Anti-tumor efficacy of CKD-516 in combination with radiation in xenograft lung cancer mouse model

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Abstract

Background: To evaluate the anti-tumor efficacy of CKD-516 in combined with irradiation (IR) and examine tumor necrosis, delayed tumor growth, and expression of molecules involved in hypoxia and angiogenesis.

Methods : A xenograft mouse model of lung cancer was established. The tumor was exposed to irradiation (IR) for 5 days per week. CKD-516 was administered with two treatment schedules (day 1 or days 1 and 5) at one hour after IR. After the administration, tumor tissues were stained with hematoxylin and eosin and pimonidazole. HIF1 , Glut-1, VEGF, CD31 and Ki-67 expression were evaluated by Immunohistochemical staining.

Results: With short-term administration, IR and CKD-516+IR (d1) significantly reduced tumor size ($p = 0.0062$ and $p = 0.0051$, respectively). CKD-516+IR groups were remarkably reduced blood vessels ($p < 0.005$). In particular, CKD-516+IR (d1) resulted in the most extensive tumor necrosis, which was significantly increased with large hypoxic area ($p = 0.02$) and decreased HIF1 , Glut-1, VEGF, and Ki-67 expressions. Long-term administration of CKD-516+IR reduced tumor size and delayed tumor growth. This combination also greatly reduced the number of blood vessels ($p = 0.0006$) and significantly enhanced tumor necrosis ($p = 0.004$). CKD-516+IR notably increased HIF1 expression ($p = 0.0047$), but significantly diminished VEGF expression ($p = 0.0046$).

Conclusion: Taken together, our results demonstrate that CKD-516 in combination with IR can significantly enhance the anti-tumor efficacy compared to CKD-516 or IR alone in lung cancer xenograft mice. Keywords: Irradiation, Tumor necrosis, Hypoxia, Squamous cell carcinoma of lung, Xenograft mice

Background

Lung cancer is one of the most common malignancies in both males and females. It is the major cause of cancer-related death worldwide [1]. Lung cancer is histologically classified into small-cell lung cancer (SCLS) and non-small-cell lung cancer (NSCLC). The latter accounts for approximately 85% of all lung cancers [2, 3]. Squamous cell carcinoma (SCC) accounts for approximately 20–30% of NSCLC. It has poor prognosis due to limited treatment options [4].

Concomitant chemotherapy combined with radiation has been regarded as the standard treatment for locally advanced stage III NSCLC [5]. However, 5-year survival rate is lower than 30% due to severe toxicities caused by the multi-modality treatment and frequent loco-regional recurrence and/or distant metastasis even after successful completion of treatment. Accordingly, it is urgent to develop a new treatment strategy that can not only enhance local effect, but also minimize side effects when anti-cancer drug is combined with radiation simultaneously or sequentially.

In contrast with normal cells that can recover rapidly in response to radiation, cancer cells are more sensitive to radiation so that they can become extinct. The anti-tumor activity of radiation is effective for

local control. It has been applied for a variety of solid tumors, including lung cancer, head and neck cancer, and cervical cancer. However, hypoxic or acidic areas in cancer tissues are known to be highly resistant to radiation. In addition, tumor response to radiation therapy varies depending on tumor size [6, 7].

Unlike normal tissue, blood vessels in tumor tissue are formed in complex structures with abnormal shapes. Such abnormal vascular structures in tumor tissue can become a hypoxic state which activates the expression of HIF1α, a hypoxia-inducible factor. Increased HIF1α induces more angiogenesis by increasing the expression of VEGF [8, 9]. Ultimately, a series of these events give rise to local progression and distant metastasis through newly created blood vessels. In fact, about 50% of cancer patients receiving radiation become resistant over the time, with low oxygen tension in tumor tissue being the leading cause of local treatment failure.

So far, lots of studies on various forms of angiogenesis inhibitors have been done to overcome the resistance to radiation by effectively suppressing hypoxia-induced tumor angiogenesis. Vascular disrupting agent (VDA) is one of angiogenesis inhibitors with unique action mechanism by selectively targeting immature blood vessels in the center of tumor. Generally, they are classified into flavonoid vascular disrupting agents and tubulin polymerization inhibitors. Flavonoid vascular disrupting agents act on cytokines such as TNF and VEGF, leading to changed actin cytoskeleton, increased vascular permeability, and endothelial apoptosis. Meanwhile, tubulin polymerization inhibitors can disrupt the tubulin network of cytoskeleton in endothelial cells, influence endothelial cell junction and actin cytoskeleton, and change vascular shape, resulting in increased vascular permeability [10].

