Enhanced Efficacy of CKD-516 in Combination with Doxorubicin: Pre-clinical Evaluation Using a Hepatocellular Carcinoma Xenograft Model

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Abstract. Aim: To evaluate the anticancer efficacy of CKD-516, a novel vascular-disrupting agent, alone and in combination with doxorubicin in the treatment of hepatocellular carcinoma (HCC). Materials and Methods: In mice bearing luciferized HCC cells, therapeutic efficacy was assessed for seven days after single administration of CKD-516, doxorubicin, or combination of CKD-516 and doxorubicin. Results: Bioluminescence-imaging (BLI) signals in the CKD-516 group abruptly decreased initially, but recovered at seven days after treatment. BLI signals in the doxorubicin group gradually decreased over the 7-day period. In the combination group, BLI signals were abruptly reduced and remained suppressed for the 7-day period. On histopathological examination, CKD-516-treated tumors showed extensive central necrosis, whereas the peripheral layers remained viable. Doxorubicin-treated tumors showed mild and scattered necrosis. Tumors from the combination group showed more extensive central and peripheral necrosis, with smaller viable peripheral layers than the CKD-516 group. Conclusion: Combination therapy can have additive effects for treatment of HCC compared with CKD-516 or doxorubicin monotherapy.

Playing an essential role in cell division and cell function, microtubule, a major type of cytoskeletal filament, has been an attractive target for anticancer drug development (1). In the course of experiments to better-understand the complexity of tubulin function and the different mechanisms of tubulin-binding, various tubulin-binding agents have been identified (2). Recently, a new class of tubulin-binding agent has been developed to target microtubules, not only in cancer cells, but also in the endothelium of tumor vessels. Classified as vascular-disrupting agents (VDAs), several novel therapeutics in this category are currently under clinical trials [e.g. combretastatin A4 phosphate (CA4P) in phase II/III; ombrabulin in phase III] (3-5). The VDAs are promising cancer therapeutics as they disrupt pre-existing tumor vessels by destroying tumor endothelium, shutting down tumor blood flow and thus leading to tumor ischemia and necrosis (3, 6).

CKD-516 is a novel tubulin-binding agent exhibiting dual functions: acting as a VDA by inhibiting tubulin polymerization and de-stabilizing pre-existing tumor vessels, and as an anti-mitotic agent by causing cell-cycle arrest at the G2-M phase (7, 8). The vascular-disrupting effect of CKD-516 was demonstrated in our previous in vitro studies with human umbilical vein endothelial cells and in in vivo studies evaluated by dynamic contrast-enhanced magnetic resonance imaging (MRI) (9). Its anti-mitotic effect was established in in vitro studies with human leukemia cells (8) and its anticancer effect was validated in a mouse xenograft tumor model bearing human colon cancer cells (7, 8).

In our previous pre-clinical study, CKD-516 performed comparably to other VDAs, demonstrating strong anticancer efficacy and thus extensive necrosis in the center regions of tumors, while leaving a layer of viable tumor cells at the
periphery of the tumor, which might be the origin of subsequent resistance or recurrence (9). We hypothesize that this phenomenon could be overcome by combining CKD-516 with other chemotherapeutic agents which are often less effective at the core of the tumor due to poor vascular perfusion and reduced drug delivery.

Hepatocellular carcinoma (HCC) is a primarily hypervascular tumor characterized by abnormal tumor vessel growth (10). One of the important therapeutic strategies for HCC is to reduce vascular in-flow combined with chemotherapeutic agents, i.e. transarterial chemoembolization (TACE) (11). Hence, HCC might be a good therapeutic candidate for VDAs or their combination with conventional chemotherapeutic agents. However, such therapeutic strategy has not been evaluated for HCC treatment. In the present study, we evaluated the therapeutic effect of CKD-516 in combination with doxorubicin, a standard chemotherapeutic for TACE, in a pre-clinical HCC xenograft model.

