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Inhibition of COX-2 and activation of peroxisome proliferator-activated receptor γ synergistically inhibits proliferation and induces apoptosis of human pancreatic carcinoma cells

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ABSTRACT

Although inhibition of cyclooxygenase-2 (COX-2) or activation of peroxisome proliferators-activated receptor γ (PPAR-γ) leads to growth inhibition in malignancies, the synergistic anti-tumor effects of combination of COX-2 inhibitor (NS-398) and PPAR-γ agonist (rosiglitazone) on the human pancreatic cancer cells remains unknown. Here, we evaluated the effects of NS-398 and/or rosiglitazone on the cell proliferation and apoptosis in a pancreatic cancer cell line, SW1990. NS-398 and rosiglitazone decreased cell proliferation in a dose- and time-dependent manner. Proliferating cell nuclear antigen (PCNA) labeling index significantly decreased in the cells treated with either NS-398 or rosiglitazone. Both NS-398 and rosiglitazone alone induced apoptotic cell death of SW1990. The combination of NS-398 and rosiglitazone exerted synergistic effects on proliferation inhibition, and apoptosis induction in SW1990 cells, with down-regulation of Bcl-2 and up-regulation of Bax expression. Our results indicate that simultaneous targeting of COX-2 and PPAR-γ inhibits pancreatic cancer development more effectively than targeting each molecule alone.

1. Introduction

Pancreatic carcinoma has been a significant health problem worldwide because of its poor prognosis and increasing incidence [1,2]. At the time of diagnosis, over 80% of the patients have advanced regional disease or distant metastasis. Therefore, curative resections cannot be considered for the majority of patients with pancreatic carcinoma. Despite multiple clinical trials using a large panel of chemotherapeutic regimens, the prognosis of advanced pancreatic carcinoma has not significantly improved [2]. Thus, it is necessary to identify more effective targets/receptors for treatment of pancreatic carcinomas.

Epidemiological studies have shown that the regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) decreases the risk of colon cancer [3,4]. NSAIDs inhibit cyclooxygenase (COX) which is key enzyme in the conversion of arachidonic acid to prostaglandins (PGs) [4]. Two isoforms of COX have been identified: constitutively expressed COX-1 and mitogen-inducible COX-2 [5]. Previous reports have shown that COX-2 mRNA and protein expression are increased in human pancreatic carcinomas and

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that high level of COX-2 is associated with increase resistant of neoplastic cells to apoptosis [6–9]. In preclinical models, selective and non-selective COX-2 inhibitors can block pancreatic cancer cell proliferation and promote apoptosis [10,11]. It has been accepted that anticarcinogenic effects of COX-2 inhibitors are due to their ability to inhibit COX-2 [12]. However, some effects of selective COX-2 inhibitors cannot be explained by simple inhibition of the COX-2 enzyme, as these drugs can also provoke responses in COX-2 negative cancer cells [13–15]. Furthermore, some NSAID derivatives that do not inhibit COX activity retain their chemopreventive action [16,17].

One putative COX-2 independent pathway might be peroxisome proliferators-activated receptor gamma (PPAR-γ) [15,18], which belongs to the nuclear hormone receptor superfamily and functions as a ligand-activated transcription factor [19]. Recent studies have shown that PPAR-γ expression can be significantly increased in various tumors including pancreatic cancer [20,21]. Ligand activation of PPAR-γ causes cell cycle arrest with terminal differentiation and apoptosis [20,22,23], and inhibits cell invasion in human pancreatic cancer cells [24,25]. NSAIDs, which function as non-selective COX-2 inhibitors, are known to be weak agonists of PPAR-γ [18]. However, to our knowledge, the effects of COX-2 inhibitor in combination with PPAR-γ ligand against pancreatic cancer have not been examined so far. In the present study, a human pancreatic cancer cell line SW1990, in which the COX-2 and PPAR-γ were expressed [26,27], was applied to examine whether inhibition of COX-2 and activation of PPAR-γ exerts synergistic anti-tumour effects on human pancreatic cancer *in vitro*.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY, USA). NS-398 and rosiglitazone were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were acquired from Sigma Chemicals (St. Louis, MO, USA). Other reagents were of analytic grade and obtained from Nanjing Chemical Reagent Co. (Nanjing, China), unless otherwise described.

2.2. Cell culture

SW1990 cells, derived from a human pancreatic ductal adenocarcinoma [28], were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

2.3. Methyl thiazolyl tetrazolium (MTT) assay

SW1990 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight in 10% FCS medium. The cells were then treated with different concentrations of selective COX-2 inhibitor NS-398 or PPAR-γ agonist rosiglitazone in serum-free conditions. Cells incubated in serum-free medium were set as control group. After incubation for 12, 24 and 48 h at 37 °C, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline [PBS]) was added to each well and incubation then continued for a further 4 h at 37 °C. 100 μl DMSO was then added into each well at 37 °C for 2 h. The optical density (OD) value was determined by using a spectrophotometer (Bio-Rad, CA, USA) at 570 nm. Each experiment was performed at least triplicate; the results were presented as the percentages relative to the controls. The 50% inhibitory concentration (IC_{50}) was calculated using the dose–response curve. The interaction of the two drugs was quantified by determining the combination index (CI) according to the method as described previously [29].