In preclinical studies, efficacy and safety profile of VDAs have been reported [11–14]. Theoretically, radiation therapy is not so effective in locally controlling the hypoxic area of tumor tissue. Because these novel agents mainly affect blood vessels locating at central area of tumor, unlike cytotoxic anti-cancer drugs or other angiogenesis inhibitors, they may have a major advantage in compensating weak activity of radiation on the center of tumor when they are combined with radiation.

CKD-516, a novel tubulin polymerization inhibitor, can selectively bind to tubulin in endothelial cells of tumor vessels and block tubulin polymerization, thereby destroying the aberrant tumor vasculature. The intracellular process can lead to rapid decrease of tumor blood flow and nutrient supply for tumor cells, consequently inducing massive apoptotic tumor cell death.

In this study, we evaluated the anti-tumor efficacy of CKD-516 alone or in combination with radiation in short-term and long-term administration schedules in an in vivo mouse model. Balb/c nude mice were used in this study because they were known to be suitable for animal models for evaluating anticancer efficacy [16]. In addition, we investigated expression of signaling molecules involved in hypoxia and angiogenesis in tumor tissues.

Methods

Cell culture and reagents

H520 (male, human squamous cell lung carcinoma) cell line was purchased from American Type Culture Collection (Manassas, USA). Cells were cultured in RPMI 1640 medium (Welgene, Korea) supplemented with 10% FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin (Gibco, Korea). Cells were maintained at 37 °C in a 5% CO_2 incubator. CKD-516, a potent tubulin polymerization inhibitor, was obtained from Chong Kun Dang Research Institute [15]. Working concentrations were freshly prepared with 1xPBS.

Animals and Xenograft model

Male Balb/c nude mice (4 weeks of age, average weight 20 g) were purchased from Orient Bio (Seoul, Korea) and maintained under specific pathogen-free condition. Mice were breeding with 20 ~ 26 ℃ temperature, 50 ± 10% humidity, 12 hour light-dark cycle, gamma lay sterilized diet (TD 2018S, Harlan Laboratories Inct, America) and autoclaved R/O water. Bedding of mice was used Aspen bedding (PG-3, LAS bedding, Germany). H520 cells ($2x10^6$) were suspended in 100 μL of serum-free RPMI 1640 medium and injected subcutaneously into the flank of mouse weighing approximately 20 g. A total of 88 mice were used for the experiment. Mice were placed in a chamber at the end of the experiment and euthanized by gradually increasing the concentration of carbon dioxide (CO_2) gas. All of surgical interventions and pre-surgical and postsurgical animal care were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the IACUC (Institutional Animal Care and Use Committee) in school of medicine, The Catholic University of Korea (Approval number: CUMS-2015-0143-01). IACUC and Department of Laboratory Animal (DOLA) in Catholic University of Korea, Songeui Campus accredited the Korea Excellence Animal laboratory Facility from Korea Food and Drug Administration in 2017 and acquired AAALAC International full accreditation in 2018.

Drug treatment and irradiation

Mice were randomized with control and treatment groups. When tumor volume in mouse reached 500 ~ 700 mm^3 in diameter, mice were divided into four groups: 1) control group, injected with phosphate-buffered saline (PBS) weekly; 2) CKD-516, injected with CKD-516 at 3 mg/kg or 5 mg/kg; 3) irradiation (IR), treated with IR at 2 Gy or 4 Gy for 5 days per week; and 4) CKD-516 + IR, treated with both CKD-516 and IR. CKD-516 was administered with two treatment schedules by day 1 (d1) or days 1 and 5 (d1, 5) at one hour after IR. Shielding device was constructed for irradiating only the subcutaneous tumor, thereby avoiding whole body IR with consideration of the action mechanism of CKD-516. A 4-mm thick device in which a 50-mL tube could be inserted was placed into the mouse holder to be used for IR. Toxicity during the period of IR was monitored based on body weight. Tumor volume was measured every other day using calipers throughout the experimental period. Tumor volume was calculated based on the following formula: tumor volume = (length x $width^2$) x 2.

