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Original Paper

The SGK1 Kinase Inhibitor SI113 Sensitizes Theranostic Effects of the $^{64}CuCl_2$ in Human Glioblastoma Multiforme Cells

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Key Words
SGK1 • GBM • $^{64}CuCl_2$ • SI113

Abstract
Background/Aims: The importance of copper in the metabolism of cancer cells has been widely studied in the last 20 years and a clear-cut association between copper levels and cancer deregulation has been established. Copper-64, emitting positrons and β-radiations, is indicated for the labeling of a large number of molecules suitable for radionuclide imaging as well as radionuclide therapy. Glioblastoma multiforme (GBM) is the CNS tumor with the worse prognosis, characterized by high number of recurrences and strong resistance to chemo-radio therapy, strongly affecting patients survival. We have recently discovered and studied the small molecule SI113, as inhibitor of SGK1, a serine/threonine protein kinase, that affects several neoplastic phenotypes and signaling cascades. The SI113-dependent SGK1 inhibition induces cell death, blocks proliferation, perturbs cell cycle progression and restores chemo-radio sensibility by modulating SGK1-related substrates. In the present paper we aim to characterize the combined effects of $^{64}CuCl_2$ and SI113 on human GBM cell lines with variable p53 expression. ***Methods:*** Cell viability, cell death and stress/authopagic related pathways were then analyzed by FACS and WB-based assays, after exposure to SI113 and/or $^{64}CuCl_2$. ***Results:*** We demonstrate here, that i) $^{64}CuCl_2$ is able to induce a time and dose dependent modulation of cell viability (with different IC_{50} values) in highly malignant gliomas and that the co-treatment with SI113 leads to ii) additive/synergistic effects in terms of cell death; iii) enhancement of the effects of ionizing radiations, probably by a TRC1 modulation; iv) modulation of the autophagic response. ***Conclusions:*** Evidence reported here underlines the therapeutic potential of the combined treatment with SI113 and $^{64}CuCl_2$ in GBM cells.

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Introduction

Copper (Cu) is a transition metal and vital micronutrient that plays a pivotal role in all the living organisms for its activity as an essential catalytic cofactor in main cellular processes, such as mitochondrial respiration, cell growth and development. Moreover, the role of copper in cancer cells metabolism has been extensively investigated in the last 20 years[1]. A strong relationship between copper levels and cancer progression has been demonstrated, allowing the identification of new targets for novel diagnostic and therapeutic agents. In the past twenty years many preclinical studies have demonstrated an effect of copper in favoring cancer development [1, 2]. Malignant gliomas represent the most frequent adult primary brain tumors and the glioblastoma multiforme (GBM) is approximately 70% of glial tumors. Despite important advances in combined therapeutic approaches, the prognosis in these patients is still poor with a median of survival after diagnosis of only 12-15 months [3]. For its elusive response to standard treatments, the development of novel therapeutic approaches for GBM remains indeed a pivotal step in the modern oncologic research. Protocols based on radionuclide therapy appear to be particularly promising for the GBM treatment [2, 4–7]. Experimental evidence has clearly demonstrated that cancer cells have a particularly high fractional content of copper inside the cytoplasmic and nuclear fraction compared to normal cells. Multiple studies have been performed to evaluate the cellular transport of copper. Copper enters the cells through the human copper transporter 1 (CTR1) and is delivered to different compartments [8]. Human copper transporter 1 (CTR1), a 190-amino-acid protein of 28 kDa with three transmembrane domains, is the primary protein responsible for importing copper in mammals and is overexpressed in a variety of cancers. This trimeric structure seems to create a central pore functioning as channel[8–10]. Several positron-emitting radionuclides with a high atomic number and/or a long half-life have been proposed both for imaging and therapeutic approach. Among these, Cu radioisotopes seem to be significantly interesting [1, 4, 11, 12]. Cu radioisotopes, as a group, comprise radioisotopes which, due to their emission properties, may be applied both for diagnostic studies (^{60}Cu, ^{61}Cu, ^{62}Cu, ^{64}Cu) and for radionuclide based therapy (^{64}Cu and ^{67}Cu)[13]. The most appealing is Copper-64 (^{64}Cu), a positron emitter with a half-life of 12.7h, that is particular useful in PET studies requiring scans many hours after the injection. Because of its emission, ^{64}Cu can also be used in therapy. This physical characteristic may be considered an advantage since it allows diagnosis and therapy with different amounts of the same radionuclide as for ^{131}I . $^{64}CuCl_2$ is a radio-pharmaceutical form used as a precursor for the *in vitro* radiolabeling of specific carriers, such as monoclonal antibodies, peptides, amino-acids, hormones and other molecules for PET imaging and/or for radionuclide therapy [2, 14, 15]. In several studies copper-64 was used in association with other therapeutic regimens in an attempt to overcome cross-resistance phenomena also dependent on Cu metabolism in tumor cells. However, cumulative toxicity has in many cases proved to be severe. In recent years, attempts have been made to develop molecular approaches able to reduce the copper therapeutic dose and resistance phenomena. Several proteins of the mTOR pathway are now known to mediate survival and chemo-radio resistance among these SGK1 [16, 17]. The serum- and glucocorticoid-regulated kinase 1 (SGK1) mediates signals of cell survival and proliferation [18, 19]. SGK1 is a serine/threonine kinase that shares structural and functional similarities with the AKT family of kinases [20]. SGK1 function is tightly dependent on mTOR phosphorylation [21]. SGK1 is regulated by insulin [22–24], IGF-1[25], glucocorticoids[26] and IL-2 [27]. Increased SGK1 expression has been documented in several human tumors[19, 28–30]. It has been demonstrated that SGK1 regulates cell survival, proliferation and differentiation via phosphorylation of MDM2, which governs p53 stability and degradation[31]. SGK1 also affects mitotic stability and miRNAs nuclear transport and maturation by regulating RANBP1 and RANGAP1, the pivotal regulators of the GTPase RAN [32, 33]. Recently, we found a family of dual SRC/ABL small molecule inhibitors, characterized by a substituted pyrazolo[3,4-d]pyrimidine scaffold, able to inhibit SGK1 and AKT kinase activity [34]. Among these molecules, SI113 has been demonstrated

