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Full Length Research Paper

In vitro interaction of cyclooxygenase-2 inhibitors with radiation in nasopharyngeal carcinoma cell line: Dependence on sequence of administration

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In nasopharyngeal carcinoma (NPC), COX-2 inhibitors have been reported to exhibit dose-dependent growth inhibition of these cancer cells, but their interaction with radiation has not yet been studied. Here, we examined the effects of selective COX-2 inhibitors SC-236 and NS-398 on the radiosensitivity of NPC cells. When incubated with COX-2 inhibitors prior to irradiation, increased radiation cell kill was documented. However, when the sequence of COX-2 administration was reversed, we observed radioprotection instead. Our findings hereby confirmed the enhancement effect of COX-2 inhibitors on radiation cell kill *in vitro*, but this effect is critically dependent on their sequence of administration.

Key words: Cyclooxygenase-2, inhibitors, nasopharyngeal carcinoma, radiosensitization, radioprotection

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is ranked the seventh most common cancer among males in Singapore. The primary treatment modality of NPC is radiotherapy and despite advances in the delivery of radiation treatment, up to 30% of patients with locally advanced disease still develop local failure (Lin et al., 2004). Hence, strategies, including the use of concurrent chemoradiation and targeted biologic agents are being employed in a bid to improve the efficacy of radiation in the treatment of local tumour (Chua et al., 2002; Ma et al., 2008).

Cyclooxygenase-2 (COX-2) is a 68 kDa enzyme that converts arachidonic acid to prostaglandins. Potentiation of radiation effects by inhibition of this enzyme has been described in several tumour types (Nakata et al., 2004). Mechanisms for this increase in radiosensitivity by COX-2 inhibition have not been fully understood, but recent experimental studies seemed to indicate the involvement of both COX-2 dependent and independent pathways (Raju et al., 2005; Dittmann et al., 2008). On a further note, it has been reported in human glioma cells that this radio enhancement effect is dependent on the sequence of inhibition such that when they were administered after irradiation, COX-2 inhibitors conferred a radioprotective effect instead (Kuipers et al., (2007). Therefore, the aim of our study was to confirm the radiosensitization effect of COX-2 inhibitors with radiation in endemic NPC and also investigate if there was an effect on radiosensitization with alternative sequencing of administration.

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Abbreviations: (NPC); Nasopharyngeal carcinoma, **(COX-2)**; Cyclooxygenase-2, **(DMSO);** Dimethyl sulfoxide, **(SF);** Surviving fraction.

MATERIALS AND METHODS

Cell lines and culture

Human NPC cell lines CNE-1, CNE-2, C666-1 (gifts from Professor Lo Kwok-Wai, The Chinese University of Hong Kong) were studied. CNE-1 and CNE-2 were established from Epstein-Barr vi-

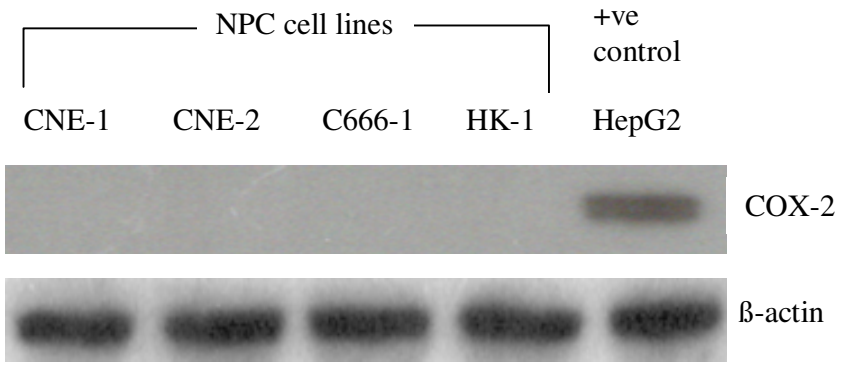

Figure 1. COX-2 protein expression in nasopharyngeal carcinoma cell lines using western blotting. HepG2: human hepatocellular carcinoma as positive control; ß-actin as internal control.

virus negative NPC, whereas C666-1, which consistently contained Epstein-Barr virus in long term cultures, was established from NPC of the endemic sub-type. Cells were grown as monolayers in 75 cm^2 flasks supplemented with Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin and incubated in a humidified 5% CO_2/95% air atmosphere at 37ºC.