Analysis of tumor hypoxic area

Hypoxyprobe™ -1 plus kit (CHEMICON, Cat#: HP2-1000) was used to evaluate the hypoxic area in tumor tissue. Paraffin-embedded tissue sections (4 μm in thickness) were deparaffinized by xylene. Endogenous peroxidase activities were blocked by immersing these sections in methanol with 3% hydrogen peroxide for 5 min followed by washing with water and 1xTBST buffer (0.1% Tween 20 added in 1xTBS). Antigen retrieval was performed by boiling sections in citrate buffer. To block non-specific binding, sections were incubated with protein blocking agent (1% BSA) for 5 min and washed with 1xTBST buffer. Sections were then incubated with FITC-MoAb1 (primary MoAb) for 30 min at room temperature using predetermined optimal dilution (1:100). After washing with 1xTBST, sections were then incubated with HPR-conjugated rabbit anti-FITC for 30 min at room temperature. After washing with 1xTBST, peroxidase activity was revealed with 3, 3'-diaminobenzidine (DAB). These sections were counterstained with hematoxylin and photographed using slide scanner. Finally, hypoxic areas were scored based on the staining intensity of tumor cells and the relative proportion of positively stained cells among total tumor cells. The staining intensity of tumor cell was graded as follows: 0, absent; 1, weak (light brown); 2, moderate (brown); and 3, strong (dark brown).

Assessment of tumor necrosis

Hematoxylin and eosin (H&E) stained sections were imaged with slide scanner to analyze the area of tumor necrosis. At least four tumor tissues per group were evaluated. H & E staining was performed using the standard protocol.

Immunohistochemistry staining

Paraffin-embedded tissue sections (4 μm in thickness) were deparaffinized by xylene. Endogenous peroxidase activities were blocked by immersing sections in methanol with 3% hydrogen peroxide for 10 min followed by washing with water and phosphate-buffered saline (PBS). Antigen retrieval was then performed by boiling sections in citrate buffer. Sections were incubated with Ki-67 (EPITOMICS, Cat#: 4203-1), Glut-1 (EPITOMICS, Cat#: 2944-1), HIF1α (Protein Tech, Cat#: 20960-1-AP), CD31 (abcam, Cat#: ab28364) and VEGF (Santa Cruz, Cat#: SC-7269) antibodies at 4 °C overnight using predetermined optimal dilution (1:100). Slides were washed with PBS and then incubated with biotinylated secondary antibody provided by Polink-2 plus HRP Detection kit for Mouse & Rabbit Antibodies with DAB Chromogen (GBI Labs, Cat#: D41-125) for 10 min at room temperature. After washing with water and PBS, the peroxidase activity was revealed with 3, 3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin and photographed using slide scanner. Finally, expression levels of Ki-67, Glut-1, HIF1α, CD31 and VEGF were scored based on the staining intensity of tumor cells and the relative proportion of positively stained cells among total tumor cells. The staining intensity of tumor cell was graded as follows: 0, absent; 1, weak (light brown); 2, moderate (brown); and 3, strong (dark brown).

Statistical analysis

Results obtained from at least three independent experiments are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to determine differences between the control and treatment groups. $P < 0.05$ was considered statistically significant. All data were analyzed using Microsoft Excel 2010 for Windows 7 (Microsoft, Seoul, Korea).

Results

Anti-tumor efficacy of CKD-516

We evaluated the anti-tumor efficacy of CKD-516 at two doses (3 mg/kg and 5 mg/kg) in H520 xenograft mice. In group 1 mice treated with 3 mg/kg of CKD-516, tumor growth was delayed until day 3. After that, tumor began to regrow. In group 2 mice treated with 5 mg/kg of CKD-516, tumor growth was inhibited from day 3. Compared to the control, at the completion of drug administration, tumor sizes were reduced by 39.5% and 81.2% in groups 1 and 2, respectively (Fig. 1A). Additionally, we stained tumor tissues with Hoechst 33342 dye to examine morphological changes of blood vessels caused by CKD-516. Under a fluorescence microscope, we found that the morphology of blood vessels in mice treated with CKD-516 showed obvious changes (Fig. 1B). Based on these results, we selected the dose of CKD-516 at 3 mg/kg for the next study.

