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Targeting reactive oxygen species to overcome cisplatin resistance in lung cancer

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Abstract

Cisplatin resistance remains a major problem in the treatment of both small cell and non small cell lung cancer (NSCLC). We have discovered that cisplatin resistant (CR) cells share one common biochemical parameter, increased reactive oxygen species (ROS), when compared to normal cells and their parental cells counterparts. Treating CR cells with either a ROS inducing agent (elesclomol), or an antioxidant agent (*N-acetylcysteine*) resulted in a significant growth inhibitory effect. This suggested that ROS levels were an important component in the survival of these cells. Co-treatment of cisplatin with NAC however resulted in and antagonistic effect, while elesclomol in combination with cisplatin treatment enhanced cisplatin sensitivity in CR cells. Our findings suggest a novel approach to overcome CR in lung tumors. Combining a ROS inducing agent with cisplatin may prove to be clinically efficacious.

Keywords: *lung cancer, cisplatin resistance, reactive oxygen species*

1. Introduction

Although lung cancer is one of the leading causes of death in the world, few new drugs have been approved by the FDA in the past 5 years for the treatment of this deadly disease. Thus far, cisplatin remains a key agent for the treatment of both non small cell lung cancer (*NSCLC)* and small cell lung cancer (*SCLC*). The majority of lung cancer patients initially respond to cisplatin treatment; however, the cancer cells can develop resistance rapidly to this drug causing therapeutic failure, hence understanding mechanisms of cisplatin resistance may lead to increased survival. Our work will not only represent a new avenue for overcoming cisplatin resistance, but also assist in the future selection of patients who will most likely benefit from this treatment.

We are primarily study resistance mechanisms in cancer, and in particular resistance to cisplatin. Cisplatin resistance (CR) involves multiple molecular mechanisms, including decreased drug accumulation, enhanced DNA repair, and upregulation of the pro-survival PI3K/AKT pathway (Siddik, 2003). We have shown that improved cisplatin sensitivity can be achieved in resistant cells by inhibiting the mammalian target of rapamycin (mTOR), a major translational regulator of growth and anti-apoptotic proteins (Wu et al., 2005). However, not all CR cells are sensitive to the cytotoxicity induced by mTOR inhibition. This insensitivity is attributed to variant signaling transduction and active autophagy. In an effort to find better therapeutic approaches, we have made a unique and important discovery in that all our CR cell lines, regardless of signaling or sensitivity to mTOR inhibitor, possess high basal levels of ROS (Reactive Oxygen Species) when compared to their parental counterparts as well as normal cells (Wangpaichitr et al., 2009). Thus, since CR cells already possess higher basal levels of ROS, further heightening of ROS by using a ROS inducing agent (elesclomol) can push the cells beyond their tolerance limit and ultimately lead to cell death (Wangpaichitr et al., 2012; Wangpaichitr et al., 2009).

Elesclomol is a small molecule known to exert its antitumor activity by increasing intracellular ROS through mitochondria (Toogood, 2008) by chelating Cu outside the cells. This elesclomol-Cu(II) complex enters the cells and generates ROS via redox cycling of Cu(II) to Cu(I) (Nagai, Vho, Cu, & Wada, 2011). Phase I and II studies of elesclomol are ongoing in metastatic melanoma, and thus far treatment has been very well tolerated (Berkenblit et al., 2007; O'Day et al., 2009; Tuma, 2008). In this brief communication we will address the role of ROS in the survival of cis-platinum resistant lung cancer cell lines.

2. Methods

2.1 Cell lines and reagents

Since it is well known that small cell lung cancer (SCLC) has a high propensity to metastasize to the bone marrow, the SCLC1 cell line was derived from the bone marrow of a SCLC patient. Tumor tissue was aspiration into heparinized tubes with mononuclear cells separated by ficoll-hypaque gradient centrifugation, and contaminated red blood cells were lysed with 1% ammonium oxalate. Tumor cells were visualized under the microscope, and were utilizing the serum-free media to deplete the culture of normal bone marrow cells and fibroblasts. SCLC grew in floating aggregates, which further facilitated fibroblasts separation. This cell line was further verified by the presence of the neuroendocrine markers, Chromogranin A (CgA) and Neuron-specific enolase (NSE), which should be presence in small cell lung cancer but not in the bone marrow cells (Carney et al., 1985; Feun, Savaraj, Solomon, Liebmann, & Hurley, 1996; Savaraj et al., 1997). SR2 is the cisplatin resistant variant derived from SCLC1 cell line which was generated by intermittent exposure to cisplatin. S is a non small cell lung cancer cell line that was established from metastatic adenocarcinoma to the brain (Carney et al., 1985; Savaraj et al., 1997). SC is a cisplatin resistant variant derived from S cell line by intermittent exposure to cisplatin. Elesclomol was kindly provided by Synta Pharmaceuticals. NAC (*N-acetyl-cysteine)* was purchased from Sigma.