to inhibit SGK1 activity, while being much less effective on AKT1, ABL and SRC[35]. SI113 induces cell death, alters the growth rate, promotes autophagy and synergizes with taxanes and radiotherapy in hepatocarcinoma and glioblastoma cells *in vitro* as well as *in vivo*, also by favoring a cellular sensibilization to these treatments[35–38]. Based on the apparent lack of toxicity and the consistent ability of SI113 to negatively modulate the chemo-radio resistance[36], in the present work we investigated the potential combined use of $^{64}CuCl_2$ and SI113 in GBMs cellular models. In our hands, in several cells with different p53 mutation status, we have been able to extrapolate the IC50 for $^{64}CuCl_2$. Moreover we determined the appropriate doses and time of exposure of SI113 and $^{64}CuCl_2$ for additive or synergical effect. We documented a potential ability for the combined treatment to affect cell viability and necro-apoptosis. In the last part of our work, finally we suggest some putative signaling pathways that may potentially explain the combined therapeutic effects observed *in vitro.*

Materials and Methods

Cell culture

Human GBM cell lines (LI)PARI, ADF and T98G were kindly provided by Dr. Marco Paggi of Regina Elena Cancer Research Institute. T98G cell line were grown in Dulbecco's modified Eagle's medium (DMEM), while ADF and LI cell lines were grown in RPMI-1640. All culture media were from Life Technologies, Inc. (Grand Island, NY). All culture media were supplemented with 10% of fetal bovine serum FBS (Sigma-Aldrich St. Louis, MO) and 1% penicillin/streptomycin (from stock solution of 10.000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich), and grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

SI113 and $^{64}CuCl_2$ treatments

SI113 was produced by one of us (Prof. Schenone). The molecule was first diluted (10 mM) in dimethyl sulfoxide (DMSO) and stored at −20°C for *in vitro* studies. The final concentration of SI113 used for viability assay was 12.5 μM. The drug was then used in association with labeled $^{64}CuCl_2$ diluted in a water solution (Sparkle S.r.l. Montecorso, Italy), at the final concentration of 5mM, corresponding to 40MBq.

Viability assay by guava "via count"

IC_{50} evaluation, cell proliferation and viability assays was performed by means of Guava ViaCount Assay.

To evaluate the dose-response effects, Lipari, ADF and T98G cell lines were seeded in 100 mmØ plates at the density of $2x10^6$ cells/well and after 24 hours were treated with increasing concentration of $^{64}CuCl_2$ (2.5, 5, 10, 20, 40 MBq) for 72 hours. In the cell count the same cell lines were seeded in 100mmØ plates at the density of $1.5x10^6$ cells/well, treated with $^{64}CuCl_2$ (40 MBq) with or without 12.5 μM of Si113 for 24-48-72 hours. Cell viability was evaluated by guava via count assay (4000-0040, Millipore) according with the manufacture 's instructions and data were analyzed by Guava system (Millipore).