2.4. Immunocytochemistry

SW1990 cells were seeded in 6-well plate containing a sterilized 20 × 20 mm glass coverslips and allowed to attach overnight. Then, the cells on the coverslips were cultured with 15 μM NS-398, 10 μM rosiglitazone, or 15 μM NS-398 and 10 μM rosiglitazone for 48 h. Thereafter, the coverslips were dislodged and washed with PBS, fixed in cold acetone for 10 min. Endogenous peroxidase activity was quenched by incubation in 3.0% H_2O_2 in methanol for 30 min. Nonspecific binding was blocked with 3% normal goat serum in PBS, and the cells were incubated with primary mouse anti-human PCNA monoclonal antibody (DAKO, Glostrup, Denmark; 1:100 dilution) overnight at 4 °C. Negative controls were performed by a 2 h pre-incubation of the primary antibody with a five-fold increased concentration of the pure blocking PCNA peptide. The cells were stained according to the avidin-biotin complex method using a commercial kit (Vectastain kit; Vector Laboratories, Burlingame, CA, USA) and visualized using 3,3′-diaminobenzidine (DAB) (Vectastain DAB kit; Vector Laboratories). Positive staining for PCNA was exhibited as orange or brown in the nuclei of SW1990 cells. Five high-power fields were randomly selected and a total of at least 1000 cells were counted by an investigator unaware of prior treatment. The PCNA labeling index was defined as the percentage ratio of labeled nuclei to total number of nuclei.

2.5. Annexin V/propidium iodide staining

Equal numbers of SW1990 cells (1×10^5) were plated in 6-well dishes and then incubated with 15 μM NS-398, 10 μM rosiglitazone, or a combination of these two drugs for 48 h. The cells were washed twice in PBS and then resuspended in 200 μl of the supplied binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing 10 μl of Annexin V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI). After incubation in the dark at room temperature for 15 min, the cells were immediately analyzed by FACScan flow cytometer (Becton Dickinson, CA, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from SW1990 cells using Trizol® (Invitrogen Co., Carlsbad, CA, USA) following the manufacturer's protocol. First strand complementary DNA (cDNA) was synthesized from 1 μg total RNA using a ReverTra Ace Kit (Toyobo, Osaka, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) transcript was used as the internal control. The primer sequences and PCR product sizes were as follows: (a) *Bcl-2* sense (5′-GTGGAGG AGCTCTTCAGGGA-3′) and *Bcl-2* antisense (5′-AGGCACCC AGGGTGATGCAA-3′), 304 bp; (b) *Bax* sense (5′-AGGAT GCGTCCACCAA-3′) and *Bax* antisense (5′-TTTCAC GGGCTCGACT-3′), 243 bp; and (c) *GADPH* sense (5′-CCACC CATGGCAAATTCCATGGCA-3′) and *GADPH* antisense (5′-TC TAGACGGCAGGTCAGGTCCAC-3′), 598 bp. PCR was performed using 5 μl of cDNA as template, 10 pM of each primer, 0.5 μl of deoxynucleoside triphosphates (dNTPs), 0.5 μl of KOD-plus Taq DNA polymerase (Toyobo), 2.5 μl of 10 × PCR buffer, and distilled water to make a total reaction volume of 25 μl. PCR was carried out by a GeneAmp PCR System (2400-R; Basel, Switzerland). The following standard conditions were used: 30 s of initial denaturation at 98 °C, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 2 s, and extension at 74 °C for 30 s. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Negative controls without DNA template were included for all PCR reaction mixtures. The mRNA bands were then visualized by UV-light and quantified by densitometric analysis with NIH Image computer program (Scion Corporation, Frederick, MD, USA). The relative expression of Bcl-2 and Bax mRNAs was normalized to that of GAPDH.

2.7. Western blot analysis

Preparation of cell lysates and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed as described in our previous reports [30]. Membranes were incubated with mouse antibody of anti-Bcl-2 or anti-Bax monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized by the enhanced chemiluminescence (ECL, Amersham, UK) method according to the instructions of the manufacturer. Membranes were routinely stripped and re-probed with mouse anti-β-actin polyclonal antibody (Sigma). Relative expressions of Bcl-2 and Bax proteins were normalized to those of β-actin.