2.2 Development of cisplatin resistance

Parental cells (SCLC1 or S) were seeded ($4x10^4$) in 6 well plates and treated with cisplatin for 24h with the ID_{50} concentration. The culture was observed daily and allowed to grow until they reach the initial density. After the cells recover from cisplatin toxicity, they were treated again with increasing dosage of cisplatin for 24 hr. These cells were seeded at 500-1000 cells per dish and culture for 5 -7 days. 3 to 4 clones were selected and expanded as well as tested for cisplatin sensitivity. Similar process of exposure was carried out for a third and fourth times to generate higher resistant clones. These clones were tested for cisplatin resistance. The resistance clone was maintained in half of ID_{50} dosage concentration (i.e. 10 fold resistant will be maintained in 5µg/ml of cisplatin).

2.3 Assay of mitochondrial ROS

Cells were collected and incubated with 5 µM of MitoSox (Invitrogen) at 37 °C for 30 min in the dark. Then the cells were washed once with PBS and centrifuged to remove impermeable reagents. Cells were resuspended in 500 µL of PBS and analyzed in Accuri Flow Cytometer (excitation at 544 nm and emission at 590 nm) for the mitochondrial ROS.

2.4 Assay of Intracellular ROS

As previously described (Wangpaichitr et al., 2009), cells were collected and intracellular H_2O_2 was measured by incubating with 10 µM of acetyl-penta-fluorobenzenesulfonyl fluorescein (APFB) (EMD) at 37 °C for 30 min in the dark. Then the cells were washed once with PBS and centrifuged to remove impermeable reagents. Cells were resuspended in 500 µL of PBS and analyzed in a fluorometer, FLUOstar OPTIMA, BMG Labtech (excitation at 485 nm and emission at 520 nm).

2.5 Growth inhibition and cytotoxicity assay by cell counter

Cells were seeded in 24-well dishes and treated with various concentrations of elesclomol alone or in combination with NAC for 72hrs. The culture medium as well as the trypsinized cells were collected and centrifuged at 400 x *g* for 5 min. The supernatant was discarded and resuspended in 1 mL of Hank's buffer and assayed for live and dead cells using a Vi-Cell cell viability analyzer (Beckman Coulter, Inc.) (Wangpaichitr et al., 2008).

2.6 Caspase activity and cell death assay

Caspase activity was analyzed with caspase fluorescein-conjugated V-D-FMK (R&D systems). Briefly, cells were treated with various concentrations of elesclomol and/or cisplatin. At 48 hr, cells were harvested, centrifuged, and the pellet was resuspended in 100 µl of staining solution (20µl of V-D-FMK fluorescein in 1ml of PBS). The suspension was incubated at 37°C for 30 min. After the staining period, cells were washed to remove unbound reagent, and resuspended in 500 µl of PBS and analyzed in a Coulter XL Flow Cytometer. A minimum of 10,000 cells were analyzed to generate caspase histograms.

2.7 Statistical analysis

All statistical analyses were performed from three separate measurements using the two-tailed *t-test* and the results were expressed as mean ±

standard deviation. A p-value of less than 0.05 was considered as statistically significant.

3. Results

3.1 Cisplatin resistant cells possess higher basal levels of mitochondrial ROS

We have previously demonstrated that CR lung cancer cells have increased basal ROS as compared to their parental cell and normal cell (Wangpaichitr et al., 2009). To further explore ROS levels inside the mitochondria, we assayed for mitochondrial ROS production using MitoSOX (Figure 1). Our data clearly indicate that CR cell lines possess intrinsically higher basal mitochondrial ROS levels than their parental cell counterparts.