Guava Nexin Assay

Apoptosis was studied in adherent LI, ADF and T98G cells. In this assay cells were treated with $^{64}CuCl_2$ (40 MBq), 12.5μM of Si113 or in association for 72 hours. 2 x 10^4 cells were incubated with 100 μl Guava Nexin Reagent (100 tests, Lot No. 14-0032, Merck Millipore), a pre-made cocktail containing V-PE and 7-AAD in buffer, in a final volume of 200 μl. After 20 min incubation at room temperature in the dark, samples were ready to be acquired on a Guava System. Four cell populations were evaluated in this assay: non-apoptotic cells AnnexinV (-) and 7-AAD (-); first apoptotic cells V (+) and 7-AAD (-); Later in apoptotic phase, annexin V (+) and 7AAD (+), necrotic/death cells Annexin V (-) and 7AAD (+). The treated cells were prepared for incubation with 1% BSA. The results include counts and percentages of cells in each of the quadrant-defined populations, as well as the mean fluorescence intensity of Annexin V and 7-AAD for each population [39, 40].

Western blot

Cells incubated with $^{64}CuCl_2$ (40 MBq) for 72 h, in the presence or absence of SI113 12.5 μM were extract with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5 % IGEPAL, 25 mM NaF,1 mM DTT, 1 mM Na_3VO_4 plus protease inhibitor cocktail 10 X Sigma-Aldrich, St. Louis, MO), separated by 12% SDS SDS-

PAGE and transferred on a nitrocellulose membrane 0.45 micron (Bio-Rad). Blocking was performed with 1X TPBS, 0.1% Tween-20 with 5% w/v nonfat dry milk. Cells treated and processed as described, were probed with: anti-MAP-LC3β(G-9) mouse monoclonal antibody (sc-376404, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), anti-p53 (DO 1) mouse monoclonal antibody (sc-126, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), anti-GAPDH(FL-335)rabbit polyclonal antibody (sc- 25778, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), Anti BECN1 (H-300) rabbit polyclonal antibody (sc-11427, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), MDM2 (N-20) rabbit polyclonal antibody (sc-813, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), p-MDM2(S166) rabbit polyclonal antibody (3521S Cell Signaling technology, Inc. USA), Caspase-3 (H-277) rabbit polyclonal antibody (sc-7148, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), Bcl-2 (N-19) rabbit polyclonal antibody (sc-492, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), CTR1 (FL-190) rabbit polyclonal antibody (sc-66847, Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

Statistical analysis

All tests were done in triplicate and experiments performed at least three times. The results are expressed as a mean ± Standard Error (SE). Differences between groups were analyzed using the Student's two-tailed *t* test (GraphPad Prism v5 software, www.graphpad.com). Asterisks denote statistical significance as indicated in the legends. The combination index (C.I.) has been calculated by means of the following equation [41]: %PCV = % Predicted Cell Viability = % Cell Viability Drug1 x % Cell Viability Drug2 x 0.01.

CI = Combination Index = % Measured Cell Viability Drug1 + Drug2 / %PCV.

Results

GBM Cell Line Characteristics

The mutation status of p53 and p21 was wild type in LI cells, whereas the gene coding for p53 presented a heterozygous G-to-A nucleotide substitution in codon 797 and a homozygous missense homozygous mutation in c.711G>A in ADF and T98G cells respectively [42, 43].

$^{64}CuCl_2$ effect on cell number and viability in Li, ADF and T98G human cancer cell lines

Li, ADF and T98G GBM cell lines were plated as indicated in the Methods section. $^{64}CuCl_2$ at different concentrations (2,5; 5; 10; 20; 40 MBq) was added for 24 h. Control cells were treated with vehicle alone. Cells were then trypsinized, and viable cells were stained and counted by means of Guava ViaCount Assay. In all three cell lines, $^{64}CuCl_2$ yielded a significant and dose-dependent reduction in the number of viable cells [Fig. 1 panel A, Li (left), panel B ADF (middle) and panel C T98G cells (right)]. The most notable effect in cell number reduction was achieved in the Li cell line, although the effect was not complete in any of the cellular lines. IC_{50} values for $^{64}CuCl_2$ (0-40 MBq, 72 hours), calculated for the 3 GBM cell lines, are listed in Table 1, and ranged from 8,85 to 37,7 MBq. In all three cell lines the 40 MBq dose was the maximum inhibitory dose. Therefore, from now on, $^{64}CuCl_2$ has been employed at the dose of 40 MBq, unless otherwise indicated.

