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The novel DNA alkylating agent BO-1090 suppresses the growth of human oral cavity cancer in xenografted and orthotopic mouse models

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Oral cancer is the fourth-most common cause of death in males and overall the sixth-most common cause of cancer death in Taiwan. Surgery, radiotherapy and chemotherapy combined with other therapies are the most common treatments for oral cavity cancer. Although cisplatin, 5-fluorouracil and docetaxel are commonly used clinically, there is no drug specific for oral cavity cancer. Here, we demonstrated that derivatives of 3a-aza-cyclopenta[*a*]indene, a class of newly synthesized alkylating agents, may be drugs more specific for oral cancer based on its potent *in vitro* cytotoxicity to oral cancer cells and on *in vivo* xenografts. Among them, BO-1090, bis(hydroxymethyl)-3a-aza-cyclopenta[*a*]indene derivative, targeted DNA for its cytotoxic effects as shown by inhibition of DNA synthesis (bromodeoxyuridine-based DNA synthesis assay), induction of DNA crosslinking (alkaline gel shift assay), and induction of DNA single-stranded breaks (Comet assay) and double-stranded breaks (γ-H2AX focus formation). Following DNA damage, BO-1090 induced G1/S-phase arrest and apoptosis in oral cancer cell lines. The therapeutic potential of BO-1090 was tested in mice that received a xenograft of oral cavity cancer cell lines (SAS or Cal 27 cells). Intravenous injection of BO-1090 significantly suppressed tumor growth in comparison to control mice. BO-1090 also significantly reduced the tumor burden in orthotopic mouse models using SAS cells. There was no significant adverse effect of BO-1090 treatment with this dosage based on whole blood count, biochemical enzyme profiles in plasma and histopathology of various organs in mouse. Taken together, our current results demonstrate that B0-1090 may have potential as a treatment for oral cavity cancer.

Cancer Therapy

Oral cavity cancer involving the lesions occurred in the tongue, oropharynx and floor of the mouth is a prevalent type of cancer in developing countries such as India, Pakistan, Bangladesh and Taiwan.[1] In south-central Asia, it is the third-most common cancer, with an average incidence rate of 1 to 10 per 100,000 people. The incidence of oral cancer is also increasing in developed countries.[2] The treatments of oral cancer include surgery, radiation or chemotherapy, depending on the stage and nature of the tumor.[3,4] Unfortunately, only marginal improvement is seen in many patients, and a complete cure is often not achieved. Half of the patients diagnosed with oral cancer succumb to death within 5 years, and for those who survive, the quality of life remains poor.[1] Thus, development of a drug specific for oral cavity cancer is needed so that substantial improvement in treatment can be achieved.

The cytotoxic and antitumor properties of alkylating agents are due to their ability to covalently bind to DNA. There are two types of DNA alkylating agents, monofunctional and bifunctional agents. Monofunctional alkylating agents mainly damage DNA by forming methylated adducts, whereas the damage induced by bifunctional alkylating agents includes monoadducts, intrastrand crosslinks and interstrand crosslinks (ICLs). However, drugs such as mitomycin C cause thrombocytopenia and leukocytopenia along with liver and lung (interstitial pneumonitis) toxicity in some patients, whereas cisplatin and carboplatin cause nephrotoxicity, ototoxicity and neurotoxicity.[5,6] Occurrence of resistance to DNA alkylating agents in cancer cells may be due to decreased uptake of the agent into cells, increased drug extrusion, drug inactivation in the cells due to chemical instability,

Key words: novel DNA alkylating agent, human oral cancer, DNA interstrand crosslinks, anticancer, xenografted, orthotopic mouse models

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or quick repair of DNA lesions.[7,8] Additionally, methylated adducts formed by drug treatment can be removed and repaired by O^6-methylguanine-DNA methyltransferase (MGMT), which is responsible for drug resistance to temozolamide and BCNU.[7,9] Although toxicity and resistance are the major problems associated with alkylating agent chemotherapy, numerous alkylating agents remain the first line drugs to treat a variety of cancers.[6,10] Newly designed alkylating agents with increased efficacy and decreased adverse side effects are needed to improve the treatment of cancer.

We previously designed and synthesized several series of bifunctional alkylating agents with improved DNA interstrand crosslinking activity.[11–13] Among them, 3a-aza-cyclopenta [α] indene derivatives were designed and synthesized based on the mechanism of action of mitomycin C, bis-carbamates and pyrrolizidine.[13] These compounds have potent cytotoxic effects on a variety of cancer cell lines *in vitro* as well as potent therapeutic efficacy in inhibiting human breast tumor MX-1 cells in xenografted mice.[13] Furthermore, we found that several 3a-aza-cyclopenta[α]indene derivatives preferentially killed oral cancer cells in culture.[13] In our study, we further explored the anticancer activity of these newly synthesized compounds against oral cancer cells.

Material and Methods

Cell lines and cell culture

Human gingival squamous carcinoma cells (OECM-1) were obtained from Dr. C. L. Meng (National Defense Medical College, Taipei, Taiwan). Human tongue carcinoma cells (SAS) (Japanese Collection of Research Biosources, Tokyo, Japan), TW-2.6, and normal oral mucosal fibroblasts (OMF) were obtained from Dr. M. Y. P. Kuo (National Taiwan University Hospital, Taipei, Taiwan). Cal 27 and SCC25 cells were purchased from American Type Culture Collection (CRL-1628). SAS-GL cells were established by infecting SAS cells with dual reporter GFP-luciferase lentivirus. KB-derived MDR (multi drug resistant)-positive cell lines, KB-VIN10 and KB-TAX50, were provided by Dr. J.-Y. Chang (National Health Research Institutes, Taiwan) and maintained in growth medium supplemented with 10 nM vincristine and 50 nM paclitaxel, respectively.[14] CHO-9 and CHO-9-3C cells with ectopic expression of MGMT, originally established by Dr. T. Coquelle,[15] were obtained from Dr. J. L. Yang of the Institute of Biomedical Sciences, National Tsing Hua University, Hsinchu, Taiwan.[16] SAS, Cal 27 and OMF cells were cultured in DMEM with 10% fetal bovine serum (FBS). KB cells were cultured in RPMI with 5% FBS. H460 cells were cultured in RPMI with 10% FBS. SCC25, TW-2.6 and CHO cells were cultured in a 1:1 mixture of DMEM and F12 with 10% FBS. Cells were incubated at 37°C in 5% CO_2.