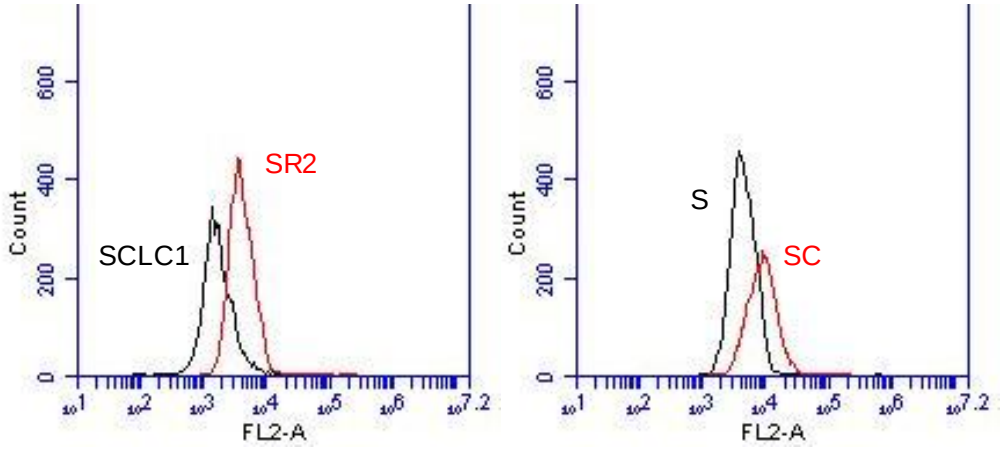

Figure 1 Cisplatin resistant cells possess higher basal levels of mitochondrial ROS. MitoSOX was used to measure and quantify mitochondrial ROS levels. Each histogram is representative of three experiments (average $P<0.05$, parental vs. resistant).

3.2 Manipulation of ROS level is cytotoxic to cisplatin resistant cells

ROS can promote cellular proliferation and inhibit apoptosis irrespective of the triggering agents (Clement & Pervaiz, 1999; Pervaiz & Clement, 2004). Moreover, ROS is known to facilitate the activation of receptor tyrosine kinase signaling as well as PI3K/AKT. These proteins play a vital role in cell growth/proliferation, survival and motility processes (Pan & Ho, 2008; Wang et al., 2007). Thus, we investigated the possibility that CR cells may require ROS for survival. To test this notion, we treated both parental and CR cells with *N-acetylcysteine* (NAC), a known antioxidant agent that should decrease ROS levels. NAC alone suppressed ROS and resulted in significant growth inhibition only in CR cells (Figure 2A and B). This supports the dependence of CR cells on ROS for growth and proliferation. Moreover, since CR cells possess higher basal levels of ROS than parental cells, CR cells maybe more vulnerable to increased oxidative stress. This concept has been previously reported by Trachootham et al. in CML cells (Trachootham et al., 2006). This group showed that by further heightening ROS; one can push the cells beyond their tolerance limit and ultimately lead to cell death. We tested this concept by generating ROS with elesclomol in our cell lines (Kirshner et al., 2008). We found that elesclomol induced significant cytotoxicity in all CR cells tested. The ID_{50} was 5-10 fold lower in resistant cells than parental cells (Figure 2C). Taken together, our data suggest that ROS levels are an important component in survival of CR cells and manipulation of ROS can selectively eliminate cisplatin resistant lung cancer cells.