COX-2 inhibition

The selective COX-2 inhibitors N-(2cyclohexyloxy-4-nitro phenyl)-methanesulfonamide (NS-398) (Sigma-Aldrich, Singapore) and 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene sulfonamide (SC-236) (Alexis Biochemicals, San Diego, CA) were used in the experiments. The inhibitors were constituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Singapore) and stored at -20ºC.

Western blotting

Whole cell lysates were prepared using the BCA protein assay kit (Pierce biotechnology, Rockford, IL), separated by electrophoresis in polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with non-fat milk, the membranes were then incubated with anti-human COX-2 antibody (Cayman Chemical, Ann Arbor, MI) overnight at 4℃. They were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies for an hour. The COX-2 bands were visualized with an enhanced chemiluminescent detection system (Amersham Bio-sciences, Buckinghamshire, UK), using beta-actin as an internal control for verification of equal protein loading and HepG2 as a positive control for COX-2 protein expression.

Clonogenic cell survival determination

To determine the cytotoxicity of the COX-2 inhibitors, specific numbers of cells were plated onto 6-well plates in triplicates and exposed to different concentrations of NS-398, SC-236 or DMSO (0.25% for SC-236; 0.2% for NS-398) for 24 h. Cells were then allowed to form colonies for10 days. Colonies of 50 cells or more were counted and surviving fractions were calculated by dividing the plating efficiency of the COX-2 inhibitor treated cells by the plating efficiency of the vehicle treated cells.

For the combination of COX-2 inhibitors with radiation, similarly, cells were exposed to SC-236 (50 uM), NS-398 (80 uM) or DMSO (0.25% for SC-236; 0.2% for NS-398) for 24 h before or after irradiation. Single doses of gamma-radiation were applied using a ^{60}Cobalt source (Gammacell 40 extractor, MDS Nordion) at a dose rate of 1.16 Gy/min. After treatment, cells were incubated for the next 10 days to form colonies and colonies of 50 cells or more were counted. Radiation survival curves were then plotted after normalizing for the cytotoxicity induced by the COX-2 inhibitors alone.

RESULTS AND DISCUSSION

COX-2 protein expression

On western blotting, all the cell lines tested did not have detectable levels of COX-2 expression (Figure 1). As CNE-1 exhibited the most competent clonogenic forming ability among the cell lines, we decided to use this cell line for subsequent experiments.

Cytotoxicity of SC-236 and NS-398

To validate the findings by Chan et al. (2005) [h], we tested the cytotoxicity of COX-2 inhibitors alone on CNE-1. Both COX-2 inhibitors tested demonstrated a dose-depedent inhibition of CNE-1 cell growth. SC-236 was a more potent inhibitor, with a dose of 50 uM (Figure 2A) obtaining 50% of growth inhibition. For NS-398, a higher dose of 80 uM was required to obtain comparable growth inhibition (Figure 2B).