2.8. Molecular docking of COX-2 and rosiglitazone

Uninhibited mouse COX-2 structure is retrieved from PDB (PDB ID: 5COX), with heme and other cofactors removed, processed with PDB2PQR [31] for protonation state at experimental pH level and then with AutoDockTools for charge assignment. Program AutoDock 4 [32] was used to carry out protein-flexible-ligand docking by using Lamarckian genetic algorithm with pseudo Solis and Wets local search algorithm. Chemical affinity and electrostatics maps were computed and centred by AutoGrid on receptor for 72 × 80 × 110 grid points at spacing of 0.375 Å containing both the cyclooxygenase site and peroxidase of COX-2 (see Fig. 6G). Docking was carried out with

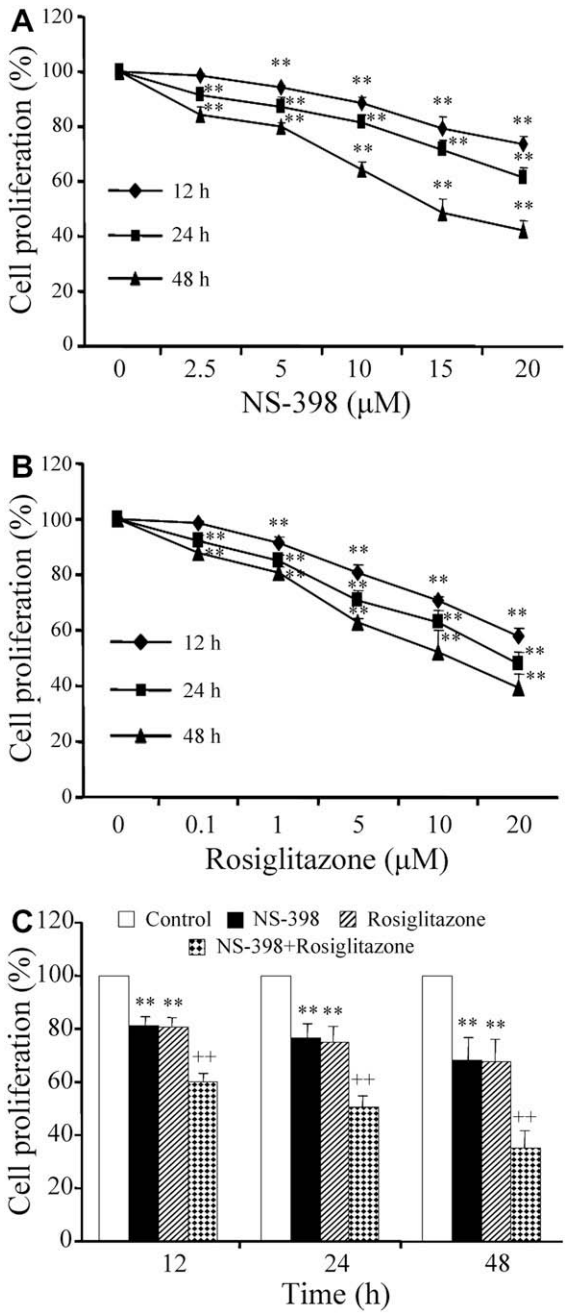

Fig. 1. Effects of NS-398 and rosiglitazone on SW1990 cell proliferation. (A) Dose- and time-dependent effects of NS-398 on cell proliferation. (B) Dose- and time-dependent effects of rosiglitazone on cell proliferation. (C) Effects of NS-398 (15 μM), rosiglitazone (10 μM), and the combination of these two drugs on cell proliferation. SW1990 cells were treated with various concentrations of NS-398, rosiglitazone, or the combinations of two drugs for 12, 24, or 48 h. The results are expressed as the percentages of the control. Data are shown as mean ± SEM from three independent experiments. $^{**}P < 0.01$ *vs.* control; $^{++}P < 0.01$ *vs.* NS-398 or rosiglitazone.

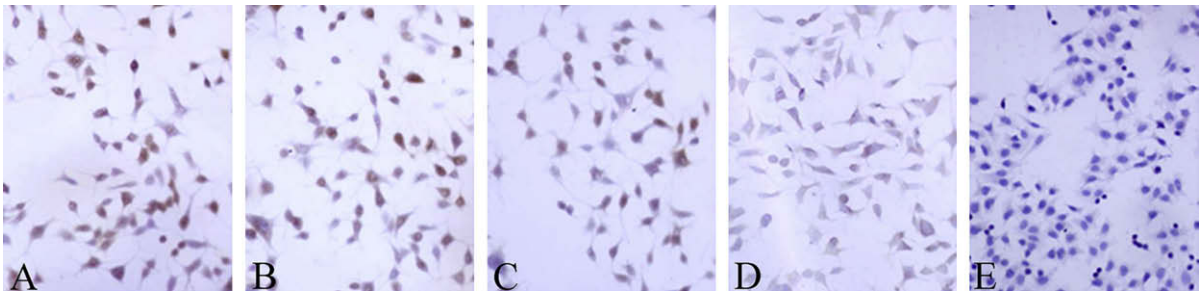

Fig. 2. Representative examples of PCNA expression by immunocytochemistry in SW1990 cells. The cells on the coverslips were cultured without (A) and with 15 μM NS-398 (B), 10 μM rosiglitazone (C), or the combination of these two drugs (D) for 48 h. When the primary antibody preincubated with the blocking PCNA peptide was applied to the untreated cells, no immunoreactive signals appeared at these cells (E). Original magnification, ×200.