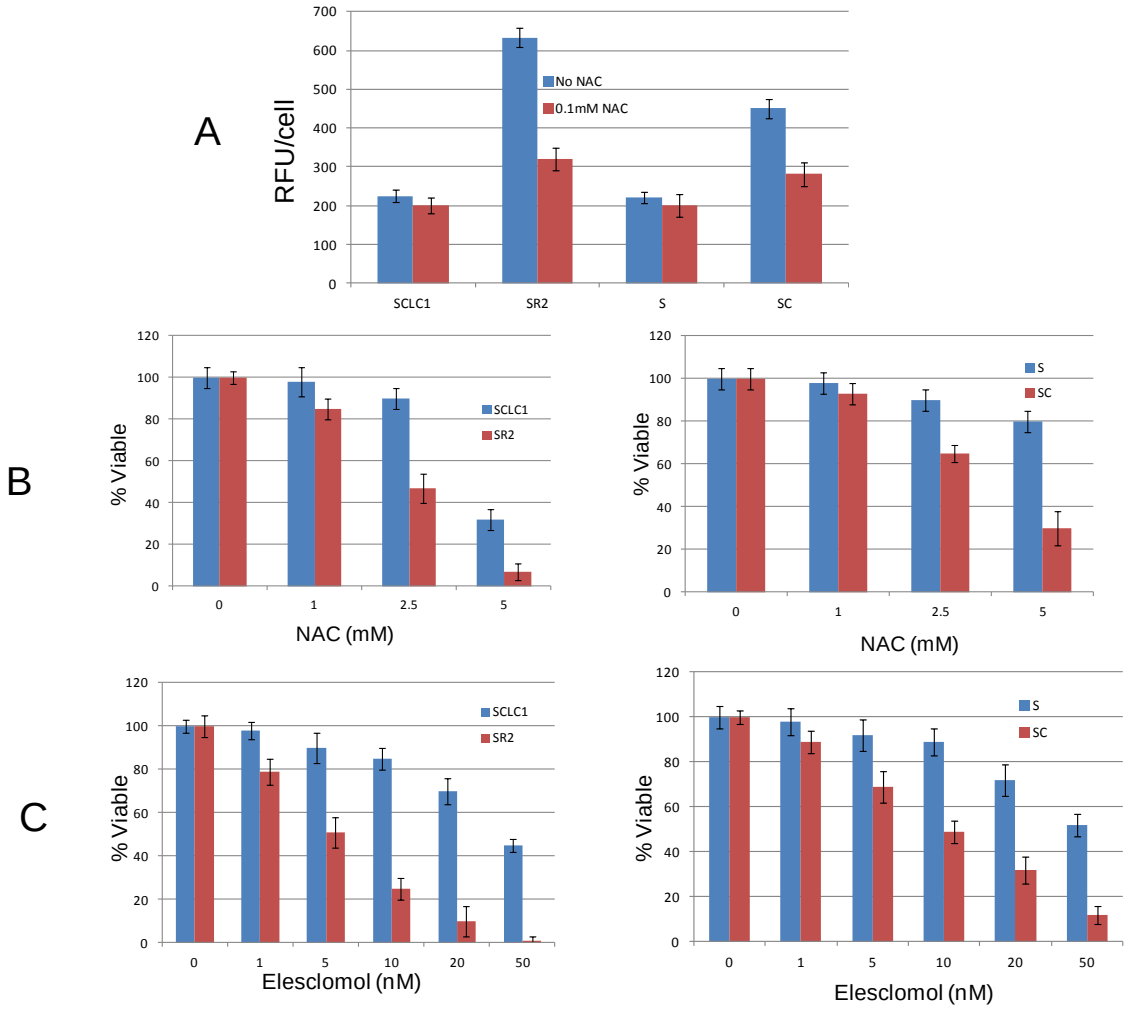

Figure 2 ROS levels are an important component in survival of CR cells .(A) Fluorometer analysis of ROS in various lung cancer cell lines detected by APFB probe indicated that NAC is more active toward CR cells. (B and C) Growth inhibitory effect of NAC or elesclomol in lung cancer cell lines (72 h). The data indicated that both agents were highly potent toward CR cells. (Mean SD of three experiments)

3.3 Elesclomol restores cisplatin sensitivity in CR cell lines.

Since elesclomol alone can inhibit cell growth, we further investigated whether addition of cisplatin can enhance cell death in CR lung cancer cells. We have treated SR2 and SC with low doses of elesclomol at 2.5nM and 5nM, respectively. At these dosages, elesclomol yielded less than 20% of growth inhibition (Figure 3B and D). We then treated CR cells with cisplatin in combination with elesclomol. As expected, significant growth inhibitor effect was observed. The addition of elesclomol reversed the ID_{50} of cisplatin resistant cells from 2.5 µg/ml to 0.1 µg/ml in SR2 (25 fold decrease), and from 3.7 µg/ml to 0.6 µg/ml in SC (7 fold decrease), (Please see Table 1 for the ID_{50} dosages of cisplatin). Moreover, addition of elesclomol did not augment the affect in the parental cell lines.