Interaction of SC-236, NS-398 with radiation

SC-236 when incubated with CNE-1 24 h before irradia-

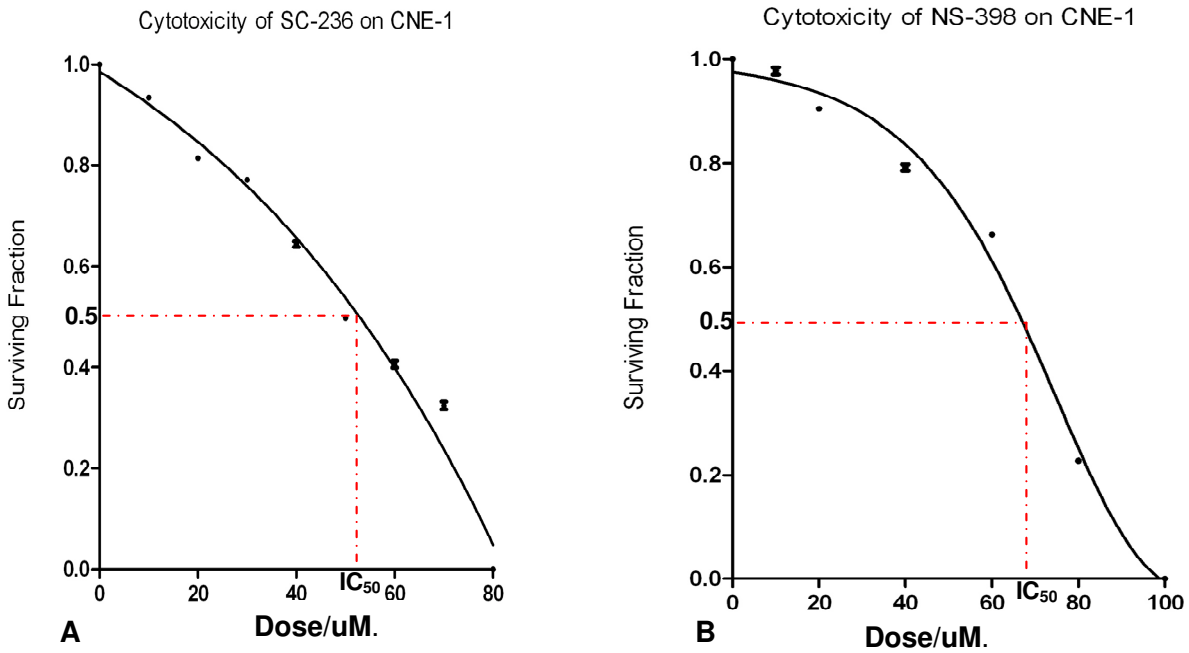

Figure 2. Cytotoxicity of SC-236 (A) and NS-398 (B) on CNE-1 cell line. Error bars represent standard error of mean for 3 independent experiments. Based on the results, we determined IC_{50} of SC-236 on CNE-1 to be 50uM. 80uM of NS-398 was chosen for subsequent experiments so as for ease of preparation with drug vehicle (DMSO).

tion at a dose of 50 uM demonstrated a clear radiosensitization effect. Surviving fraction (SF) at 2 Gy was 0.55 (Control) versus 0.37 (SC-236), representing a radio enhancement ratio of 1.49. Conversely, when the sequence of SC-236 administration was reversed, we did not observe similar radiosensitization effect but instead, there was decreased radiation cell kill with SF_{2Gy} being 0.55 (Control) versus 0.68 (SC-236), radio enhancement ratio of 0.81 (Figure 3A, $p < 0.001$).

When repeated with NS-398 given at a dose of 80 uM, similar findings were observed. NS-398 adminis-tered 24 h before irradiation demonstrated a radio enhancement ratio of 1.48 (SF_{2Gy} of 0.59 (Control) versus 0.40 (NS-398)) and when given after irradiation, the radio enhancement ratio was 0.77 (SF_{2Gy} of 0.59 (Control) versus 0.77 (NS-398)) (Figure 3B, $p < 0.001$).

DISCUSSION

COX-2 inhibition had been shown to radiosensitize several tumour cells in both *in vitro* and *in vivo* models. In NPC cell line CNE-1, we have demonstrated a radiosensitization effect, which however, is dependent on the sequence of administration of COX-2 inhibitors with radiation, similar to the findings of Kuipers et al. (2007) in glioma cell lines.

Similar to that study, we also observed radioprotection when the COX-2 inhibitors were added after irradiation and to the best of our knowledge, our study is the only one so far to confirm their findings. Kuipers et al. (2007) postulated the radioprotective effect to prolonged cell cycle arrest by the COX-2 inhibitors, thus allowing for more DNA repair to occur. We have obtained preliminary data, using microarray analysis, that addition of COX-2 inhibitors after irradiation may result in the activation of STAT1 gene, a known marker for radioresistance in head and neck cancers and renal cell carcinomas (Khodarev et al., 2004;Hui et al., 2009).