Experimental compounds

Derivatives of 3a-aza-cyclopenta[*a*]indenes (BO-1090, BO-1100, BO-1130 and BO-1131) (Fig. 1*a*) were synthesized as described previously.[13] These derivatives were used in our study because they selectively kill oral cancer cell lines compared to other cancer cell lines.[13]

Cytotoxicity assay

The cytotoxicity assay was performed by using the cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Penzberg, Germany) followed the protocol as similar to our previous report.[13,17] Apoptosis assay was performed by using an annexin V–FITC apoptosis detection kit (Calbiochem, La Jolla, CA) as described.[17]

DNA damage assays

DNA synthesis was assayed with a 5-bromo-2′-deoxyuridine (BrdU) labeling and detection kit from Roche. Briefly, SAS cells were incubated overnight in 96-well plates and then treated with various concentrations of BO-1090, BO-1100, BO-1130 or BO-1131 for 24 hr, followed by incubation with 10 μM BrdU for 4 hr. At the end of the incubation, the cells were fixed, and the BrdU content of the cells was determined.[18] Analysis of BO-1090 induced DNA strand breaks and ICLs in cells were performed by Comet and modified Comet assays as previously described.[13,17] *In vitro* alkaline gel electrophoresis adopted from our previous studies[13,17] was used to demonstrate the formation of ICLs by direct interaction of BO-1090 and plasmid DNA. The appearance of γH2AX foci (marker for double-stranded DNA breaks) in nuclei was detected by immunofluorescence staining following the protocol as previously described.[17]

Western blotting analysis

Logarithmically growing SAS cells were treated with 2 μM BO-1090. At the indicated times, cells were harvested and lysed, and then intracellular proteins were separated by SDS gel electrophoresis. The western blotting protocol was adopted from a previous study.[19] Antibodies against caspases 3, 7, 8 and 9 (1:1000 dilution), poly (ADP-ribose) polymerase (PARP) (1:1000) and chk2 and phospho-chk2 (1:1000) were obtained from Cell Signaling; anti-p53 was obtained from Upstate (1:500), and anti-CDC25A (1:200) was obtained from Santa Cruz Biotechnology. Secondary antibodies against rabbit (1:1000) and mouse (1:3000) immunoglobulin were purchased from Cell Signaling.

Cell-cycle analysis

Cell cycle analysis was performed following the protocol as described.[17] A BrdU-based pulse-chase assay was performed using an *in situ* cell proliferation kit from Roche with minor modifications of the manufacturer's instructions.

Tumor suppression analysis in a xenografted mouse model

Animals were housed and maintained as per Academia Sinica animal welfare guideline.[17] SAS (3×10^6 cells) or Cal 27 (5×10^6 cells) cells were suspended in 100 μL of PBS and subcutaneously inoculated into the flank of mice. When the tumor size reached about 100 mm^3 for SAS on the 8th day and

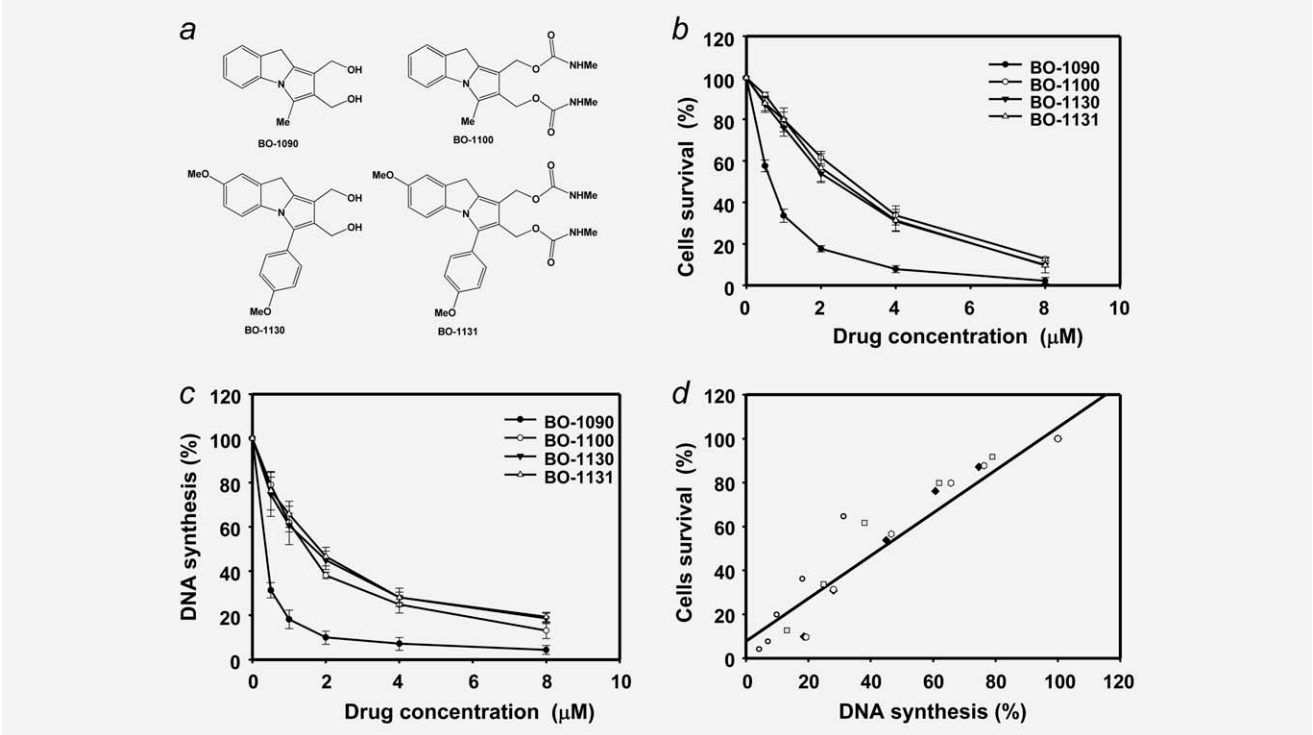

Figure 1. Correlation of cell survival with DNA synthesis inhibition by BO-1090, BO-1100, BO-1130, and BO-1131 in SAS oral cancer cells. (*a*) Structures of 3a-aza-cyclopenta[α]indene derivatives. (*b*) *In vitro* cell-survival assay. SAS cells were treated with the indicated concentrations of drugs for 72 hr. Cell viability was determined with the cell proliferation reagent WST-1. (*c*) DNA synthesis–inhibition assay. SAS cells were treated with the indicated concentrations of drugs for 24 hr. The DNA synthesis activity was determined with BrdU-based ELISA tests. (*d*) Correlation between cytotoxicity and DNA synthesis inhibition. The survival rates of SAS cells treated with various concentrations of tested drugs (shown in *b*) were plotted against the percentage of DNA synthesis activity (*c*) at the same concentration. All the determinations in (*b*) and (*c*) were performed in triplicate and are shown as the mean ± S.E.