SI113 potentiates in a time dependent manner the radiation-induced growth inhibition of 64CuCl2 in GBM cell lines

The data described up to now, point to a detrimental but non complete effect of $^{64}CuCl_2$ on GBM growth and survival. We therefore explored the possibility that SI113-dependent inhibition of SGK1 might synergize with the positrons and β-radiations of $^{64}CuCl_2$, as verified for the traditional linear RT in HCC and GBM [36, 37]. We evaluated the effect of SI113 on $^{64}CuCl_2$ radiosensitivity of LI, ADF and T98G cells 24h after plating. Cells were exposed to either no radiation (0 MBq-mock alone) or $^{64}CuCl_2$-dependent radiation (40 MBq), with or

Tab. 1. IC_{50} values for $^{64}CuCl_2$ in the GBM cell lines

$^{64}CuCl_2$ IC50 values		
Cell Lines	Activity	Concentration
Li	8,85MBq	1mM
ADF	23,6MBq	3mM
T98G	37,7MBq	4mM

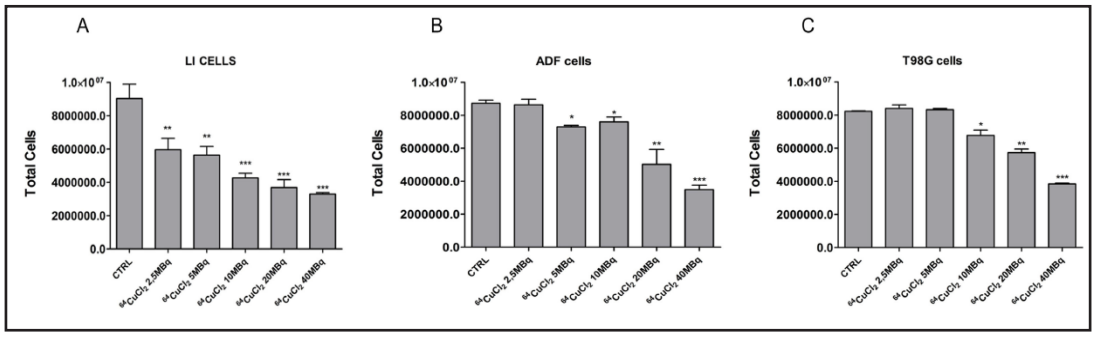

Fig. 1. Dose-response effects in LI, ADF and T98G human GBM cell lines under $^{64}CuCl_2$ treatment. A. LI human Glioblastoma cell line. B. ADF human Glioblastoma cell line. C. T98G human Glioblastoma cell line. Bar graph represents the number of cells treated with increasing concentration of $^{64}CuCl_2$ (2.5, 5, 10, 20, 40 MBq) for 72h. Data were acquired and analyzed by means of Guava ViaCount Assay. Results represent the mean ± S.E. of three independent experiments.*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