Radiomodulators were often thought to only exhibit specific desired properties in one direction. When COX-2 inhibitors were first studied, they were thought to only exhibit radio enhancing properties. However, both the studies by Kuipers et al. (2007) and ourselves have indicated otherwise. Taken together, there is clearly a need to obtain more understanding of the mechanistic interactions of COX-2 inhibitors and radiation before implementing their use in clinical practice.

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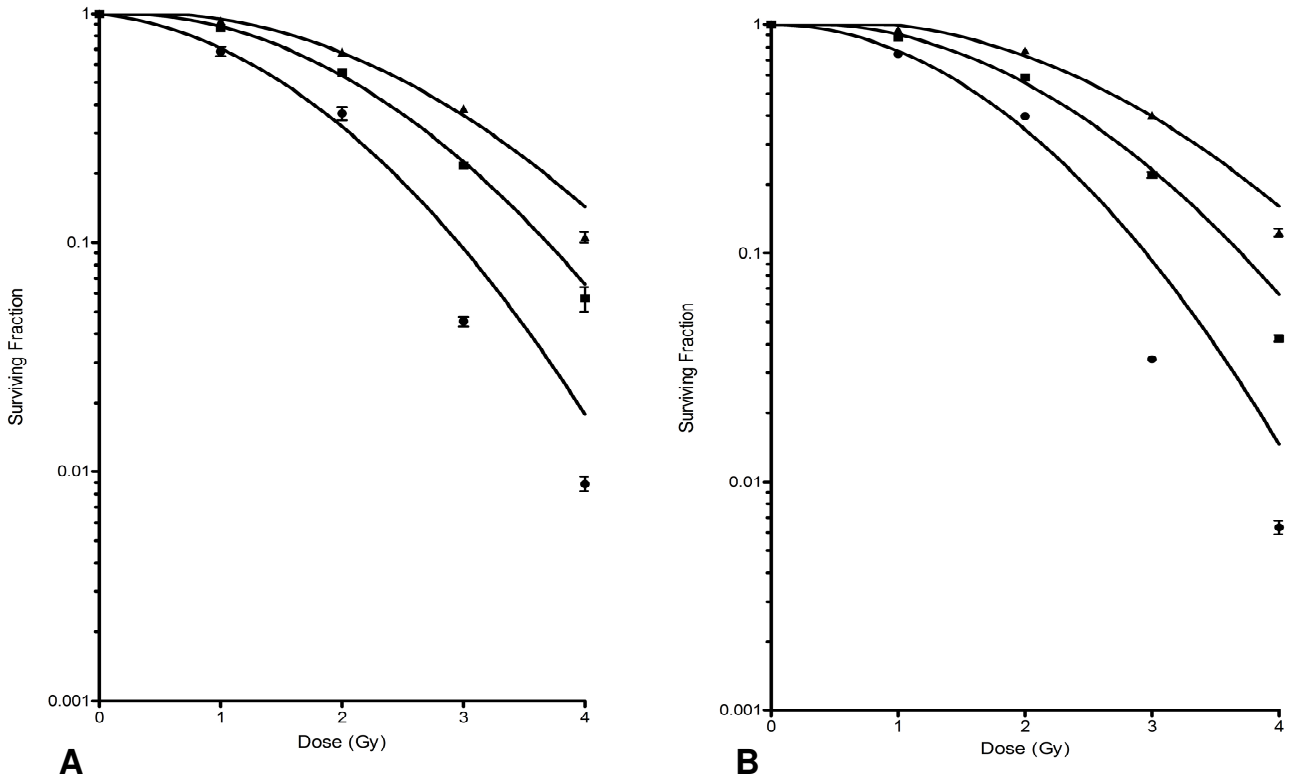

Figure 3. Cell survival curves of CNE-1 following incubation with SC-236.
Surviving fractions of CNE-1 were obtained after irradiation at various doses (0-4Gy) following exposure to 50 uM of SC-236 (A); 80 uM of NS-398 (B) 24 h before irradiation (●; 24 h after irradiation (▲) or vehicle alone (0.25% DMSO,(■). Error bars represent standard error of mean for 3 independent experiments, cell survival curves were fitted based on the linear quadratic model. Differences between survival curves were analyzed using Statistical Package for Social Sciences (SPSS, Chicago, Illinois, USA) statistical software.

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