50 mm^3 for Cal 27 on the 12th day, mice were randomly divided into four groups. The first group received vehicle, the second group was treated with 40 mg/kg body weight BO-1090, the third group received 60 mg/kg BO-1090, and the fourth group was treated with 4 mg/kg cisplatin. The dose of cisplatin used was following the literature reports[20–22] in which cisplatin at 2 to 6 mg/kg cisplatin once or twice a week effectively suppressed a variety of xenografted oral and other cancer cells. Beyond this concentration range, cisplatin caused renal and hepatic toxicity in mice. BO-1090 was given intravenously (i.v.) five times on alternating days 8, 10, 12, 14 and 16 for SAS model and on days 12, 14, 16, 18 and 20 for Cal 27 model. Cisplatin was injected intravenously three times with a 3-day gap between injections, *i.e.*, days 8, 12 and 16 for SAS model, and days 12, 16 and 20 for Cal 27 model. BO-1090 was prepared in 20% dimethyl sulfoxide and 15% Tween 80 in 65% normal saline, which was also the vehicle control. Tumor volume (mm^3) was measured with calipers and calculated according to the following formula: tumor volume = (length × $width^2$)/2. Mouse body weight was also measured on alternating days and was used as an indicator of the systemic toxicity of the treatment.

Whole blood, biochemical parameters and histopathology

One day after the last intravenous injection, blood were drawn from heart and then animal were sacrifice to obtain organs. Hematological and biochemical parameters and histopathology of liver, kidney, lung and spleen in mice treated with BO-1090 and cisplatin were examined at the Taiwan Mouse Clinic and the Pathology Core of the Institute of Biomedical Sciences, Academia Sinica, respectively. Blood parameters were analyzed with an Abbott Cell-Dyn 3700 machine, and biochemical parameters were analyzed with a Fuji Dri-Chem Clinical Chemistry Analyzer FDC 3500.

Orthotopic oral cancer model

SAS cells were infected with GFP-luciferase encoding dual repoter GFP and luciferase genes to generate stably expressing SAS cells. A suspension of 1×10^5 of SAS-GL cells in 10 μL PBS were injected into 6-week-old male NOD-SCID mice ($n = 5$ per group) around the buccal mucosa. On the 4th day onward, the 1st control group received vehicle, the 2nd group received intravenous BO-1090 40 mg/kg and the 3rd group 60 mg/kg for seven times. Bioluminescent imaging was

Table 1. Summary of the IC_{50} values ± the S.E (μM) of various 3a-aza-cyclopenta[α]indenes and cisplatin to human oral cavity cancer cell lines and a human lung cancer cell line[1]

Cell line	BO-1090	BO-1100	BO-1130	BO-1131	Cisplatin
OECM-1	0.43 ± 0.03	0.78 ± 0.07	0.97 ± 0.06	1.11 ± 0.05	3.45 ± 1.23
SAS	0.74 ± 0.02	2.47 ± 0.03	2.07 ± 0.05	2.22 ± 0.27	5.22 ± 0.20
Cal 27	0.51 ± 0.01	1.61 ± 0.41	1.24 ± 0.10	2.61 ± 0.11	1.99 ± 0.42
TW-2.6	1.68 ± 0.17	7.81 ± 0.74	3.42 ± 0.61	6.54 ± 0.28	10.90 ± 1.86
SCC-25	2.34 ± 0.27	3.56 ± 0.64	3.14 ± 1.08	5.17 ± 0.62	4.16 ± 0.76
OMF	17.49 ± 0.45	20.49 ± 0.96	11.51 ± 1.46	26.94 ± 4.54	33.28 ± 3.07
H460	5.15 ± 0.86	15.21 ± 0.12	7.33 ± 0.35	18.7 ± 0.62	36.54 ± 2.34

[1]Cytotoxicity was determined with a WST-1 assay as described in Materials and Methods.

performed weekly until sacrifice. *In vitro* bioluminescent imaging of SAS-GL cells was performed with an IVIS Imaging System (IVIS Spectrum). Images and measurements of bioluminescent signals were acquired and analyzed using Living Image software (Xenogen, CA).

Results

Oral cavity cancer cells are sensitive to 3a-aza-cyclopenta[α]indene derivatives

Cytotoxicity of the tested compounds (BO-1090, BO-1100, BO-1130 and BO-1131 (Fig. 1*a*) to several human oral cavity cancer cell lines (OECM-1, SAS, Cal 27, TW-2.6 and SCC-25), a human lung cancer cell line (H460), and OMF was determined with a WST-1 assay. The IC_{50} values of all tested compounds are summarized in Table 1. Cisplatin, one of the drugs most commonly used to treat patients with oral cancer, was included for comparison. Consistent with our previous study,[13] the four tested compounds were more cytotoxic than cisplatin in inhibiting the growth of oral cancer cell lines *in vitro*. Additionally, all tested compounds including cisplatin were several times less cytotoxic to normal mucosal fibroblasts than to cancer cell lines, indicating the selectivity of these novel compounds for cancer cells. Furthermore, we confirmed that all oral cavity cancer cell lines were more sensitive to these bifunctional alkylating agents than human lung cancer H460 cells (Table 1). Among the four tested compounds, BO-1090 was the most potent in killing oral cavity cancer cells (Fig. 1*b*). Compared to cisplatin, BO-1090 was 4- to 7-fold more effective than cisplatin in killing oral cavity cancer cells. OMF cells were 8–40-fold less sensitive to BO-1090 than oral cavity cancer cells.

Derivatives of 3a-aza-cyclopenta[α]indenes target DNA for its sensitivity to cancer cells

Because the 3a-aza-cyclopenta[α]indene derivatives were designed to be bifunctional DNA alkylating agents, we analyzed DNA synthesis inhibition and DNA damage to demonstrate that these compounds target to DNA. A BrdU incorporation-based ELISA assay was adopted to determine the inhibitory activity of tested compounds on DNA synthesis. As shown in Figure 1*c*, all four tested compounds significantly inhibited DNA synthesis in SAS cells. The IC_{50} values for DNA synthesis inhibition by BO-1090, BO-1100, BO-1130 and BO-1131 were 0.17 ± 0.02, 1.48 ± 0.05, 1.56 ± 0.40 and 1.78 ± 0.41 μM, respectively. Consistent with the cytotoxicity data, BO-1090 was the most potent DNA synthesis inhibitor among these four compounds. A linear correlation with a correlation coefficient (r^2) of 0.92 was obtained when the relative survival was plotted against DNA synthesis inhibition in SAS cells treated with the tested compounds (Fig. 1*d*), suggesting that DNA was the major target of the 3a-aza-cyclopenta[α]indene derivatives. Similar effects were observed in OECM-1 cells (data not shown).

A comet assay was then used to determine whether treatment of cells with BO-1090 causes DNA damage. As shown in Figure 2*a*, DNA strand breaks were induced in a dose-dependent manner in SAS cells exposed to 0.5 μM to 2 μM BO-1090 for 24 hr. Irradiation of SAS cells with X-rays at 20 Gy served as a positive control. Additionally, we evaluated whether BO-1090 causes DNA ICLs by mixing this agent with isolated plasmid DNA (pEGFP-N1) and subjecting the mixture to alkaline agarose gel electrophoresis. As shown in Figure 2*b*, BO-1090 induced significant ICLs that could not be dissociated into single strands in alkaline conditions. In this experiment, melphalan was used as a positive control. BO-1090 was more effective than melphalan in inducing ICLs. Furthermore, after treatment with 10 μM melphalan, plasmid DNA became a smear on the gel (Fig. 2*b*), suggesting that this treatment may caused significant DNA strand breaks. To determine whether BO-1090 induced DNA ICLs in SAS cells, we performed a modified comet assay.[13] If cellular DNA contains ICLs, the tail moment induced by ionic irradiation should be reduced in an alkaline single-cell gel electrophoresis comet assay (Supporting Information Fig. 1). As summarized in Figure 2*c*, treatment of cells with BO-1090 or melphalan for 1 hr inhibited the X-ray irradiation-induced tail moment in a dose-dependent manner. These results indicated that both BO-1090 and melphalan caused ICLs of cellular DNA in SAS cells. Our results also showed that BO-1090 was more potent than melphalan in inducing ICLs in cultured cells.