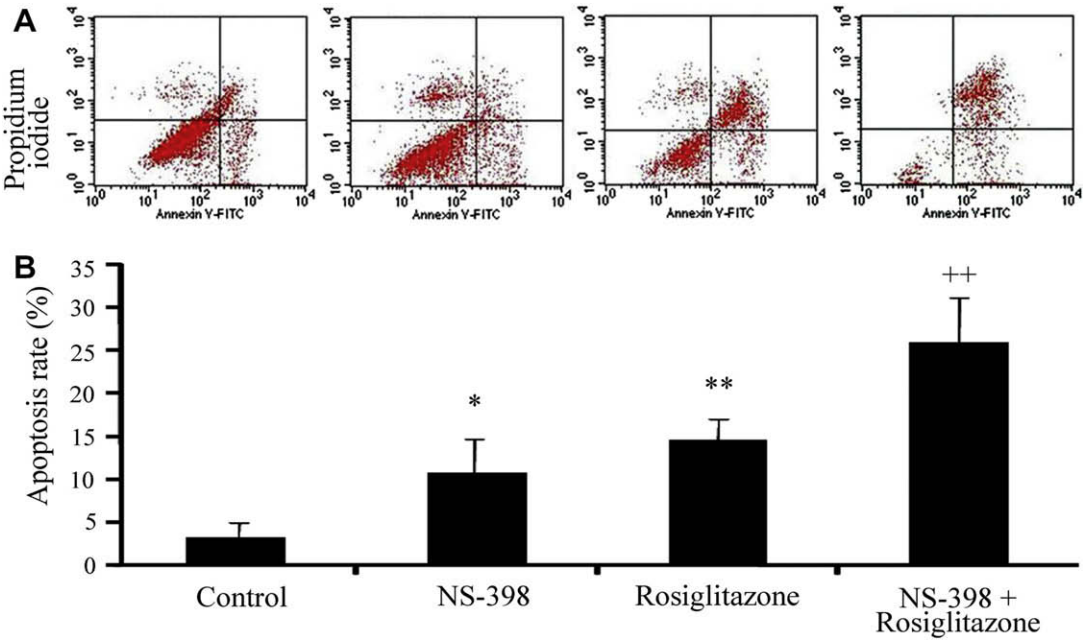

Fig. 3. Effects of NS-398 and rosiglitazone on the apoptosis of SW1990 cells. Cells were treated with 15 μM NS-398 and/or 10 μM rosiglitazone for 48 h. Apoptosis was measured by annexin V-FITC/PI staining followed by flow cytometric analysis. Upper panel (A) shows representative of three separate experiments undertaken. The histogram at the bottom (B) represents the mean ± SEM of apoptosis rates obtained from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control; and $^{++}P < 0.01$ *vs.* NS-398 or rosiglitazone.

"ga_num_evals" set to 60,000,000 and "ga_pop_size" set to 200, sufficient for reaching converging results for the DOF of flexible ligand. Each run comprises of 256 trials. In the end of the run, all docked conformations were clustered using a tolerance of 2 Å root-mean-square deviation (RMSD).

The structures of rosiglitazone and NS-398 are retrieved from PubChem Compound database (CID: 77999 and 4553, respectively), drawn and also geometrically minimized with software Ghemical. Both of them are docked onto COX-2 with same set of parameters described above.

We treat docking in this paper as a tool not to obtain a "good" structure, but as a sampling tool to explore the free energy landscape of the binding of the ligand to the macromolecular complex. In AutoDock 4, each trial in a run is in independent from the other trials and starts from some random positions. Those positions are updated according to genetic algorithm to emerge a "final" docking position. As we have a pretty large number of trials in a run, many possible binding positions and ligand binding poses could be revealed. Positions and poses where dwells clusters of low energy and large size are regarded as favourable binding modes between the ligand and the complex.