Anti-tumor efficacy of short-term treatment with CKD-516 alone or in combination with radiation

We evaluated the anti-tumor efficacy of short-term administration with CKD-516 alone or in combination with radiation. At 24 hours after the completion of administration schedule, CKD-516 did not reduce tumor size. However, IR reduced it by 27.8% compared to the control (Fig. 2A). When CKD-516 was combined with IR, tumor size was reduced by 28.6% in CKD-516 + IR (d1) and 27.9% in CKD-516 + IR (d1, 5). We also checked tumor size at 72 hours after the end of drug administration. CKD-516 reduced tumor size more at 72 hours than that at 24 hours. Both IR alone and CKD-516 + IR (d1) delayed tumor growth. On the contrary, in CKD-516 + IR (d1, 5), tumor did grow again at 72 hours (Fig. 2A). When tumor sizes were compared among four groups at the end of treatment, IR alone and CKD-516 + IR (d1) significantly reduced the tumor by 55.5% ($p = 0.0062$) and 58.5% ($p = 0.0051$), respectively. Notable body weight loss was observed in IR alone, CKD-516 + IR (d1), and CKD-516 + IR (d1, 5) groups by 15.4%, 13.7%, and 11.5%, respectively. In contrast, no changes in body weight were observed in the CKD-516 alone group (Fig. 2B). We counted the number of blood vessels and tumor necrosis area as well as tumor volume. As shown in Fig. 2C, the number of blood vessels stained with CD31 antibody was significantly diminished in CKD-516 group (52.1%, $p = 0.0001$) compared with the control. Both CKD-516 + IR (d1) and CKD-516 + IR (d1, 5) groups showed much more reduction in the number of blood vessels by 64.8% ($p < 0.005$) and 59.1% ($p = 0.00016$), respectively. We also analyzed tumor necrosis area in tumor tissue stained with H & E. IR

significantly induced necrosis by 60.4% compared to the control (p = 0.004). CKD-516 + IR (d1) produced the most extensive tumor necrosis which was significantly increased by 66.0% (p = 0.02) compared to the control (Fig. 2D). However, tumor necrosis in CKD-516 alone or CKD-516 + IR (d1, 5) group did not significantly differ from that in the control.

Sustained tumor necrosis and hypoxia after short-term treatment with CKD-516 in combination with radiation

We investigated post-treatment effects of IR, CKD-516, and their combinations on tumor necrosis and hypoxic environment. The calculated tumor necrosis area (%) was the largest in IR alone (37.3%) at 24 hours from the beginning of treatment. However, no further change was detected at 72 hours (37.1%) (Fig. 3A). Both CKD-516 and CKD-516 + IR (d1) produced more tumor necrosis area by 41.3% (p = 0.049) and by 47.1% (p = 0.004), respectively, at 72 hours. At 24 hours after the end of treatment, calculated hypoxic area was in the following order: 56.6% in CKD-516 + IR (d1), 42.0% in CKD-516 + IR (d1, 5), and 34.4% in IR alone. At 72 hours, the hypoxic area was rapidly declined from 34.3% to 6.9% in IR alone (p = 0.0003). However, it was increased from 56.6% to 64.9% in CKD-516 + IR (d1).

Expression of hypoxia-related molecules in mice after short-term treatment of CKD-516 in combination with radiation

We evaluated protein expression levels of hypoxia-related molecules (HIF1α, Glut-1, VEGF, and Ki-67) affecting the maintenance of hypoxic microenvironment in mice treated with CKD-516, IR, or their combinations (Fig. 4A). The expression of HIF1α, a classic marker for hypoxic condition, was the highest in CKD-516 group (57.6%) at 24 hours after drug administration (Fig. 4B). However, at 72 hours after treatment, it was the highest in IR group (68.1%). VEGF expression in IR alone was increased by 34.8% at 72 hours. In CKD-516 + IR (d1) group, it was significantly diminished from 22.0% to 7.0% (p = 0.019). The expression of Glut-1 was decreased as much as 20–30% for the analyzed area in all treatment groups at 24 hours after the end of drug administration. In IR alone, Glut-1 expression was decreased by 50.2% from 24 hours to 72 hours. It was greatly diminished in CKD-516 + IR (d1) group (81%, p = 0.0039). Ki-67 expression was the lowest (16.3%) in the CKD-516 alone group among four groups at 24 hours after the beginning of drug administration. However, at 72 hours, its expression was significantly declined in CKD-516 + IR (d1) and CKD-516 + IR (d1, 5) groups (86%, p = 0.0036 and 50.8%, p = 0.027, respectively).