We then determined whether exposure of elesclomol can increase apoptosis when combined with a low dose (0.25ug/ml) of cisplatin (Figure 4). The apoptotic effect (as indicated by caspase activity) increased from 20% to 42% in CR cell lines, while only increasing by 2% in parental cells. Our data clearly demonstrate that elesclomol is not only more cytotoxic for cisplatin resistant cells, but that treatment also increased cisplatin toxicity in CR cell lines.

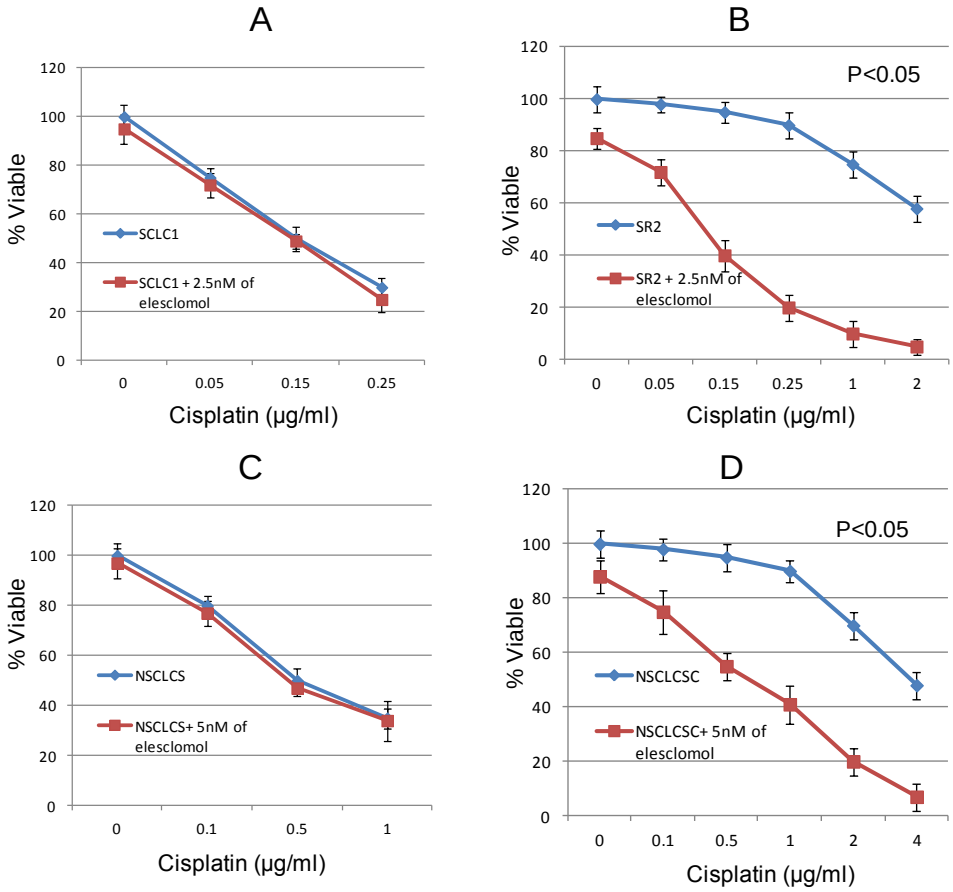

Figure 3 Elesclomol overcomes cisplatin resistance in CR cells. Growth inhibitory effect of cisplatin in combination with elesclomol in lung cancer cell lines (72 h). (A and C) parental cells were treated with cisplatin alone or in combination with elesclomol. (B and D) CR cells were treated with cisplatin alone or in combination with elesclomol. (Mean SD of three experiments)