Materials and Methods

Animal model. Human hepatocellular carcinoma cells (Hep3B) were purchased from the Korean Cell Line Bank (Seoul, Korea) and were used at the second to fourth passages. Hep3B cells were transfected with a lentivirus containing the firefly luciferase reporter gene and a highly expressing reporter clone was isolated to establish Hep3B-luc cells. Hep3B-luc cells were cultured in Dulbecco’s modified Eagle’s medium (Welgene, Seoul, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO, Seoul, Korea).

All animal procedures were performed according to our Institutional Animal Care and Use Committee approved protocol (SNUH-IAACU #12-0245). Male Balb/c-nude mice (n=27), aged six weeks and weighing 20-25 g, were used. Hep3B-luc cells were suspended at 1x10⁶ cells/0.1 ml in DMEM and subcutaneously injected into the right flank of animals.

Treatment. CKD-516 (Chong Kun Dang Pharm, Seoul, Korea) was provided by the manufacturer (7, 8). Doxorubicin (Hana Pharm, Seoul, Korea) from clinical packaging vials were used. The animals were divided into four groups: a control group treated with saline (n=6), a doxorubicin group (2 mg/kg, n=7), a CKD-516 group (7.5 mg/kg, n=7), and a combination group (n=7) in which doxorubicin (2 mg/kg) was initially administered followed by CKD-516 (7.5 mg/kg) at a 4-h interval. The animals were treated with a single administration of therapeutic agents at approximately two weeks after tumor implantation when the tumor diameter had reached 5-6 mm in diameter. All therapeutic agents were dissolved in saline (0.1 ml) and injected intraperitoneally.

Mice were monitored daily for up to seven days after treatment. The extracted tumors were perfused with PBS, fixed in 4% paraformaldehyde solution, and embedded in paraffin. The sections were切成 at a thickness of 5 μm at the largest tumor area.

To evaluate the extent of tumor necrosis/apoptosis, a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) stain assay was performed with a commercial kit (Roche, Mannheim, Germany). The TUNEL stain is a method to stain cells with DNA damage (i.e. DNA fragmentation) which is caused from either apoptosis or non-apoptotic DNA damage, such as necrotic cell death (12, 13). Hematoxylin and eosin (H&E) stain was also performed for the sampled representative regions of the tumor tissue. The percentage area of necrosis/apoptosis was calculated using NIH-Image J software (NIH, Bethesda, MD, USA). After drawing a free-hand ROI completely around the tumor, the number of pixels in the tumor area was counted. Within the selected tumor area, the number of pixels within the necrosis/apoptosis area which was stained with TUNEL (i.e. brown-stained area) was counted. The percentage area of necrosis/apoptosis (%) was calculated by dividing the area of the TUNEL-stained portions (pixels) by the area of the total tumor (pixels).

To evaluate the change in tumor vasculature, immunohistochemical staining of a vascular endothelial antigen, CD31, was performed using a primary rat antibody against mouse CD31 (BD Pharmingen, San Diego, CA, USA) and a secondary goat anti-rat antibody (1:1000, Molecular Probes, Eugene, OR, USA). To determine the microvessel density (MVD) (14), the three hot spots with the most intense neovascularization were selected while screening in a low-power field (×40). Microvessel counts of three areas for each hot spot were performed in a high power field (×100). Any brown-stained endothelial cells or endothelial cell clusters (identified from anti-CD31 staining) clearly separated from adjacent microvessels were counted as one microvessel, irrespective of the presence of the vessel lumen. The mean microvessel count of all measured areas was determined to be the MVD.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Comparison of the longitudinal curves of the normalized BLI signal measurements was performed. The mean values of the BLI signal between treatment groups obtained at different time points were compared using a two-way repeated
measures analysis of variance (ANOVA) with a post-hoc Fisher’s least significant difference (LSD) test. One-way ANOVA with a post-hoc Fisher’s LSD test was also performed to compare the mean values of the percentage area of the necrosis/apoptosis and the MVD between treatment groups. A paired *t*-test was performed to compare the tumor volume and body weight changes between before treatment and 7 days after treatment. A *p*-value of less than 0.05 was considered to be significant. A computer software package (GraphPad Prism, version 5.0, GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