2.9. Statistical analysis

Statistical analyses were performed using the SPSS software package (version 11.0; SPSS Inc., Chicago, IL, USA). Data were shown as mean ± SEM. Comparisons between groups were analyzed using Student's *t-test* or one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of NS-398 and rosiglitazone on the proliferation of SW1990 cells

MTT assay was used to analyze metabolic activity in proliferating cells. Either NS-398 or rosiglitazone inhibited the cell proliferation in a dose- and time-dependent manner (Fig. 1A and B). The IC_{50} for NS-398 and rosiglitazone at 48 h was 15.91 ± 0.82 and 13.70 ± 0.56 μM, respectively. The inhibitory effects on the cell proliferation were significantly enhanced when SW1990 cells were treated simultaneously with 15 μM NS-398 plus 10 μM rosiglitazone (Fig. 1C). To quantify the synergy, the MTT data were used to calculate the CI using Isobologram analysis. The CI values were 0.89 ± 0.17, 0.74 ± 0.14 and 0.70 ± 0.15, at 12, 24 and 48 h, respectively, which indicated that the synergism for the drugs combination was also time-dependent.

3.2. Effects of NS-398 and rosiglitazone on PCNA expression in SW1990 cells

To further confirm the proliferation inhibition by NS-398 and rosiglitazone, the expression of PCNA in SW1990 cells was evaluated by immunocytochemistry. As shown in Fig. 2A, nuclear staining for PCNA was evident in control group. NS-398 and rosiglitazone decreased PCNA expression in SW1990 cells (Fig. 2B–D). When the antibody preincubated with the blocking PCNA peptide was applied to the cells, no immunoreactive signals appeared (Fig. 2E). PCNA labeling indexes were 59.4 ± 2.9% and 57.7 ± 3.3% in the cells treated with NS-398 and rosiglitazone, respectively, which were significantly lower than 71.7 ± 4.0% in the control cells ($P < 0.01$). Additionally, the combination of NS-398 and rosiglitazone resulted in a statistically significant decrease in the PCNA labeling index (42.3 ± 2.9%) compared with the effects of each drugs alone ($P < 0.01$).

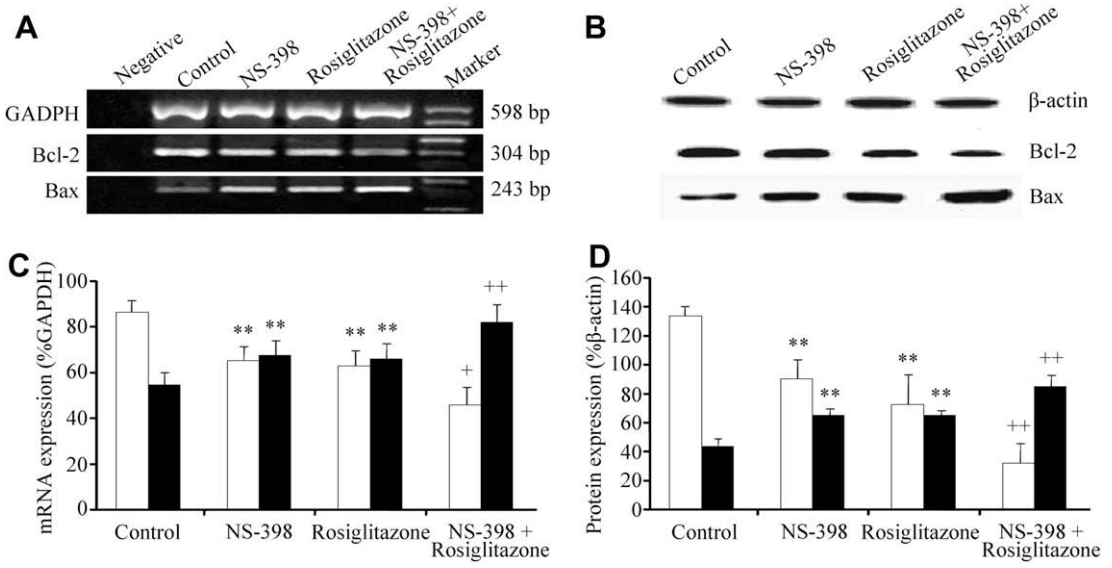

Fig. 4. Effects of NS-398 and rosiglitazone on the Bcl-2/Bax mRNA and protein expression in SW1990 cells. Cells were treated with 15 μM NS-398 and/or 10 μM rosiglitazone for 48 h. (A) Bcl-2 and Bax mRNA were detected by RT-PCR analysis. Negative control indicates no DNA template. (B) Bcl-2 and Bax protein expression was detected by western blot analysis. (C) Quantitative detection of Bcl-2 (white bars) and Bax (black bars) mRNA levels in SW1990 cells compared with GAPDH. (D) Quantitative detection of Bcl-2 (white bars) and Bax (black bars) protein levels in SW1990 cells compared with β-actin. Data are shown as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control; and $^{+}P < 0.05$, $^{++}P < 0.01$ *vs.* NS-398 or rosiglitazone.