Delayed tumor growth after long-term treatment of CKD-516 in combination with radiation

We evaluated delayed tumor growth, tumor necrosis, and tumor hypoxia after long-term treatment with a combination of CKD-516 with IR compared to results from short-term treatment with a combination of CKD-516 and IR. Because weight loss and skin rash due to IR were frequently observed in short-term treatment, IR dose was decreased from 4 Gy to 2 Gy in the long-term combination schedule. Regarding tumor growth inhibition (TGI), both IR alone and CKD-516 + IR remarkably reduced tumor size (56.2%, p = 0.0091 and 71.2%, p = 0.007, respectively) at the end of administration (Fig. 5A and Table 1). Additionally, we found sustained tumor growth delay even at 72 hours after the end of treatment, especially in the group of CKD-516 + IR (33.0% vs. 37.6%). No significant differences in body weight were found between CKD-516 and control groups (Fig. 5B). However, both IR alone and CKD-516 + IR groups displayed gradual decrease of body weight when the administration schedule progressed. We measured the number of blood vessels at 72 hours after the end of administration. Compared to the control, CKD-516, IR, and CKD-516 + IR significantly decreased the number of blood vessels by 38.4% (p = 0.003), 72.9% (p = 0.0002), and 84.2% (p = 0.0006), respectively (Fig. 5C). Conversely, tumor necrosis area was significantly expanded to 67% in IR group, 82% in CKD-516 group, and 84% in CKD-516 + IR group compared to the control (p = 0.02, p = 0.005, and p = 0.004, respectively) (Fig. 5D).

Table 1
Tumor growth inhibition by CKD-516 combined with IR in long-term administration

Group (n = 10)	Radiation (Gy/kg/day)	CKD-516 (mg/kg/day)	TGI (%)	
			24 h	72 h
Vehicle	-	-	-	-
IR	2	-	51.9	56.2*
CKD-516	-	3	-	-
CKD-516 + IR	2	3	61.0	71.2*

Expression of hypoxia-related molecules in mice after long-term treatment of CKD-516 in combination with IR

HIF1α expression was significantly increased by 64.0% in CKD-516 group (p = 0.0022) and by 65.0% in CKD-516 + IR group (p = 0.0047) compared to the control (Fig. 6A). VEGF expression levels in both IR and CKD-516 groups were similar to those in the control. However, it was significantly diminished by 41.0% in CKD-516 + IR group (p = 0.046) (Fig. 6B). Glut-1 expression was increased in both IR and CKD-516 groups. However, it showed no significant change in CKD-516 + IR group (Fig. 6C). Ki-67 expression was greatly diminished by 4.3%, 4.4%, and 5.2% in IR, CKD-516, and CKD-516 + IR groups, respectively (data not shown).

Discussion

Chemotherapy combined with IR has been widely accepted as the standard treatment for locally advanced stage III NSCLC. However, hypoxic and acidic areas in the center of the tumor can lead to tolerance to radiation which is a major cause of treatment failure. In order to overcome IR-induced tolerance of hypoxic conditions, many studies have combined VDA or angiogenesis inhibitor with IR [17–21]. Although VDAs can cause rapid occlusion in the central tumor vessel, drug resistance to VDA can appear immediately. It might be attributed to remaining cancer cells acquiring nutrients and oxygen from marginal area of tumor [22]. Since tumor growth becomes restored within a few hours after the administration of VAD [23, 24], it is very important to combine VDA with other treatments to improve its anti-tumor efficacy. CKD-516 has been proven to have excellent activity in disrupting tumor vasculature in preclinical studies [25, 26, 27]. Its safety has also been confirmed in early clinical studies [28]. Recently, several investigators have demonstrated synergistic anti-tumor efficacy by combining CKD-516 with other cytotoxic agents such as doxorubicin or gemcitabine in hepatocellular carcinoma and lung cancer xenograft mice [29, 30]. We confirmed the tumor formation on SCC cell lines (SK-MES-1, HCC-95, H520), and the H520 cell line with the best tumor formation was used in this study. We evaluated the anti-tumor efficacy with expression changes of hypoxia-related signaling molecules in SCC xenograft mice after short- and long-term administration of CKD-516 alone or in combination with IR.