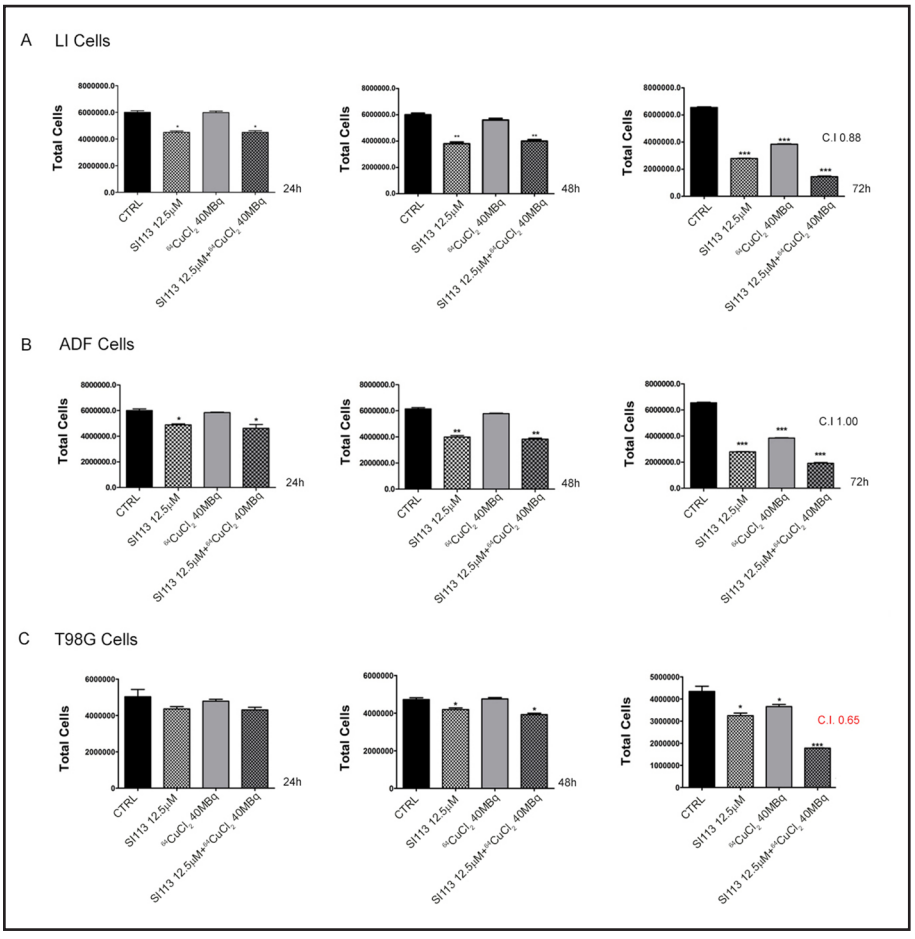

Fig. 2. Viability assay in co-treated cell lines. A. LI, B. ADF and C. T98G human GBM cell lines, treated with $^{64}CuCl_2$ (40 MBq) with or without SI113 (12.5µM) for 24-48-72 h. Results represent the mean ± S.E. of three independent experiments.*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

without the combined treatment with SI113 (12.5µM) for 24h, 48h and 72h, and assayed for cell viability by means of Guava ViaCount Assay. In each of the cell lines analyzed, SI113, as a single agent, significantly reduced the number of viable cells already at 24h with a clear-cut time-dependent kinetics, as expected. On other hand, the sole $^{64}CuCl_2$ treatment appeared effective only at 72h. When both agents were used, the combination of SI113 (12.5µM) and $^{64}CuCl_2$ (40MBq) reduced the number of viable cells significantly more than either agent alone (Fig. 2 Panel A, B, C). Interestingly, at 72 h the calculation of the combination index