Because γH2AX protein expression is a hallmark of DNA double-stranded breaks (DSBs),[23] we then examined the

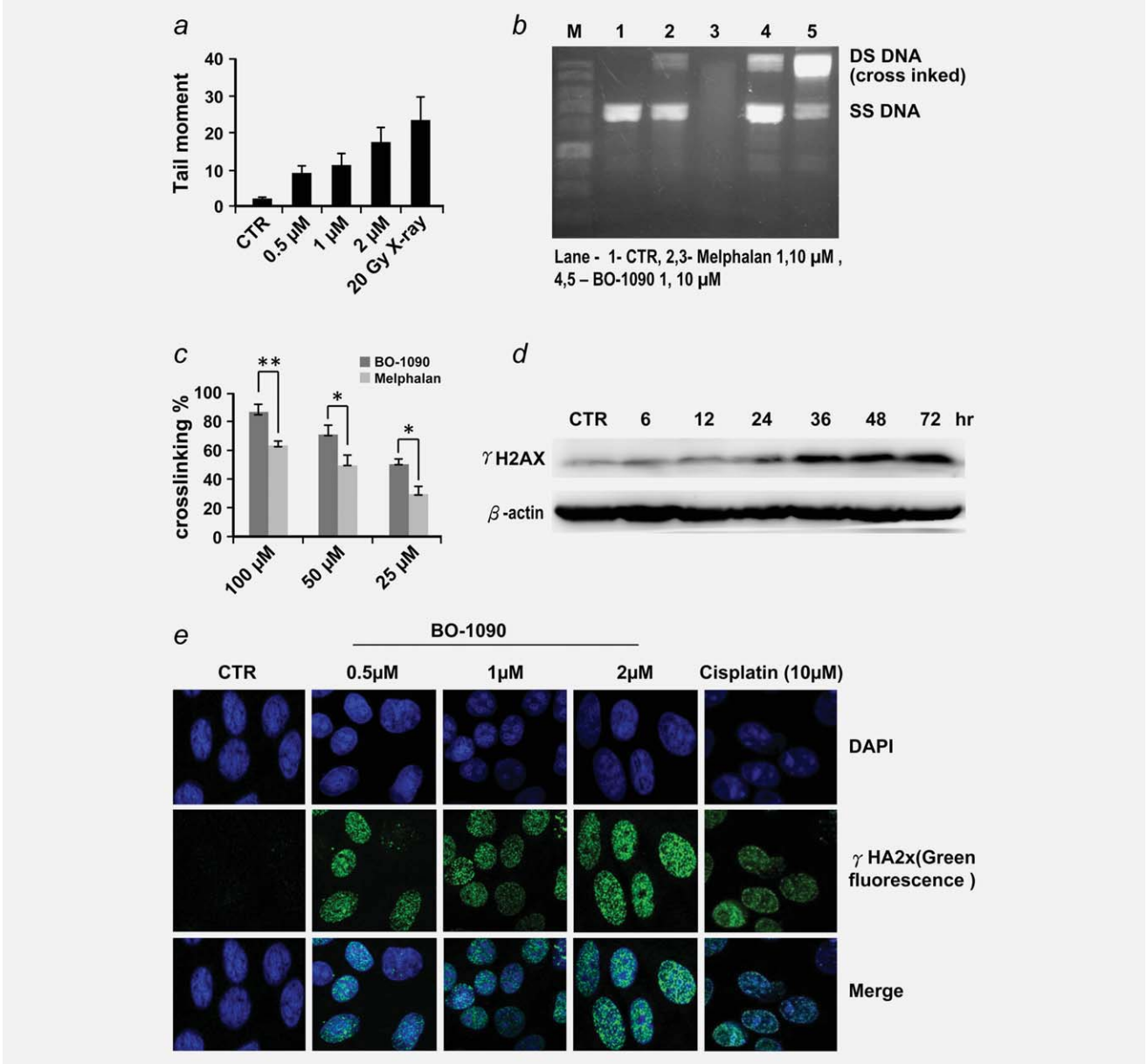

Figure 2. DNA damage induced by BO-1090 in SAS cells. (*a*) DNA strand breaks induced by BO-1090. SAS cells were treated with BO-1090 for 24 hr. DNA strand breaks were evaluated by the tail moment as determined by a comet assay. (*b*) *In vitro* DNA ICL formation. DNA plasmid pEGFP-N1 was incubated with BO-1090 or melphalan and subjected to alkaline agarose gel electrophoresis. The gel was stained with ethidium bromide, and DNA bands were visualized under UV light. (*c*) *In vivo* DNA ICLs in SAS cells induced by BO-1090 and melphalan. SAS cells were treated with the indicated concentrations of BO-1090 or melphalan for 1 h. The ICLs of cellular DNA were determined with a modified comet assay. $^{**}p < 0.01$ and $^{*}p < 0.05$ between BO-1090 and melphalan treatment (Mean ± SEM). (*d*) Western blot analysis of γ-H2AX protein. SAS cells were treated with 2 μM BO-1090 for the indicated times. After electrophoretic separation of cellular proteins, γ-H2AX was visualized by western blotting. β-actin served as a loading control. (*e*) Immunofluorescent staining of γ-H2AX in SAS cells. SAS cells were treated with the indicated concentrations of BO-1090 or 10 μM cisplatin for 24 hr. The cells were fixed and stained with primary antibody against γ-H2AX and secondary antibody conjugated to Alexa Fluor 488. DNA was counterstained with DAPI.

appearance of γH2AX in BO-1090-treated SAS cells with western blotting and immunofluorescence staining. As shown in Figure 2*d*, the protein level of γH2AX gradually increases in SAS cells after expose to 2 μM of BO-1090 from 24 hr to 72 hr. We also observed the obvious appearance of γH2AX foci in the nuclei of SAS cells treated with various doses of BO-1090 for 24 hr (Fig. 2*e*). Approximately 80, 90 and 98% of cells were γH2AX positive (more than five foci per cell) in