Results of the present study confirmed that high dose of CKD-516 (5 mg/kg) reduced tumor volume and increased tumor necrosis significantly more than a low dose (3 mg/kg). There was no noticeable change in body weight after low dose treatment. However, gradual weight loss was observed after high dose treatment. Therefore, we used the administration dose of CDK-516 at 3 mg/kg for subsequent experiments. Based on preclinical data that VDA administration following IR was more effective for inhibiting tumor growth in breast cancer model [17], CKD-516 was also given one hour following IR in the present study.

After short-term treatment of CKD-516, IR, or their combinations for 1 week, both IR alone and CKD-516 + IR (d1) significantly reduced the tumor by more than 50%. In particular, CKD-516 + IR (d1) inhibited tumor growth up to 72 hours even after the end of administration. However, tumor did grow again in CKD-516 + IR (d1, 5). There was less tumor necrosis and hypoxia with higher expression of Glut-1 and Ki-67 in CKD-516 + IR (d1, 5) group compared to those in CKD-516 + IR (d1) group. Interestingly, we found that expression of Ki-67 in rim area of tumor tissue in CKD-516 + IR (d1, 5) was increased (data not shown). Tumor is likely to regrow in CKD-516 + IR (d1, 5) at 72 hours after the end of drug administration.

In our study, CKD-516 + IR in combination significantly reduced blood vessels and CKD-516 + IR (d1) produced the most extensive tumor necrosis. In a previous study with hepatocellular carcinoma xenograft model, CKD-516 caused necrosis at the central area of tumor and markedly reduced CD31 expression [30], consistent with our results. We investigated delayed effects on tumor necrosis and hypoxia by CKD-516, IR, and their combinations up to 72 hours after the end of administration. Both CKD-516 and CKD-516 + IR (d1) significantly enhanced tumor necrosis between 24 and 72 hours. Especially, CKD-516 + IR

(d1) induced the largest hypoxic area among all treatment groups at 24 hours and enlarged the area at 72 hours. A prior literature has reported that CA-4-P, one of VDAs, can lead to tumor necrosis up to 120 hours even after the end of treatment. It tends to decrease hypoxia at 24 hours [31]. We also checked that the expression of hypoxia-related molecules. CKD-516 + IR combination decreased HIF-1, Glut-1, and VEGF expression levels continuously from 24 to 72 hours, particularly CKD-516 + IR (d1). Additionally, CKD-516 + IR (d1) markedly reduced Ki-67 expression up to 72 hours. Our data indicated that CKD-516 (d1) combined with IR (d1-5) was the most effective treatment schedule for reducing tumor size and inducing tumor necrosis. CKD-516 (d1) already could generate central tumor necrosis by vascular occlusion with decreased expression of VEGF, Glut-1, and Ki-67. Hence, additional administration of CKD-516 (d5) did not seem to have a synergism with IR.

Our results are contrary to prior literatures showing that expression levels of hypoxia-related proteins such as HIF1α, VEGF, and Glut-1 are increased in the presence of hypoxic condition [8, 9, 32, 33]. Meanwhile, another study has shown that potent VEGF inhibitors including sunitinib and ziv-aflibercept can produce tumor necrosis and decrease expression levels of CD31 and Ki-67 in renal cell carcinoma PDX model [34]. The most likely explanation of our result was that CKD-516 + IR rapidly reduced tumor blood flow with excessive hypoxic condition, consequently leading to massive apoptotic tumor cell death with decreased expression of VEGF, Glut-1, and Ki-67.

In the present study, losses of body weight and skin rash were recorded in all IR-administered groups, showing no significant difference from each other. Prior in vivo studies have shown that if body weight of C57BL/6 mice is reduced by about 13 to 20%, their survival probability is lowered [35]. Furthermore, Balb/c nude mice responded more sensitively to IR than C57BL/6 mice [36]. One study has shown that when IR is given to colon cancer xenograft mice at 2 Gy for 5 days, tumor volume is reduced. However, body weight is not changed [37]. Therefore, we decreased IR dose to 2 Gy and applied it for the long-term treatment.