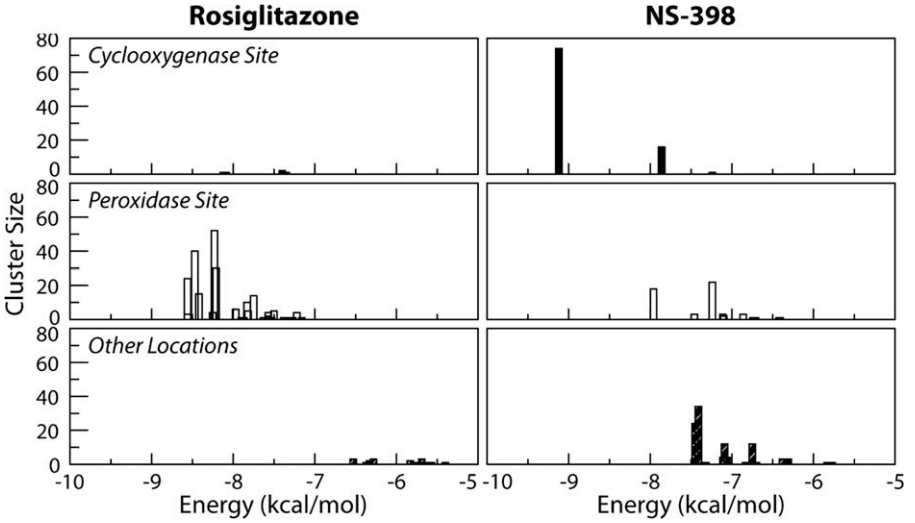

Fig. 5. Clustering histogram of docking results showing both energy and cluster size.

3.3. Effects of NS-398 and rosiglitazone on the apoptosis of SW1990 cells

When cells undergo apoptosis, a phosphatidylserine residue normally on the inside of the plasma membrane flips to the outside and are specifically recognized by annexin V [33]. Counterstaining by PI allows the discrimination of apoptotic from necrotic cells. Necrotic cells can be stained only with PI, whereas early apoptotic cells with annexin V, and late apoptotic cells with both annexin V and PI. As shown in Fig. 3A, the lower right panels correspond to apoptotic cells which have high FITC and low PI signals. Both NS-398 and rosiglitazone induced apoptosis in SW1990 cells; the combination of two drugs further enhanced the apoptosis ratio (Fig. 3B).

3.4. Effects of NS-398 and rosiglitazone on the expression of Bcl-2 and Bax in SW1990 cells

To determine which gene will be regulated by NS-398 and rosiglitazone during apoptosis, the expression of Bcl-2 and Bax mRNA were measured using RT-PCR. Exposure of SW1990 cells to NS-398 or rosiglitazone decreased the expression of anti-apoptotic gene Bcl-2, and increased the pro-apoptotic gene Bax (Fig. 4A and C). The down-regulation of Bcl-2 and the up-regulation of Bax by NS-398 or rosiglitazone were also confirmed in protein level using western blot analysis (Fig. 4B and D). In addition, the combined application of NS-398 and rosiglitazone resulted in a statistically significant decrease in the Bcl-2 expression with the simultaneous increase in the Bax expression compared with the effects of either of drugs.

3.5. Binding of COX-2 with rosiglitazone and NS-398

From clustering histogram (Fig. 5), rosiglitazone and NS-398 are shown to have different docking location preference for COX-2.

Results are being categorized into three types according to their location: results docked into cyclooxygenase site, or into peroxidase site, or other locations. Most results in the third category, other locations, are identified to be due to software/force field artefacts (e.g. binding to a single non-secondary-structure loop), which are ignored.

More than 1/3 (#1 + #3 = 90, structure of cluster #1 is shown in Fig. 6A) of all trials of NS-398 is docked into the cyclooxygenase site. NS-398 is in contact with residues which are known to interact with other COX-2 selective inhibitors. Results docked into peroxidase site (#2 + #5 + #7 + . . . = 55) count for slightly more than half of the number of docking results located at the cyclooxygenase site.

For rosiglitazone, a dominant number of docking results (#1#10 = 189) are located at the peroxidase site. Each cluster differs from each other by their positions. Results located at cyclooxygenase site occur rarely, ranked among results at peroxidase site. Some of them can be viewed from Fig. 6B–D (#11, #13, and #20, respectively).

The binding energy of NS-398 (−9.93 kcal/mol) from docked structure is also lower than rosiglitazone by 1–2 kcal/mol (Fig. 6E and F).

NS-398 and rosiglitazone differ in their structural composition and structure, which makes them having different preference in binding location. Cluster #11 and #13 of rosiglitazone rejects one possible answer to such preference: rosiglitazone is unable fit into the cavity at cyclooxygenase site and results in positive binding energy.

Above results strongly suggest if rosiglitazone is found inhibitive to COX-2, it is quite likely via peroxidase site (Fig. 6G), despite lacking of known structural models.