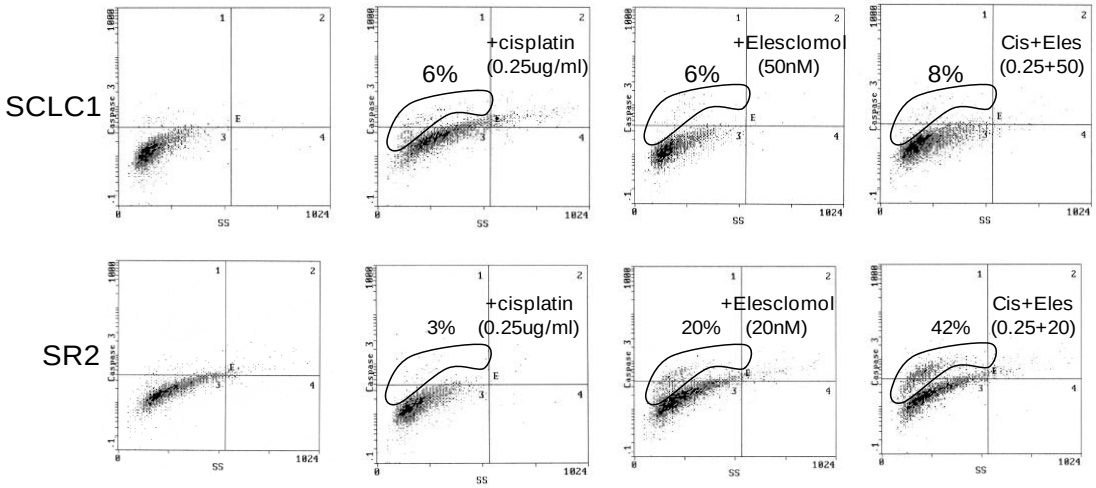

Figure 4 Elesclomol stimulates greater caspase activity in cisplatin resistant cells when combined with cisplatin. SCLC1 and SR2 were treated with cisplatin alone or elesclomol alone and in combination for 48h followed by caspase activity probe FMK-Fluoroscein. Note: The percentage of caspase activation is correlated with percentage of cell death in both SCLC1 and SR2 (data not shown). Each dot plot are representative of two experiments.

Table 1 ID50 dosages of cisplatin in SCLC1 vs. SR2 and S vs. SC

SCLC1	0.15±0.02
SR2	2.5±0.10
S	0.5±0.05
SC	3.7±0.35

3.4 NAC combination does not restore cisplatin sensitivity in CR cell lines

As shown above, NAC has anti-tumor activity in cisplatin resistant cells by significantly suppressing ROS expression. Hence, we tested whether lowering ROS using NAC can increase cisplatin sensitivity in CR cells. We treated cisplatin resistant cells with NAC in combination with cisplatin for 72 hrs to assess whether inhibition of ROS can hyper-sensitize CR cells to cisplatin (Figure 5). While NAC exposure alone elicited a growth inhibitory effect, co-treatment of NAC and cisplatin did not restore sensitivity to cisplatin. Instead, this combination treatment resulted in an antagonistic effect. These results indicate that combining cisplatin with an antioxidant agent may not to be a potent way of killing cisplatin resistant cells.

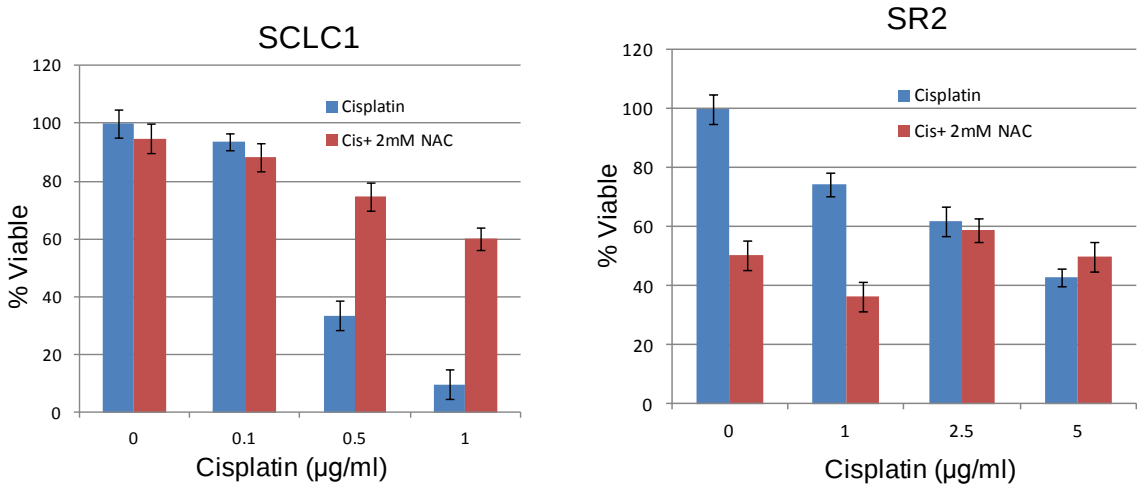

Figure 5 NAC combination does not overcome cisplatin resistance in CR cells. Growth inhibitory effect of cisplatin in combination with NAC for 72 hrs. (Mean SD of three experiments)