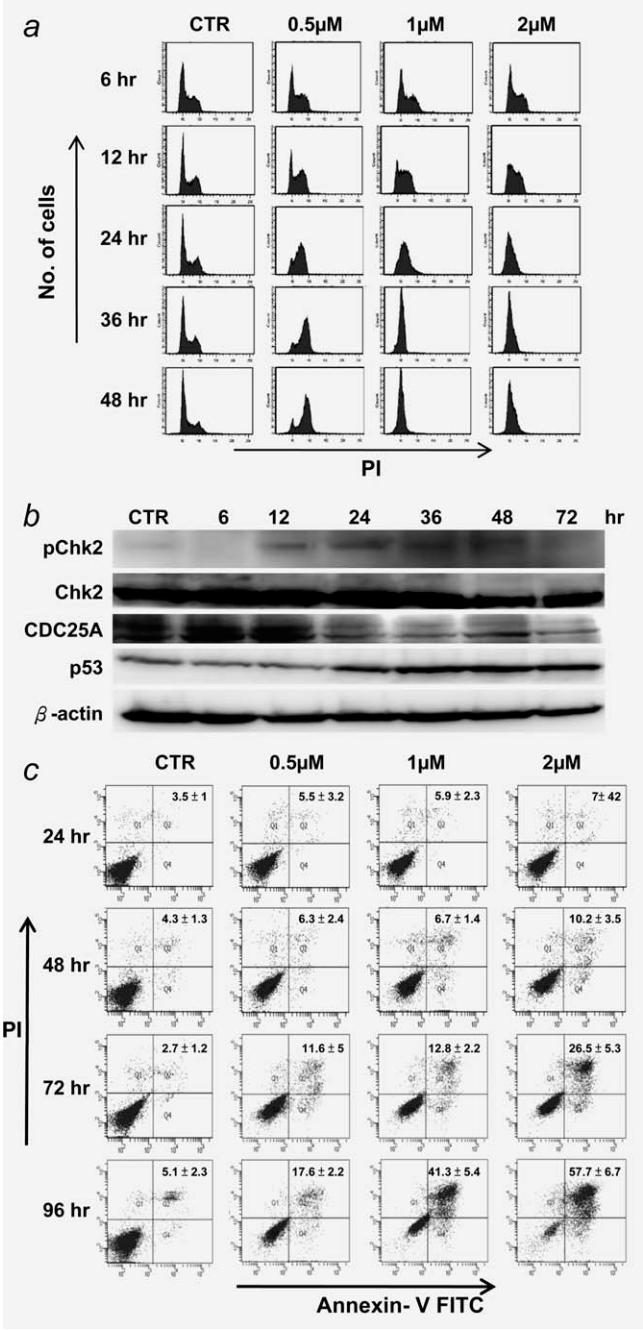

Figure 3. Cell-cycle interference and apoptosis induced by BO-1090 in SAS cells. (*a*) Cell-cycle distribution. SAS cells were treated with the indicated doses of BO-1090 for 6 to 48 hr. Phases of the cell cycle were analyzed by flow cytometry. (*b*) Western blot analysis of pCHK-2, p53 and CDC25A expression. SAS cells were treated with 2 μM BO-1090 for 6 to 72 hr. Proteins from whole-cell extracts were then separated on SDS polyacrylamide gels and immunoblotted. β-actin served as a loading control. (*c*) Annexin V–based apoptosis assay. SAS cells were treated with BO-1090 for 24 to 96 hr, and apoptosis was analyzed using the annexin V-FITC apoptosis detection kit. Annexin V–positive cells were determined by flow cytometry.

SAS cells treated with 0.5, 1 and 2 μM BO-1090, respectively. Similarly, treatment of 10 μM cisplatin in SAS cells for 24 hr produced 80–85% γH2AX-positive cells (Fig. 2*e*). These results clearly indicated that BO-1090 is a potent alkylating agent that targets to DNA and leads to DNA strand breaks and ICLs.

BO-1090 disrupts cell-cycle progression and triggers apoptosis

Because treatment of cells with BO-1090 caused DNA breaks, ICLs and inhibited DNA synthesis, we therefore investigated its effects on cell-cycle progression. We treated SAS cells with various concentrations of BO-1090 (0.5, 1 and 2 μM) for different amounts of time (6 to 48 hr) and determined the cell-cycle phase using flow cytometry. As shown in Figure 3*a*, 0.5 μM BO-1090 treatment gradually caused an arrest of the cells at the G2/M phase. With 1 and 2 μM BO-1090, we first observed a slight accumulation in S-phase cells, which then largely arrested at G1. Since SAS cells were fast growing cells, a slightly more control cells were at the G1 at the end of incubation could be due to the cells reaching to near confluence. The cell-cycle distribution of SAS cells treated with BO-1090 was summarized in Supporting Information Table 1. To confirm that BO-1090 disturbed cell-cycle progression, we prelabeled the cells with 10 μM of BrdU for 60 min. Because only S-phase cells incorporate BrdU into their DNA, we could chase the progression of the S-phase cells with flow cytometry in cultures treated with BO-1090. Consistent with the results shown in Figure 3*a*, most of the S-phase labeled cells were arrested at the G2/M phase by treatment with 0.5 μM BO-1090, whereas they were arrested at the G1 phase by 1 and 2 μM BO-1090 (Supporting Information Fig. 2). To determine if the cells arrested at G1 were indeed at G1 or were at the G1/S boundary, we labeled SAS cells with BrdU for 1 hr at the end of BO-1090 treatment. As shown in Supporting Information Figure 3, BrdU was incorporated into DNA in SAS cells 48 hr after treatment with 1 and 2 μM BO-1090. At this time, they appeared to be in G1, suggesting that 1–2 μM BO-1090 arrested the cells at the G1/S boundary. In western blotting analysis, after 12 hr, activated Chk2 (pChk2) was observed in SAS cells treated with BO-1090. pChk2 persisted through 48 hr and declined by 72 hr (Fig. 3*b*). The level of p53 was significantly enhanced along with increased pChk2, but CDC25A was remarkably reduced by 24 hr (Fig. 3*b*).

Because DNA damage and cell-cycle disruption usually trigger apoptosis, we conducted an annexin V–based apoptosis assay using flow cytometry. Apoptosis was induced in a dose- and time-dependent manner in SAS cells treated with 0.5, 1 and 2 μM BO-1090 for 24 to 96 hr (Fig. 3*c* and Supporting Information Fig. 4). We performed immunoblotting to determine the pathways of caspase-mediated death. Active forms of caspases 3, 7, 8 and 9, and PARP appeared at 24 to 48 hr after treatment of SAS cells with BO-1090 (Supporting Information Fig. 5).