**Results**

**Serial BLI for therapeutic effects.** Seven days after single-drug administration, the mean values of the normalized BLI signal increased continuously in the control group, reflecting an active tumor growth for Hep3B-luc cells (Figure 1A). In the doxorubicin-treated group, these values decreased gradually, indicating that the cytotoxic effect of doxorubicin affected tumor growth for up to seven days (Figure 1B). In the CKD-516-treated group, the normalized signal rapidly dropped as early as 4 h after treatment which lasted for two days, followed by a significant recovery from three days to seven days, suggesting a rapid vascular shutdown effect and associated cytotoxic effect at the early stage of treatment and a rebound phenomenon at the late stage of treatment (Figure 1C). However, in the combination-treated group, the normalized signals significantly dropped at an early stage of treatment (from 4 h to three days) and showed minimal recovery at seven days after treatment (Figure 1D). These results reflect the additive effects of combination therapy in

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Figure 1. Serial bioluminescence imaging (BLI) of hepatic tumor response. The BLI signal was measured at baseline and 4 h, 1 day, 2 days, 3 days, and 7 days after single administration of therapeutic agents. All measured BLI signals at each time point are normalized to baseline measurement (Signal<sub>time</sub>/Signal<sub>baseline</sub>). A: Control group (i.p. normal saline); B: Doxorubicin group (i.p. 2 mg/kg); C: CKD-516 group (i.p. 7.5 mg/kg); D: Combination group (i.p. doxorubicin followed by CKD-516); E: Plot of normalized BLI signals (mean values and standard deviation) of each group over the treatment period.
Figure 2. Areas of necrosis/apoptosis on histology. Histological specimens of hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (magnification ×10) show areas of necrosis/apoptosis of tumors. On TUNEL staining, brown-stained areas are indicative of apoptotic/necrotic cells with DNA damage. Necrosis/apoptosis is not observed in the control tumor. In the doxorubicin-treated tumor, areas of necrosis/apoptosis are scattered throughout the tumor. In the CKD-516-treated tumor, necrosis/apoptosis occurs predominantly in the central tumor. The tumor treated with the combination shows areas of necrosis/apoptosis occurred both in the central and peripheral portions of the tumor.
Figure 3. Immunohistochemistry specimens with antibody against CD31. On the microscopic specimens (magnification ×20 and ×100), the control tumor and doxorubicin-treated tumor exhibit abundant tumor microvessels which have a patent brown-stained endothelium. The CKD-516- and combination-treated tumors exhibit paucity of patent microvessels.
that CKD-516 induces a rapid treatment effect and doxorubicin may prevent the rebound phenomenon of VDAs.

These results were supported by statistical analysis (Figure 1E). Using a two-way repeated-measure ANOVA, the longitudinal curves of the mean values of normalized BLI signals significantly differed between each treatment group (between subject effect, p < 0.001). Fisher’s LSD tests at each time point revealed that the normalized BLI signals of both the combination-treated group and the CKD-516-treated group were significantly lower than those of the doxorubicin-treated group at 4 h, one day, and two days after treatment (all p-values < 0.05). However, at seven days after treatment, the BLI signal of the CKD-516-treated group returned to basal levels, whereas that of the combination-treated group was stable and significantly lower than those of the doxorubicin- (p=0.002) and the CKD-516- (p < 0.001) treated groups. There were no significant differences in tumor volume values between all treatment groups before, and seven days after treatment (p > 0.05 in all groups, paired t-test).