4. Discussion

It is suggested that elevated ROS from chemotherapy treatment is one of the hallmark in cancer cells and may be inherited during the carcinogenic process, and promote proliferation. We have found that in tumor cells, selected for cisplatin resistance by repeated exposure, the basal levels of ROS amplified (Wangpaichitr et al., 2009). Since cisplatin as well as other chemotherapeutic agents are known to generate ROS (Arner et al., 2001; Sasada et al., 1999; Witte, Anestal, Jerremalm, Ehrsson, & Arner, 2005), we hypothesized that in order to survive the repeated assault by cisplatin, certain cells adjust to survive in high ROS levels. Thus, ROS can be used as a selective target for future treatment of cisplatin resistant cells.

We and others have shown that excessive accumulation of ROS can lead to cell death in chronic myeloid leukemia (CML) cells (Trachootham et al., 2006; Wangpaichitr et al., 2009). This is due to the fact that these cells already possess higher basal levels of ROS and by further heightening ROS, these cells are pushed beyond their tolerance limit and ultimately die.

In this communication, we have shown that increasing ROS using the ROS generating agent, elesclomol, can selectively kill cisplatin resistant cells. Importantly, the addition of elesclomol also greatly enhanced the cytotoxic effect of cisplatin toward the resistant cell lines. It is however unclear why elesclomol can augment the cisplatin toxicity in the resistant cells. We have previously shown that

the copper transporter (hCTR1) which is known to be regulated by the redox system can also transport cisplatin into the cells (Kuo, Chen, Song, Savaraj, & Ishikawa, 2007; Song et al., 2004). Therefore, it is possible that induction of ROS by elesclomol may alter cellular redox staging, resulting in greater cisplatin uptake. These underlining mechanisms are currently under investigation.

Treating cisplatin resistant cells with NAC alone suppressed ROS and resulted in 50% growth inhibition. This result supports the idea that CR cells required ROS for growth and proliferation. However, co-treatment with NAC and cisplatin resulted in an antagonistic effect. This is plausible, since one of cisplatin's cytotoxic mechanisms is through ROS generation. Thus, by suppressing ROS using an antioxidant, the cytotoxic effect of cisplatin is attenuated. Moreover, we did not observe a significant growth inhibitory effect when treating parental cells with NAC. Combination treatment also antagonized the cytotoxic effect of cisplatin in the parental cells.

5. Conclusion

Lung carcinoma is one of the leading causes of cancer deaths in the world and is the second common cancer after liver cancer in Thailand. Interestingly, the data from tumor registry also showed that lung cancer is most prevalent in the Northern part of the country i.e. Chiang mai (Vatanasapt, Sriamporn, & Vatanasapt, 2002). National Statistical Office indicated that the number of regular smokers now stands at 10.6 million, representing 22.5% of the population (National Statistic Office of Thailand, 2004).

Treatment for early stage lung cancer is surgery but most patients have locally advanced or metastatic disease at the time of diagnosis. For these patients, chemotherapy or chemotherapy with radiation therapy remains the major treatment. Cisplatin or its less nephrotoxic analog carboplatin is incorporated in all the first line chemotherapeutic regimens used for both small cell lung cancer and non small cell lung cancer. Cisplatin causes DNA damage which reflects its therapeutic cytotoxic properties. This drug affects tumor cells more than normal cells due to the fact that tumor cells are rapidly dividing and constantly synthesizing new DNA. It has also been shown that heavy smoking stimulates DNA repair capacity in response to DNA damage caused by cisplatin, hence further leading to the development of resistance to these chemotherapy regimens. Moreover, the majority of lung cancer patients initially will respond to cisplatin treatment; however, the cancer cells can develop resistance rapidly to this drug causing therapeutic failure.

Our findings suggest another novel approach to selectively kill cisplatin resistant lung tumors which intrinsically produce higher ROS. The knowledge gained from this work can be used as a platform to investigate other tumor types that are resistant to cisplatin.

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