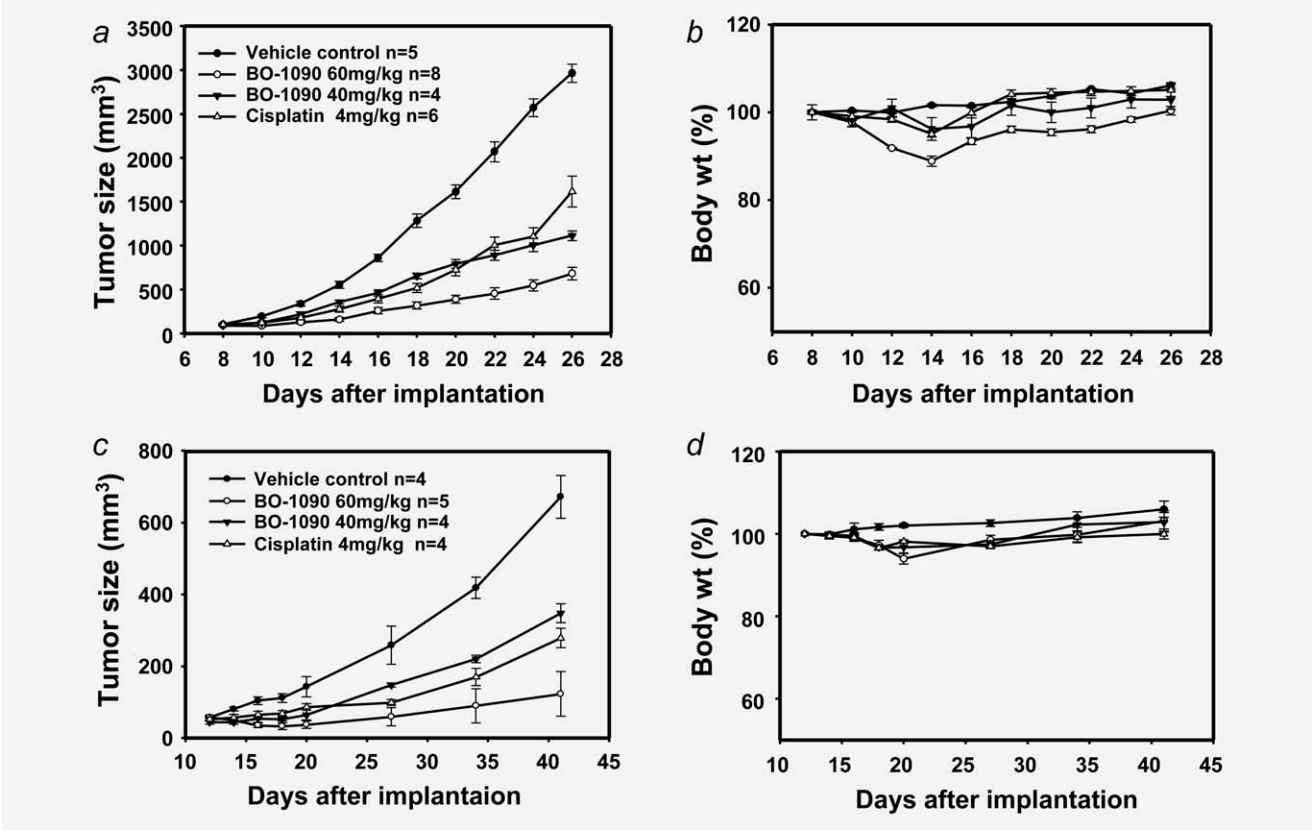

Figure 4. Therapeutic effects of BO-1090 and cisplatin in xenografted mouse models. (*a* and *c*) Suppression of tumor growth. BALB/c nude mice bearing SAS or Cal 27 oral cancer xenografts were divided into four groups and Control group treated with vehicle, BO-1090 (40 or 60 mg/kg body weight) or cisplatin (4 mg/kg body weight). Treatment schedule is mentioned in material and methods. Tumor volumes were measured with calipers and are shown as the mean ± S.E. (*b* and *d*) Effects of drug treatment on body weight. The body weights of SAS xenografted mice (*b*) and Cal 27 xenografted mice (*d*) were measured at the indicated times.

BO-1090 inhibits the growth of SAS cells in xenografted and orthotopic mice

Because BO-1090 showed promising cytotoxic activity on SAS and Cal 27 cells i*n vitro*, we investigated its therapeutic efficacy in xenografted mouse models. SAS tongue carcinoma cells and Cal 27 cells were implanted subcutaneously into the flanks of nude mice. When the tumor size reached about 100 mm^3 in SAS xenografts and 50 mm^3 in Cal 27 xenografts, mice were treated with BO-1090 or cisplatin. SAS and Cal 27 xenografted mice treated with BO-1090 (40 or 60 mg/kg, Q2D × 5 by i.v. injection) or cisplatin (4 mg/kg, Q4D × 3 by i.v. injection) showed significant tumor reduction in comparison to the vehicle control (Fig. 4). In the SAS xenograft model (Fig. 4*a*), 63% ($p < 0.001$) and 78% ($p < 0.001$) tumor burden reduction was observed in mice treated with 40 and 60 mg/kg BO-1090 on day 26, respectively, whereas 46% ($p < 0.001$) reduction was seen in mice treated with 4 mg/kg cisplatin. Similarly, BO-1090 significantly suppressed the growth of Cal 27 cells in xenografted mice (Fig. 4*c*), *i.e.*, 49% ($p = 0.003$) and 82% ($p < 0.001$) reduction of tumor burden was seen in Cal 27 xenografted mice treated with 40 and 60 mg/kg BO-1090, respectively, on day 42. Cisplatin (4 mg/kg) reduced the Cal 27 tumor burden by 59% ($p = 0.001$). Although there was no significant difference in tumor reduction between mice treated with 40 mg/kg BO-1090 and those treated with 4 mg/kg cisplatin, the tumor suppression in both SAS and Cal 27 xenografted mice treated with 60 mg/kg BO-1090 was significantly more effective than mice treated with 4 mg/kg cisplatin (Figs. 4*a* and 4*c*). The body weight reduction in SAS- and Cal 27-xenografted mice treated with 60 mg/kg BO-1090 was about 13 and 6 %, respectively (Figs. 4*b* and 4*d*), suggesting that the doses used in our animal studies caused no significant toxic effects. As shown in Supporting Information Table 2, mice treated with 40 or 60 mg/kg BO-1090 did not have significant hematological damage, except for slight leukocytopenia, which was observed in mice treated with 40 and 60 mg/kg BO-1090, and slight leukocytosis, which was seen in mice treated with 60 mg/kg BO-1090. However, mice treated with 60 mg/kg BO-1090 showed slightly increased ALT, AST, LDH and CPK (Supporting Information Table 3), suggesting minor hepatotoxicity. However, no significant liver damage was observed in histological sections (Supporting Information Fig. 6). Biochemical and histological indicators also showed that BO-1090 did not

have renal toxicity (Supporting Information Table 3 and Supporting Information Fig. 6). Additionally, we did not find any major pathological or inflammatory changes with macroscopic or microscopic examination of liver, lungs, kidney, spleen or heart (Supporting Information Fig. 6). However, consistent with other reports, reduced white blood cell count and protein leaking was observed in cisplatin-treated mice, confirming its renal toxicity.