No mice showed signs of debilitation during the 7-day post-treatment period. The changes in mice body weight before and seven days after treatment were not significant in the control, doxorubicin-, and CKD-516-treated groups (p > 0.05, paired t-test), but was significant in the combination-treated group (22.8±1.8 g vs. 21.6±1.6 g, p = 0.007, paired t-test).

Histopathological findings. We performed a series of histopathological studies to validate the BLI imaging results. Quantification of the necrosis/apoptosis area of the tumor using the TUNEL assay at seven days after treatment revealed that the combination group had the highest percentage area of necrosis/apoptosis (56.5±18.7%), followed by the CKD-516-treated group (34.3±28.0%), the doxorubicin group (26.2±15.5%), and the control group (6.3±6.6%). One-way ANOVA revealed significant differences between each group (p < 0.001). Compared to the control group, the doxorubicin-, CKD-516-, and combination-treated groups had significantly higher percentage areas of necrosis/apoptosis (p < 0.05, Fisher’s LSD test). Among the treatment groups, the combination group had significantly greater necrosis/apoptosis than the doxorubicin group (p = 0.006, Fisher’s LSD test) and the CKD-516 group (p = 0.038), while there was no significant difference between the doxorubicin group and the CKD-516 group (p = 0.434), which may suggest a greater cytotoxic effect of the combination treatment compared CKD-516 or doxorubicin monotherapy.

Microscopically on H&E staining, necrosis was scattered throughout the tumor in the doxorubicin-treated group, whereas in the CKD-516-treated group, necrosis occurred predominantly in the central tumor portion (Figure 2). In the group treated with the combination, necrosis occurred predominantly in the central tumor but also occurred in the peripheral tumor. This may explain the additive efficacy of the combination therapy in that CKD-516 induces a rapid, large centralized necrosis while doxorubicin induces further peripheral necrosis, and inhibits rebound tumor growth at the tumor periphery.

MVD on immunohistochemistry. Histological specimens from anti-CD31 staining and the quantification of MVD of the tumors harvested at seven days after treatment showed that the MVD was highest in the doxorubicin-treated group (25.7±5.5), followed by the control group (22.5±5.3), the CKD-516-treated group (14.0±3.7) and the combination-treated group (13.6±4.0) (Figure 3). One-way ANOVA revealed significant differences between groups (p < 0.05). Post-hoc multiple comparison tests revealed that the MVD did not differ between the doxorubicin-treated and control groups (p = 0.226) nor between the CKD-516-treated and combination-treated groups (p = 0.865), while the MVD values of the CKD-516-treated and combination-treated groups were significantly greater than those of other two groups (p < 0.003, between the control group and CKD-516-treated group; p = 0.002, between the control group and combination-treated group; p < 0.001, between doxorubicin-treated group and CKD-516-treated group; p < 0.001, between doxorubicin-treated group and combination-treated group).

Discussion

Compared to other solid tumors, HCC responds poorly to conventional chemotherapy due to its high resistance to various chemotherapeutic agents; thus, new therapeutic strategies are still being sought (15). The tumor vasculature is a potential therapeutic target for the treatment of HCC (16). Catheter-based chemotherapies, such as TACE or chemoembolization with drug-eluting beads, which are based on a combined strategy of blocking tumor blood supply and delivering chemotherapeutic agents, have been widely used in clinical practice (11).

Tumor vessels of HCC are formed by sinusoidal capillarization and neoarterialization (17). Tumor vessels are usually composed of a network of immature, tortuous vessels with a high proportion of proliferating endothelial cells (16). Out of the three major components of the endothelial cytoskeleton, i.e. microtubules formed by tubulin heterodimers, actin microfilaments, and intermediate filaments (18), the structural integrity of a normal vessel wall relies on both microtubules and actin microfilaments (19). However, the structural integrity of tumor vessels relies on microtubules, because actin microfilaments are not well-developed in immature tumor vessels (20). A previous study demonstrated that CKD-516 selectively disrupts microtubules without affecting actin (9). Therefore, CKD-
516 can selectively impair the endothelial integrity of tumor vessels and shut down the blood flow to deprive the tumor of oxygen and nutrients, eventually leading to ischemia and necrosis of HCC.