4. Discussion

Pancreatic carcinoma remains one of the most malignant cancers with poor prognosis, mainly due to its clinicopathological features and resistance to chemotherapy.

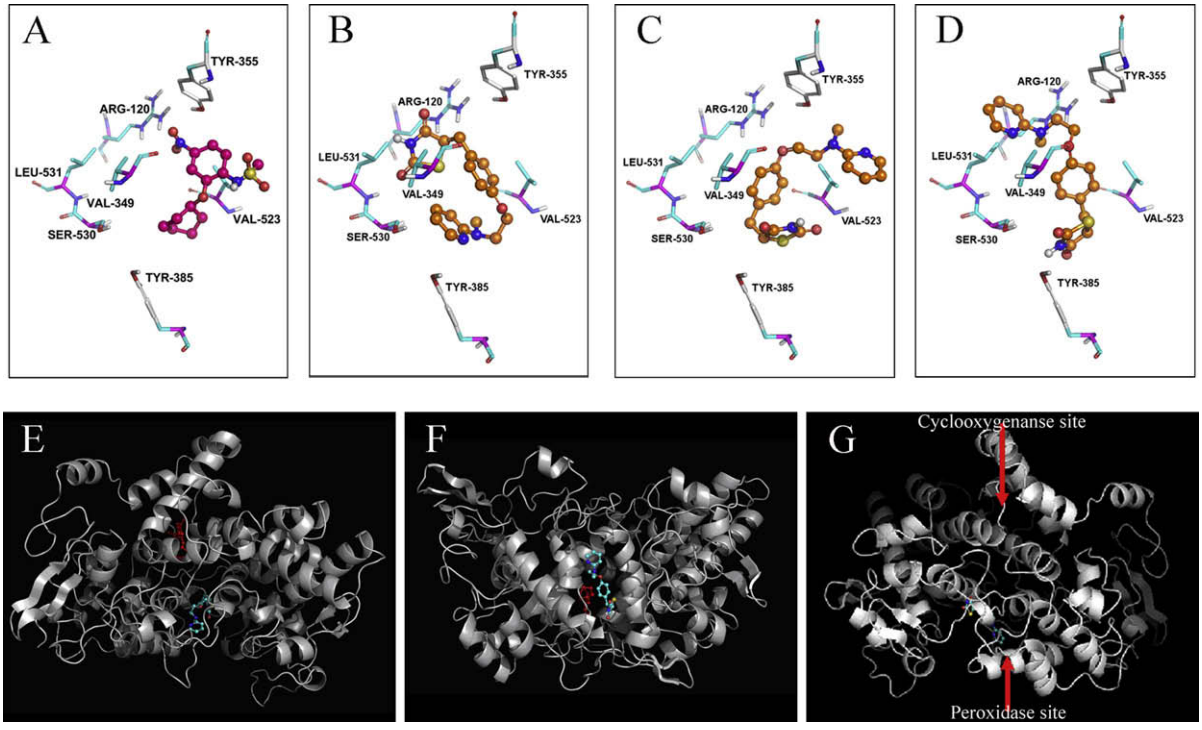

Fig. 6. Binding of COX-2 with NS-398 and rosiglitazone. (A) NS-398 in cyclooxygenase site of murine COX-2 (Cluster #1). (B) Rosiglitazone in cyclooxygenase site of murine COX-2 (cluster #11). (C) Rosiglitazone in cyclooxygenase site of murine COX-2 (cluster #13). (D) Rosiglitazone in cyclooxygenase site of murine COX-2 (cluster #20). (E) Rosiglitazone in peroxidase site of murine COX-2 (side view, red is NS-398 in cyclooxygenase site). (F) Rosiglitazone in peroxidase site of murine COX-2 (bottom view, viewed from peroxidase site, red is NS-398 in cyclooxygenase site). (G) Two actives of COX-2 (5COX): cyclooxygenase site and peroxidase site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Therefore, it is very important to ascertain potential novel therapeutic and chemopreventive agents. A molecular target of chemoprevention that has been studied extensively in the last decade is COX-2. In the present study, we demonstrated that a selective COX-2 inhibitor, NS-398, significantly decreased cell proliferation in a time- and dose-dependent manner, which supports previous findings that COX-2 inhibitors suppress cell proliferation and induce apoptosis *in vitro* [6,10,11,13], therefore *in vivo* decrease tumor growth and enhance survival rate [11]. To clarify the mechanism by which NS-398 inhibits the proliferation of pancreatic carcinoma cells, expressions of PCNA were detected by immunocytochemistry. Our results showed that NS-398 significantly reduced PCNA expression in SW1990 cells. PCNA is a nuclear protein that is synthesized in late G1 and S phases of the cell cycle and that is used to assess changes in the growth status of cells [34,35]. The modulation of PCNA expression provides an important indicator of early changes in the cellular proliferation which may be related to agent exposure. However, our present studies did not rule out the possibility of NS-398 modulating other targets, because another COX-2 inhibitor, celecoxib, was shown to suppress cell proliferation and induce apoptosis via a COX-2 independent pathway [36,37].