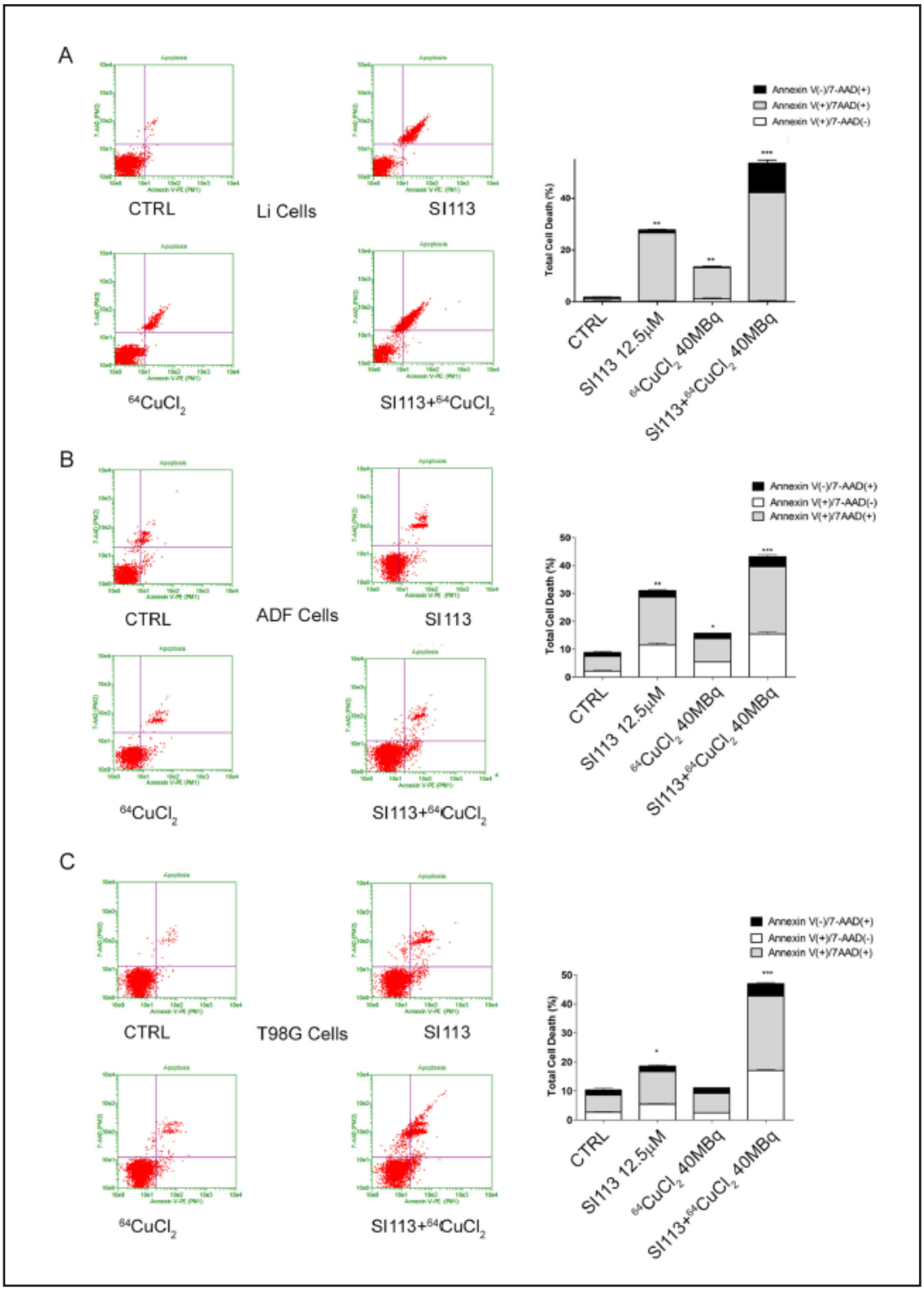

Fig. 3. Apoptosis assay in GBM cells. A.LI, B.ADF and C.T98G cells, treated with $^{64}CuCl_2$ (40 MBq) with or without SI113 (12.5μM) for 72h. The percentage of cells stained with either Annexin V, or 7-ADD, or both (calculated by means of Guava Annexin assay) is shown in the graph. Histograms depict the percentage of early apoptotic Annexin V(+)/7-AAD(−), late apoptotic Annexin V(+)/7−AAD(+) and necrotic/dead Annexin V(−)/7-AAD(+) cells. Results represent the mean ± S.E. of three independent experiments.*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

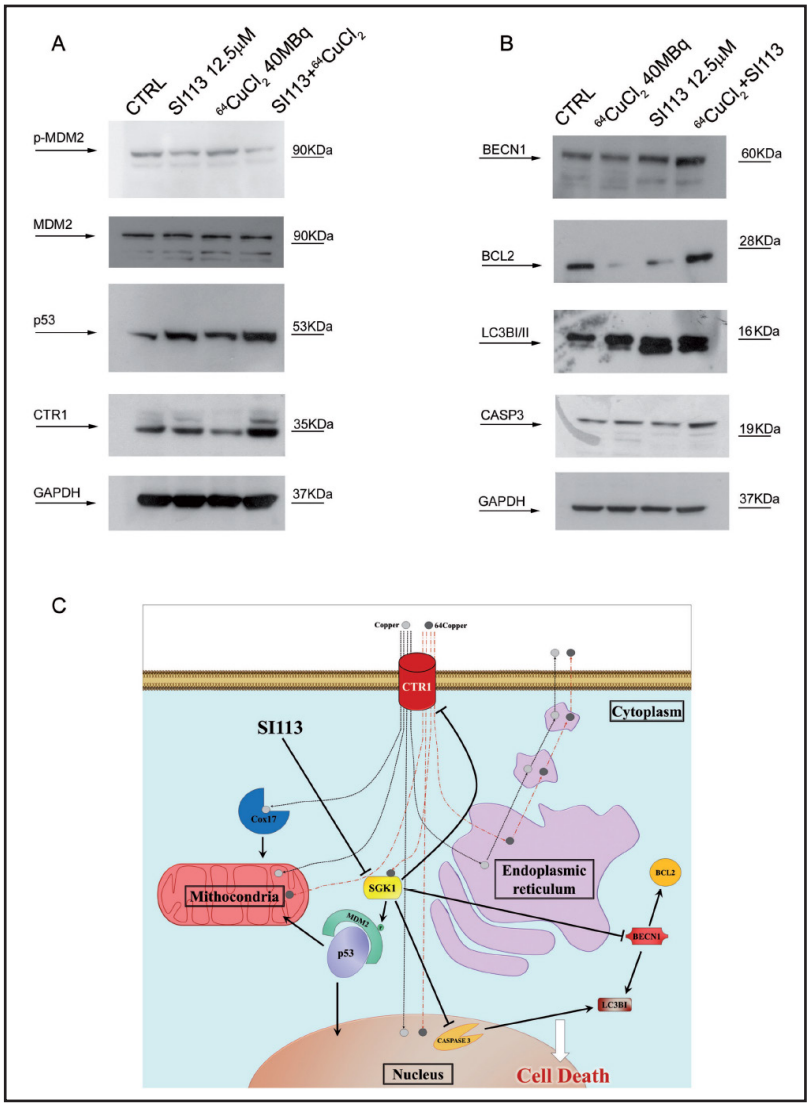

Fig. 4. Cell Signaling analysis in treated GBM cells. Immunoblot of 50 µg Li cell extracts for A. p-MDM2, MDM2, p53, CTR1 and B. BECN1, BCL-2, LC-3BI/II, CASP-3, 72 h after single or combined treatment. As loading control, GAPDH was determined in the same blots using an anti-GAPDH antibody. C. schematic Cartoon depicting the molecular effects resulting from the SI113-dependent SGK1 inhibition and concomitant $^{64}CuCl_2$ administration.

demonstrated full additivity for Li and ADF (C.I. 0.88 and 1.00 respectively) and complete synergy for the T98G a (C.I. 0.65) for SI113 and $^{64}CuCl_2$.