In the present study, CKD-516 therapy induced extensive central necrosis of tumor but also led to rebound growth of viable peripheral tumor cells. In addition, the MVD was significantly decreased in the tumors treated with CKD-516 and doxorubicin. These findings indicate that CKD-516 may be an effective VDA against HCC. The extensive central necrosis induced by CKD-516 could be a unique advantage of VDAs over other chemotherapeutic agents, which might not be as effective in the poorly-perfused central portion of tumors. The inadequate delivery of drugs and the hypoxic environment of the central tumor portion can limit the effect of conventional chemotherapeutic agents (21).

In contrast, the rebound growth of viable peripheral tumor cells might pose a major drawback to CKD-516 treatment, as was observed with other VDAs (3-5). This could be attributed to the regional difference of vasculature in that the central tumor portion is composed predominantly of immature tumor vessels and the peripheral tumor portion is composed of both normal host vessels and immature tumor vessels. The normal vessels in tumor periphery can be spared from the vascular-disrupting effect of CKD-516 which is primarily based on selective impairment of microtubules of tumor vessels (22). In addition, oxygen and nutrients can be supplied to the tumor periphery from the surrounding tissues by diffusion, which may increase the survival rates of viable tumor cells at the tumor periphery (6, 22).

To overcome the rebound growth of viable peripheral tumor cells, the combination of conventional cytotoxic drugs with VDAs may be a practical strategy. The viable tumor cells in the periphery are generally highly proliferative, which can increase their susceptibility to conventional cytotoxic chemotherapy. The environment of the tumor periphery is highly oxygenated and well-perfused, which are factors that can enhance the effect of cytotoxic chemotherapy. Synergistic or additive effects of combination therapy have been previously found. For example, when combined with doxorubicin, CA4P, a tubulin-binding VDA prototype drug, exhibited an additive effect against medullary thyroid carcinoma and melanoma (23, 24). In our study, we demonstrated the additive efficacy of a treatment combining CKD-516 and doxorubicin, which was greater than the effect of therapy with CKD-516-only or doxorubicin-only. When we combined CKD-516 and doxorubicin, the tumors showed extensive central necrosis because of the vascular-disrupting effects of CKD-516 and peripheral necrosis from the cytotoxic effect of doxorubicin (with a correspondingly smaller peripheral layer of viable cells). These findings indicate that CKD-516 and doxorubicin have complementary anticancer mechanisms.

Currently, doxorubicin is one of the most widely used drugs for treatment of HCC, especially in catheter-based chemotherapy. Our findings suggest that the clinical efficacy of monotherapy with CKD-516 or doxorubicin may be limited and that the combination of these drugs may be a more efficacious approach for the treatment of HCC. In our animal study, the combination treatment of CKD-516 and doxorubicin was well-tolerated with no signs of debilitation, despite a slight decrease in body weight over seven days. As this is the first pre-clinical study focusing on the treatment effects of CKD-516 and the combination of CKD-516 and doxorubicin against HCC, further investigation is warranted to determine the pharmacokinetics/pharmacodynamics of these regimens and to ascertain the optimal doses and dosing schedules of CKD-516 and doxorubicin. In particular, additional studies are needed to evaluate the applicability of these treatments in catheter-based chemotherapy, to evaluate the long-term efficacy and tolerability of these regimens.

In conclusion, we provide evidence that combination of CKD-516 and doxorubicin is a more effective treatment of HCC than CKD-516 or doxorubicin monotherapy, as the effects of CKD-516 and doxorubicin complement each other. This knowledge can help broaden the application of VDAs and provide alternative approaches in the treatment of HCC.

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