In the long-term treatment schedule, single IR and CKD-516 + IR significantly inhibited tumor growth with markedly reduced tumor. Moreover, CKD-516 + IR sustained tumor growth delay up to 72 hours even after the end of treatment. A previous report has shown that combination of CKD-516 and gemcitabine can greatly enhance its anti-cancer efficacy [29]. Another study has reported that tumor growth is restored from 3 days after a single administration of CKD-516. However, tumor growth is effectively inhibited until day 7 when CKD-516 is combined with doxorubicin [30]. These results strongly support our data that CKD-516 + IR combination could significantly reduce tumor volume by effectively delaying tumor growth rate. However, considering the continuous decrease of body weight during long-term administration, IR dose should be readjusted in the next study.

In the current study, IR and CKD-516 + IR significantly decreased the number of blood vessels and CKD-516 + IR expanded central tumor necrosis with larger range, similar to results obtained from the short-term treatment schedule. Either CKD-516 or CKD-516 + IR significantly increased HIF1α expression. However, CKD-516 + IR strikingly diminished VEGF as well as Glut-1 expression. Ki-67 expression was also

noticeably reduced when CKD-516 was combined with IR (data not shown). Glut-1, a glucose transporter regulated by HIF1α, plays a role in inhibiting cell death together with other glucose degradation proteins [38]. Previous studies have shown that Glut-1 and VEGF expression can increase along with increase of HIF1α expression [32, 33]. When Glut-1 expression is diminished, Ki-67 expression is down-regulated [39]. However, in our study, Ki-67 expression tended to increase in the peripheral margin compared to that in the central portion of tumor (data not shown). Several studies have revealed that Ki-67 expression tends to decline throughout 24 hours after administration of OXi4503, a tubulin binding agent, but increase again in the margin of tumor at day 5 [24]. Resistance to VDA due to rebound proliferating cancer cells at tumor margins is a major obstacle to reflect anti-tumor activity. Further studies are needed to precisely understand the underlying mechanisms. Not only overall decreased Ki-67 expression, but also prolonged inhibition of VEGF and Glut-1 expression by long-term administration of CKD-516 + IR may be closely related to sustained hypoxic environment and central tumor necrosis.

Conclusion

Taken together, our study demonstrated that CKD-516 in combination with IR could effectively enhance the anti-tumor efficacy compared to CKD-516 or IR alone in SCC xenograft in vivo model. Further studies are needed to indicate a new research direction to overcoming some limitations of VDA.

Abbreviations

SCLC
Small-cell lung cancer
NSCLC
Non-small-cell lung cancer
SCC
Squamous cell carcinoma
IR
Irradiation
VDA
Vascular disrupting agent
d1
day 1
d1, 5
day 1 and day 5
HIF-1α
Hypoxia inducible factor-1 alpha
VEGF
Vascular endothelial growth factor
Glut-1

Glucose transporter type 1
CD31
Cluster of differentiation 31 (Platelet/endothelial cell adhesion molecule-1)
PDX
Patient-Derived Xenograft

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflict of interest was reported by the authors.

Author's Contribution

MY Kim and JY Shin were conception and design the study. MY Kim was performed the overall experiments conducted in this study and writing this manuscript. JY Shin contributed to analysis and interpretation of data. JO Kim and CK Jung were analysis of IHC data and review the manuscript. KH Son was performed together with the overall animal experiment and review the manuscript. JH Kang was conception and designs the study, review and edit, approval of manuscript. Each author has read and approved the final version of the manuscript

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Figures

Figure 1

CKD-516 has different anti-tumor efficacy depending on its dose. Tumor volume (A) in H520 xenograft mice after treatment with two different doses of CKD-516. They were divided into three groups (vehicle, untreated; CKD-516, 3 mg/kg; and CKD-516, 5 mg/kg) when tumor size reached 600-800 mm3. CKD-516 was administered via intraperitoneal injection (arrow: administration of CKD-516). Caliper was used to measure tumor volume and body weight every other day (tumor volume [mm3] = {(length [mm]) × (width [mm])2}/2). (B) Morphological changes of blood vessel induced by CKD-516. After completion of the administration schedule, heochst33342 was injected intravenously into the tail vein at 72h (at 27 days). Mice were then sacrificed and blood vessels were examined by fluorescence microscopy (n = 6 per group). Mean ± SE are presented. * denotes $p < 0.001$.