Significant reduction of SAS and Cal 27 xenografts by BO-1090 motivated us to study the efficacy of this agent in an orthotopic oral cancer model. SAS-GL cells were implanted into the buccal mucosa of NOD-SCID mice. One advantage of orthotopic xenograft was to determine the efficacy of BO-1090 in metastatic inhibition. Starting on the fourth day, mice were treated with vehicle or 40 or 60 mg/kg BO-1090 seven times, every other day. Weekly IVIS images were taken to ascertain the efficacy of the drug. As shown in Figures 5*a* and 5*b*, mice treated with 60 mg/kg BO-1090 showed a significant average tumor reduction of 60% compared to the vehicle control ($p = 0.014$). However, 40 mg/kg BO-1090 provided an average reduction of about 15%, which was not statistically different from the control ($p = 0.129$). Furthermore, we observed that orthotopically implanted SAS tumors migrated first to the salivary gland. We also measured the photons emitted by cells other than those of the tumor mass, which we considered metastatic tumors. Mice treated with 60 mg/kg BO-1090 had a marked reduction (76%) in metastatic tumors, whereas 40 mg/kg BO-1090 did not significantly affect the size of metastatic tumors compared to vehicle control mice (Fig. 5*c*). Orthotopic tumors were also examined for cleaved caspase 3 activity. The number of positive cells per high-power field was increased in a dose-dependent manner in orthotopic tumors in mice treated with 40 and 60 mg/kg BO-1090 (Figs. 5*d* and 5*e*).

Discussion

Therapeutic drugs should be specific and selective for cancer cells to minimize overall toxicity.[24] Cisplatin, one of the most commonly used therapeutic agents for treating oral cancer, shows renal toxicity and ototoxicity along with the development of resistance in cells. These crucial factors are responsible for treatment failure.[25] Derivatives of 3a-aza-cyclopenta[-]indene (BO-1090, -1100, -1130 and -1131) were rationally designed to have increased affinity and specificity toward the DNA in cancer cells. We have shown that these 3a-aza-cyclopenta[α]indene derivatives are effective against the growth of different cancer cells in xenografted mice.[13] Among the cancer cell lines used in our previous study,[13] these compounds were more cytotoxic to human oral cavity cancer cells than to human lung cancer, prostate cancer and neuroblastoma cells. Here, we further confirmed that all oral cavity cancer cell lines tested were more susceptible to the tested compounds than human lung cancer cells and normal OMF cells, implying that these newly synthesized may have potential to be developed as therapeutic agents against oral cavity cancers. Among these compounds, BO-1090 was 2- to 4-fold more effective compared to other tested compounds in a variety of oral cancer cell lines. Because the ability of BO-1090 to block DNA synthesis was greater than that of other derivatives, BO-1090 was mainly used in our study.

ICL formation by drugs alters DNA structure, leading to catastrophic consequences for replication and transcription in cells if not repaired.[7,26] Impairment of DNA synthesis by ICLs is one of the main reasons for the inhibition of cell proliferation and subsequent cell death when the damage is beyond repair by the cellular machinery.[26–28] The cytotoxicity of nitrogen mustard drugs is well correlated with its ability to form ICLs,[26] whereas cisplatin is cytotoxic mainly due to intrastrand crosslinking.[26,28] Thurston *et al.*[29] have demonstrated that the cytotoxicity in several cell lines is directly correlated with ICLs that are induced by therapeutic drugs. Our study showed that BO-1090 is more potent in inducing ICLs than melphalan, a known ICL agent (Figs. 2*b* and 2*c*). We also observed a good correlation between the effect of DNA synthesis inhibition and the survivability of cells treated with tested compounds. These results clearly indicate that the main target of the tested agent is DNA.

It is well documented that the majority of cancers treated with DNA crosslinking agents, such as mitomycin C and cisplatin, become irreversibly arrested at the G2 phase.[27] In our study, treatment of SAS cells with BO-1090 at doses lower than the IC_{50} value initially delayed the progress through the S phase and then arrested the cells at G2 from 24 hr through 48 hr of incubation. It is likely that after 24 hr, cells may recover from the replication block and enter G2, which is very sensitive even to slight damage.[27] However, BO-1090 at doses higher than the IC_{50} value caused a significant amount of cell death. The remaining attached cells were apparently at the G1/S boundary. The arrest of cells at the G1/S boundary by BO-1090 is not unusual. High concentrations of mitomycin C and cisplatin also arrest cells at G1.[30,31] DNA crosslinking drugs such as 9-amino-1-nitroacridines arrest HeLa S3 cells at the S phase.[27] Elimination of CDC25A *via* polyubiquitination and proteasome-mediated degradation is associated with DNA damage-induced G1/S arrest.[32] SAS cells carry wild-type p53 with a single mutation that does not interfere with normal p53 function.[33] After treating SAS cells with BO-1090, we observed a significant increase in activated Chk2 (pChk2) and p53 and a remarkable decrease in CDC25A (Fig. 3*b*). Similarly, DNA-damage responses induced by cisplatin include activation of the ATR, Chk2 and p53 pathways[34] and cause cells to arrest at G1 or G1/S.[35] Furthermore, pChk2 responds to stalled replication and DSBs.[36] Thus, it is likely that Chk2 activation play crucial role in the response to DNA damage induced by BO-1090, including DNA synthesis inhibition and the generation of DSBs.

DSBs are intermediates formed during the repair of ICLs, *i.e.*, due to stalled DNA replication forks during the S phase of the cell cycle in restarting of replication.[7,26] If cells fail to