The SI113/$^{64}CuCl_2$ association boosts the late apoptosis and necrosis in GBM cell lines

In order to support the theranostic potential of the association between SI113 and $^{64}CuCl_2$, we carried out an apoptosis assay by means of Nexin Guava approach, that allowed the definition of early phase apoptosis, [Annexin-V (+) / 7-AAD (-)], late phase [Annexin-V (+) / 7-AAD (+)] and necrosis [Annexin-V (-) / 7-AAD (+)]. Li, ADF and T98G cells were labelled with Annexin-V-Fluorescein and 7-AAD, and the percentage of the described subpopulation was determined by flow cytofluorometry (Fig. 3, panel A,B,C left). A significant increase in late stage apoptosis (necro-apoptosis) appeared evident in all the SI113-treated GBM cell lines, as indicated (Fig. 3, panel A,B,C right, second column), whereas only in the Li and ADF cells the $^{64}CuCl_2$ treatment slightly affected the late stage (Figure 3, panel A,B,C right, third column). However, when the two agents were combined, a potent induction of late apoptosis and necrosis occurred. Interestingly, in ADF and T98G cells a substantial and significant induction of early apoptosis was also recorded (Fig. 3, panel A,B,C right, fourth column).

SI113/$^{64}CuCl_2$ and protein expression in Li, ADF and T98G cells

We used mono-dimensional SDS–polyacrylamide gel electrophoresis followed by Western blot to evaluate the expression of proteins that are SGK1 targets and/or substrates, in

the effort to explain the molecular mechanism underlying the pharmacological cooperation between $^{64}CuCl_2$ and the SI113. After treatment with the single agents or co-treatment of Li cells with SI113 (12.5μM) and/or $^{64}CuCl_2$ (40MBq) for 72 h, the expression of p-MDM2, MDM2, p53, CTR1, BECN1, BCL2, LC3BI/II, and CASP3 was examined (Fig. 4, panels A and B). In the SI113 treated samples, expression of BCL2, as well as the phosphorylation of p-MDM2 (serine 166) were reduced, consistently with previously published observations [31, 35, 44], whereas the expression of p53, BECN1 and LC3BI/II was increased, as expected [31, 40]. In the $^{64}CuCl_2$ treated cells we only recorded modifications affecting p53, BCL2 and TCR1. Surprisingly in the co-treated cells we observed an important and cooperative p-MDM2 reduction and a consequent increase in the p53 expression, in addition, we documented, for the first time, an additive effect of the co-treatment on the CTR1 expression. Interestingly when we focused on the autophagic marker, we found that SI113, not $^{64}CuCl_2$, used as a single agent, determined an important increase in the BECN1/BCL2 expression, with no modification in the expression of LC3BI/II. When both agents were used together, the effects on BECN1/BCL2 expression were still observed. However, in addition, an increase of cleaved-caspase 3 expression was documented.

Discussion

Copper is necessary for the accelerated metabolism in neoplastic cells and for the tumor angiogenesis process. High copper levels have been demonstrated in several types of human cancer, including prostate, breast and brain tumor [45]. The most important copper's transporter into cells is CTR1, a 190-amino acid protein of 28kDa, harboring three trans-membrane domains. TCR1 controls the process of internalization by determining the achievement of a copper concentration suitable for cell growth, proliferation and development. The copper homeostasis is a dynamic process determined, in several types of cells by the ligand-dependent endocytosis of CTR1 followed by its degradation [46, 47], that is required to avoid the accumulation of toxic levels of the metal. The organs showing the highest accumulation of $^{64}CuCl_2$ are brain, liver, pancreas and kidney [48]. Given its β-emission, ^{64}Cu is also suitable for therapeutic applications. This physical behavior may thus be considered an advantage, allowing diagnosis and therapy with different amounts of the same radionuclide. On the other hand the β-emission provides a radiation burden to the patient exposed to $^{64}CuCl_2$ during diagnostic procedures [49, 50]. Other studies have also documented that CTR1 and the other Cu-transporters are responsible for platinum and taxanes resistance. A down regulation of CTR1 has been associated to multidrug resistance [51]. Recently, SGK1 has been shown to be involved in taxanes and platinum resistance [32, 52]. SGK1 is also responsible for proliferative and differentiating processes and allows epigenomic regulation in the cell [27, 31, 33]. The role of Sgk1 in controlling autophagic processes in tumor systems and response to oxidative stress is also well known [40]. Recently the SGK1 inhibitor, SI113, has shown a potential role in the treatment of several cancers, showing an absence of short-term toxicity. Interestingly, SI113 has demonstrated ability to synergize with radiotherapy in hepatic tumor models and glioblastoma, acting presumably as a cell sensitizer toward β-particles [36, 39, 40].