Figure 2

After treatment with CKD-516 (day 1 or days 1 and 5) combined with daily IR for five consecutive days, tumor growth was retarded, vascularity was declined, and tumor necrosis occurred in xenograft mice. Graphs of tumor volume (A) and body weight (B). Xenograft mice were divided into five groups depending on the administration schedule: vehicle (untreated), IR alone (4 Gy/day), CKD-516 alone (3 mg/kg), and CKD-516 (3 mg/kg, day 1 or day 1 and 5) combined with IR. We consecutively irradiated tumor mass at 4 Gy for 5 days from day 20 to day 24 when tumor size reached 600-800 mm3. We examined two treatment schedules of CKD-516 (day 1 or days 1 and 5) at 1 h after irradiation via intraperitoneal injection (arrow:

administration of CKD-516). Mice were sacrificed at 24 h (25 days) and 72 h (27 days) after the completion of administration schedule. Caliper was used to measure tumor volume and body weight every day (tumor volume [mm3] = {(length [mm]) × (width [mm])2}/2) (n = 6 per group). (C) The number of blood vessels was counted by IHC with CD31 antibody in collected tumor tissues. Results are shown in the graph. (D) Histopathological analysis of tumor necrosis in mouse tissue with H & E staining. Mean ± SE are presented. * denotes $p < 0.05$, ** denotes $p < 0.001$, *** denotes $p < 0.0001$.

Figure 3

Short-term treatment of CKD-516 combined with IR induces persistent tumor necrosis and hypoxia. Necrosis area by H&E staining (A) and hypoxic area by pimonidazole staining (B) are shown. Tumor tissues obtained from sacrificed mice were stained with H & E or pimonidazole. Necrosis and hypoxic area were analyzed using Panoramic MIDI (3DHISTECH Ltd, Hungary) and then compared among five groups: vehicle (untreated), IR alone (4 Gy/day), CKD-516 alone (3 mg/kg), and CKD-516 (3 mg/kg, day 1 or days 1 and 5) combined with IR. Data are presented as mean ± SE. * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

Figure 4

Sustained hypoxia by short-term treatment of CKD-516 combined with IR is associated with expression of hypoxia-related molecules. (A) Immunohistochemical staining was performed with HIF1α, VEGF, Glut-1, and Ki-67 antibodies in all treatment groups. Density was analyzed according to the degree of staining. (B) Results are displayed quantitatively in the graph (Panoramic MIDI, 3DHISTECH Ltd, Hungary). Mice were divided into five groups according to the dosing and treatment schedule: vehicle (untreated), IR alone (4 Gy/day), CKD-516 alone (3 mg/kg), and CKD-516 (3 mg/kg, day 1 or day 1 and 5) combined with IR. Mean ± SE are presented. * denotes $p < 0.05$, ** denotes $p < 0.01$.

Figure 5

Tumor growth is suppressed and delayed in H520 xenograft mice when CKD-516 is combined with IR by long-term administration. Graphs of tumor volume (A) and body weight (B) after long-term treatment of IR alone and CKD-516 alone are shown. H520 cells were injected subcutaneously into the right forearm of nude mice. Depending on the administration method, mice were divided into four groups: vehicle (untreated), IR alone (2 Gy/day), CKD-516 (3 mg/kg) alone, and CKD-516 (3 mg/kg, day 1) combined with IR. IR was administered at 2 Gy every 3 weeks with 5 days per cycle and CKD-516 was administered via intraperitoneal injection at 1 h after IR on the first day of cycle (arrow: CKD-516 administration). At the end of each cycle, no treatment was given for 2 days. Mice were sacrificed at 72h (22 days) after the end of administration schedule. Caliper was used to measure tumor volume and body weight (tumor volume

[mm3] = {(length [mm]) × (width [mm]) 2}/2). (C) CD31 was used to stain mouse tumor tissue using IHC. The number of blood vessels was measured for each group. (D) The area of tumor necrosis was analyzed by H & E staining (n = 10 per group). Mean ± SE are presented. * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

Figure 6

Changes in expression of hypoxia-related molecules after three cycles of CKD-516 combined with IR. Expression levels of hypoxia-related molecules including HIF1α, VEGF, and Glut-1 are presented qualitatively (top) and quantitatively (bottom) after IHC staining using Panoramic MIDI (3DHISTECH Ltd, Hungary). Mice were divided into four groups according to administration methods: vehicle (untreated), IR alone (2 Gy/day), CKD-516 alone (3 mg/kg), and CKD-516 (3 mg/kg, day 1) combined with IR (n = 10 per group). Mean ± SE are presented. * denotes $p < 0.05$, ** denotes $p < 0.01$.

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