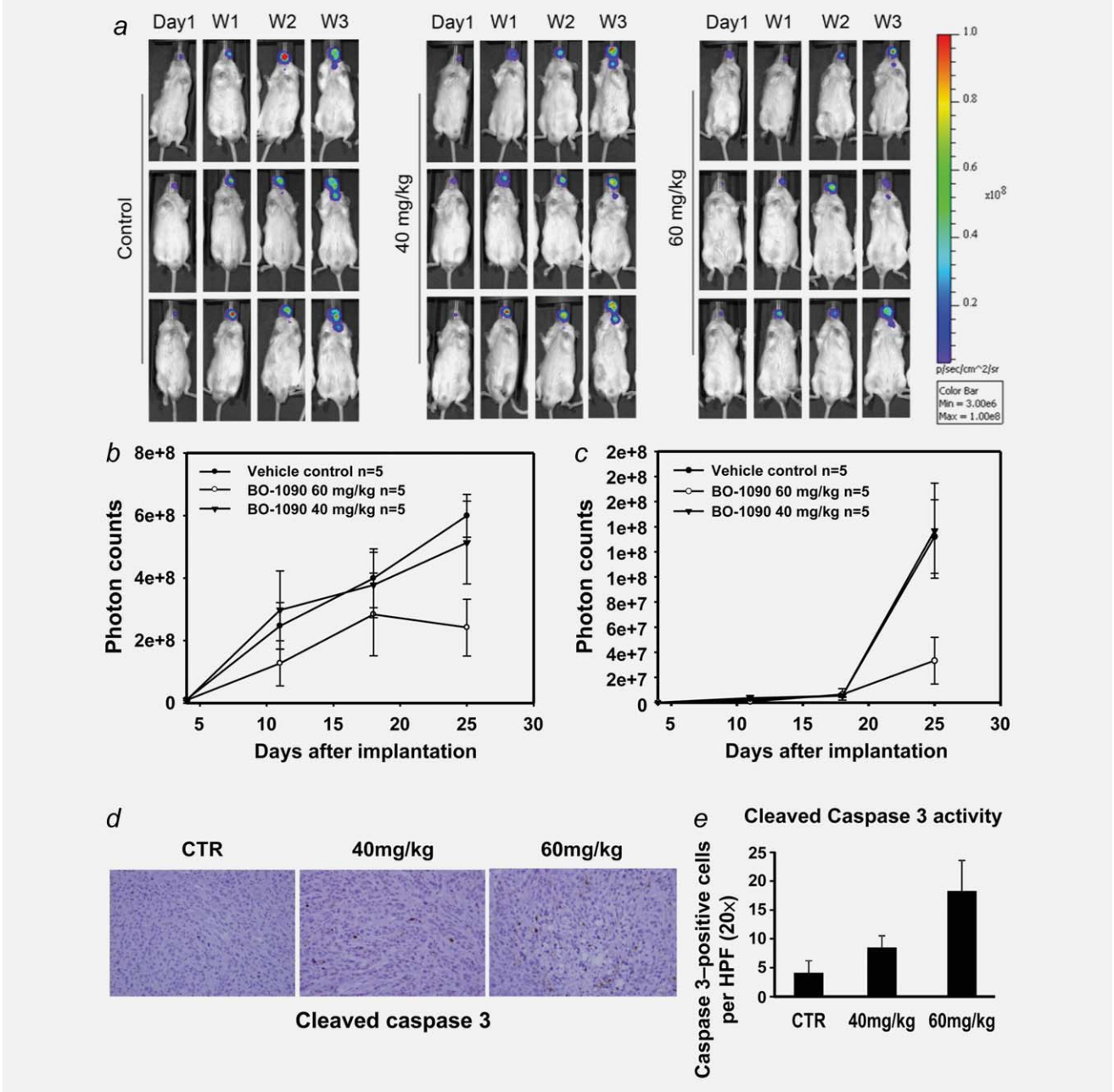

Figure 5. Therapeutic effects of BO-1090 on orthotopic mice bearing SAS-GL oral cancer cells. (*a*) IVIS images of oral cancer orthotopic mice. Mice that were orthotopically bearing SAS-GL oral cancer cells were treated with 40 or 60 mg/kg body weight of BO-1090. The IVIS images were taken weekly [for three weeks (W1, W2 and W3)]. (*b*) Suppression of orthotopic oral cancer by BO-1090. The photon counts taken weekly are the average of the five mice in each group. (*c*) Suppression of metastasis of orthotopic oral cancer by BO-1090. The photon counts outside the tumor mass were considered to indicate metastatic tumors. (*d*) Analysis of cleaved caspase 3. Animals were sacrificed on the day after the seventh (last) injection, and tumor tissues were fixed, sectioned, and immunohistochemically stained with an antibody against cleaved caspase 3 The brown color indicates cleaved caspase 3–positive cells. (*e*) Quantitative data of cleaved caspase 3–positive cells in orthotopic tumors. The percentage of cleaved caspase-3–positive cells was determined from 20 high-power fields (HPF) (as shown in *d*). The data are shown as the mean ± S.E.

repair DSBs, the death process will be initiated.[26] γH2AX, which was detected by immunoblotting and immunostaining, is a well-documented marker of DSBs.[23] In our study, DSBs initiated by BO-1090 appeared after 12 hr of incubation with the drug and persisted until 72 hr, indicating that lesions produced by BO-1090 are beyond repair and subsequently trigger the death process as observed in a variety of DNA damaging agents.[28]

Alkylating agents such as temazolamide, BCNU and ACNU fail to produce therapeutic efficacy in cancer types that overexpress MGMT, an enzyme that repairs DNA damaged by alkylating agents.[9] When BO-1090 and temazolamide were used against parental Chinese hamster ovary cells (CHO-9) and MGMT-overexpressing CHO-9-3C cells, temazolamide reduced the cell proliferation in CHO-9 but not in CHO-9-3C cells (Supporting Information Fig. 7). However, BO-1090 reduced the cell proliferation in both cell types almost equally. Thus, BO-1090 may be functionally similar to mitomycin C, which causes alkylation at N^7 and N^2 and ICLs[28,37] instead of inducing simple alkylation at the O^6 position.[37] So this agent can be substitute for cell which is resistant to TMZ or BCNU. These results also support the view that the cytotoxic effects of BO-1090 are mediated through its reaction with DNA and formation of ICLs.

Drug resistance is a major concern in cancer therapy. Multidrug resistance and DNA repair are of prime concern with DNA alkylating agents.[7,8] Intriguingly, BO-1090 was more effective in Taxol- and vincristine-resistant KB cell lines (Supporting Information Table 4), which exhibit the pg170/MDR1 resistance phenotype,[14] than in parental KB cells. The resistance ratio (ratio of IC50 of resistant to parental cell) of BO-1090 was 0.93 for Taxol-resistant and 0.731 for vincristine-resistant cells, indicating that BO-1090 may bypass the MDR1 effect. Further investigation is being carried out to determine its effectiveness against resistant cells.

In the SAS xenograft model, 40 mg/kg and 60 mg/kg BO-1090 were more effective than 4 mg/kg cisplatin in suppressing tumor growth in nude mice. However, the efficacy of 4 mg/kg cisplatin was between 40 and 60 mg/kg BO-1090 in the Cal 27 xenograft model. These results correlated with the *in vitro* cytotoxicity of BO-1090 and cisplatin to SAS and Cal 27 cells. SAS cells were about eight times more susceptible to BO-1090 than to cisplatin, whereas Cal 27 cells were four times more susceptible to BO-1090 than to cisplatin. The relatively high doses of BO-1090 were used in *in vivo* study, implying the rapid metabolism of BO-1090 in mice. Pharmacokinetic study will be done in the future. In general, hematopoietic toxicity is a major dose-limiting toxicity factor for alkylating agents aside from toxicity toward other organ systems. However, drugs such as cyclophosphamide and ifosfamide are less hematopoietically toxic than other alkylating agents,[38,39] which is consistent with our observation in BO-1090 treated mice (Supporting Information Table 3). Even though the levels of some biochemical enzymes were higher in BO-1090-treated mice than in the control mice, we did not observe significant histological alterations. The less effective of BO-1090 against oral cavity cancer in othotopic model than xenografted model is likely due to some inherent technical problem with orthotopic model, such as we have found some clotting formed on the control tumor. The clot blocked the photon coming out, and hence decreased total photon number of the tumor and affected the final out comes.

In summary, BO-1090 exhibited cytotoxicity against various oral cancer cell lines *in vitro* and also showed efficacy and limited toxicity in oral cancer subcutaneous xenograft and orthotopic models. Thus, BO-1090 has the potential to be developed for use in oral cancer, either alone or in combination with other agents.

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