In the present study we have determined the maximum cell killing dose of $^{64}CuCl_2$, in three cell lines of glioblastoma with different mutational status for p53. The maximum effective dose corresponded to 40 MBq in all the cell lines. However, the different kinetics of response to copper-64, provided a wide IC_{50}-range from 8.85 MBq to 37.7 MBq. Among the cell lines tested, Li cells showed the greatest slope, suggesting an higher sensitivity to copper-64, probably associated to the presence of a wild type p53 form in Li cells. Indeed the status of p53 has been widely associated to the copper-64 dependent response [8]. We therefore decided to use the maximum dose of 40 MBq in all the other cells. In a time-course experimental set, we determined that the maximum effect of response occurred at 72 h. Interestingly this timing paralleled the observed response to SI113 in previous experiments

[35, 36]. In order to test the possible co-operation between the SI113-dependent SGK1 inhibition and copper-64 administration, we carried out a co-treatment test, confirming that the combination of SI113-dependent SGK1 inhibition and copper-64 administration was more effective in inducing cell death than either agent alone. A double profile of additivity (Li and ADF) and synergy (T98G) in all three cell lines examined, regardless of the mutational state of p53 was also determined. At this point, we focused on the analysis of cell survival, in order to determine whether the SI113/Copper-64 combination could have significant effects on apoptosis even on the remaining cultured cells. For this reason, we focused on the analysis of the vitality profile, through a FACS-based approach, on cells still adherent after 72h treatment. The results were particularly interesting. Indeed, in all 72h-treated lines, the combination of the two drugs resulted in a powerful synergistic effect with a substantial increase in late apoptosis [Annexin-V (+) / 7-AAD (+)]. In Li cells, the necrotic component was also greatly increased in the double treatment [Annexin-V (-) / 7-AAD (+)], whereas in the T98G cells a clear up-regulation of the earliest phases of apoptosis were evident [Annexin-V (+) / 7-AAD (-)]. In the effort to comprehend the molecular mechanisms that could support the effects of co-operation between SI113 and Copper-64, we conducted some signaling studies, focusing on proteins, involved in survival, cellular Cu-bioavailability and radio-resistance. The first relevant data was generated by observing the behavior of p-MDM2 and p53. Both are involved in apoptotic response and cellular damage and are two well-known effectors of the SGK1-dependent response. As expected, SI113 was able to lower the phosphorylation of MDM2 on serine 166, with a consequent increase in p53 cellular abundance [31, 36]. The combination of the two treatments further amplified this response. This may explain the decrease in cell proliferation and survival, but also the highest susceptibility to copper-64, since high levels of p53 are required for a more efficient copper-64 dependent theranostic effect. We also realized that the combination of SI113 and Copper-64 caused a tremendous increase in the levels of CTR1, thus ensuring a greater bioavailability of copper-64 in the cell with possible enhancements of radio-toxicity. Indeed the down regulation of CTR1 is associated with drug resistance phenomena [51], hence its greater expression could explain the increased susceptibility towards drugs and radiations. Furthermore, in our analysis we demonstrated that the combination of the two drugs boosts autophagic-dependent cell death by increasing the levels of LC3BI and caspase 3. In our experiments, Beclin-1 was also increased as expected in presence of a strong autophagic response [38, 40]. In the same conditions, the observed increase in the expression of BCL-2 may be interpreted as an initial attempt to activate a survival response to stress. However, the increased combination of both caspase3 and LC3BI / II is the hallmark of the important cell death observed in these conditions. A schematic cartoon is provided to summarize what has been described so far. Taken together, our data suggest a possible therapeutic combination for the treatment of glioblastomas, based on a copper-dependent radiometabolic approach and the simultaneous SI113-mediated inhibition of SGK1. This first experimental effort points to define a new approach in the treatment of tumors characterized by strong chemo-radio resistance. In previous animal studies SI113 was well tolerated, showing no tissue toxicity[44]; on the other hand, liver and kidney toxicity are described during treatment with $^{64}CuCl_2$. Therefore further studies are required to determine the therapeutic effects and toxicity *in vivo* of the combined treatment.

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Disclosure Statement

The authors declare no commercial or financial conflict of interest.

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