. *Clin Cancer Res* **13**, 1955–1960.
- Tse, A.N., Sheikh, T.N., Alan, H., Chou, T.C., and Schwartz, G.K. (2009). 90-kDa heat shock protein inhibition abrogates the topoisomerase I poison-induced G2/M checkpoint in p53-null tumor cells by depleting Chk1 and Wee1. *Mol Pharmacol* **75**, 124–133.
- Tsutsumi, S., Scroggins, B., Koga, F., Lee, M.J., Trepel, J., Felts, S., Carreras, C., and Neckers, L. (2008). A small molecule cell-impermeant Hsp90 antagonist inhibits tumor cell motility and invasion. *Oncogene* **27**, 2478–2487.
- Yano, A., Tsutsumi, S., Soga, S., Lee, M.J., Trepel, J., Osada, H., and Neckers, L. (2008). Inhibition of Hsp90 activates osteoclast c-Src signaling and promotes growth of prostate carcinoma cells in bone. *Proc Natl Acad Sci U S A* **105**, 15541–15546.
- Zhao, R., *et al.* (2005). Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell* **120**, 715–727.

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