We recently demonstrated that blockade of cholecystokinin-2 (CCK-2) receptor and COX-2 synergistically induces cell apoptosis, and inhibits the proliferation of human gastric cancer cells [38]. The presence of both CCK-2 receptor and gastrin was also observed in many pancreatic tumors. However, the roles of them during cancer development remain controversial [39,40]. Previous results have shown that failure to detect gastrin immunoreactivity in conditioned media from a panel of 14 human pancreatic carcinoma cell lines argues against the general occurrence of an autocrine loop involving gastrin and the CCK-2 receptor [41]. Also, recent studies showed that activation of PPAR-γ induces growth arrest and apoptosis in human pancreatic cancer [20,22–25]. Therefore, here we are interesting in examining the effect of simultaneous targeting of COX-2 and PPAR-γ on the proliferation and apoptosis of human pancreatic cancer cells. The present results showed that NS-398 in combination with rosiglitazone synergistically inhibited cell proliferation and induced apoptotic cell death *in vitro*. Our results are consistent with the previous studies that simultaneous targeting of COX-2 and PPAR-γ synergistically inhibited breast cancer development [42].

Although COX-2 and PPAR-γ mediate different signaling pathways, they also modulate common molecular targets and therefore synergistically inhibit cancer development [42]. It has been reported recently that COX-2 and PPAR-γ are inversely correlated in the human breast cancers [43]. Activation of PPAR-γ caused COX-2 inhibition [44] or the down-regulation of COX-2 expression [45], whereas inhibition of COX-2 resulted in PPAR-γ activation [46] or up-regulation of PPAR-γ expression [47]. Furthermore, COX-2 inhibitors acted as partial PPAR-γ agonists [18], whereas PPAR-γ agonists partially inhibited COX-2 and PGE_2 synthesis [48,49]. These observations may explain that combinational treatment with COX-2 inhibitor, NS-398, and PPAR-γ ligand, rosiglitazone, exerts synergistic anti-tumour effects in our present study. In addition, our molecular docking site of COX-2 and rosiglitazone shown that single rosiglitazone molecule has a possibility to enter the cyclooxygenase active site of COX-2, and binding energy is close to what NS-398 achieves. However, lacking of structural proof, it is still difficult to know exactly how rosiglitazone inhibits COX-2 in the present time. Our previous study showed that another selective COX-2 inhibitor, celecoxib, inhibited L-type calcium channels, which could play anti-tumor effects on pheochromocytoma (PC12) cells [50]. Chang et al. demonstrated that ligand-dependent PPAR-γ growth suppression is attenuated by gastrin, at least in part, through attenuation of PPAR-γ activity and through an increase in PPAR-γ protein degradation in colorectal cancer cells [51]. Thus, it is possible that growth inhibition by NS-398 and rosiglitazone in human pancreatic cancer cells through PPAR-γ pathway partially, and it is also possible that PPAR-γ mediate the gastrin/CCK-2 receptor indirectly or directly, but unlikely via calcium channels.

It is well known that tumor genesis is due to the disruption of the balance between cell proliferation and apoptosis [52]. In the present study, both NS-398 and rosiglitazone significantly induced apoptosis in SW1990 cells. It has been demonstrated that apoptosis is modulated partially by Bcl-2 family including apoptosis inhibiting gene products (Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-w) and apoptosis accelerating gene products (Bax, Bak, Bcl-XS, Bim) [53]. In our present results, both RT-PCR and western blot analysis revealed the down-regulation of Bcl-2 expression with the simultaneous up-regulation of Bax expression in SW1990 cells treated with NS-398 or rosiglitazone. Therefore, COX-2 inhibitor and PPAR-γ agonist modulated the ratio of the expression of Bax/Bcl-2, and increased Bax protein expression in SW1990 cells.

In conclusion, both NS-398 and rosiglitazone had growth inhibitory and apoptosis inductive effects on the pancreatic cancer cell line through down-regulation of Bcl-2 with the simultaneous up-regulation of Bax expression, which suggests that COX-2 inhibition as well as PPAR-γ activation is new molecular targets for effective therapy against pancreatic cancer. In addition, our results show for the first time that NS-398 and rosiglitazone exerted a synergistic effect on the inhibition of proliferation and the induction of apoptosis of SW1990 cells. The synergistic effect may enable the use of a specific COX-2 inhibitor at lower and safer concentrations and may pave the way for a more effective treatment in human pancreatic cancer, although further studies are required to elucidate whether the synergistic effect of COX-2 inhibitor and PPAR-γ ligand also exerts in the pancreatic cancer *in vivo*.

Conflicts of interest statement

None declared.

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