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Seleno-cyclodextrin sensitises human breast cancer cells to TRAIL-induced apoptosis through DR5 induction and NF-κB suppression

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ABSTRACT

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) exhibits potent antitumour activity via membrane receptors on cancer cells without deleterious side-effects for normal tissue. Unfortunately, like many other cancer types, breast cancer cells develop resistance to TRAIL; therefore, TRAIL-sensitising agents are currently being explored. In this study, we report that seleno-cyclodextrin (2-selenium-bridged β-cyclodextrin, 2-SeCD), a seleno-organic compound with glutathione peroxidase (GPx)-mimetic activity, sensitises TRAIL-resistant human breast cancer cells and xenograft tumours to undergo apoptosis. *In vitro*, 2-SeCD reduces the viability of cancer cells by inducing cell cycle arrest in G_2/M phase. Furthermore, 2-SeCD efficiently sensitises MDA-MB-468 and T47D cells but not untransformed human mammary epithelial cells to TRAIL-mediated apoptosis, as evidenced by enhanced caspase activity and poly-ADP-ribose-polymerase (PARP) cleavage. From a mechanistic standpoint, we show that 2-SeCD induces the expression of TRAIL receptors DR5 but not DR4 on both mRNA and protein levels in a dose-dependent manner. Moreover, 2-SeCD treatment also suppresses TRAIL-induced nuclear factor-κB (NF-κB) pro-survival pathways by preventing cytosolic IκBα degradation and p65 nuclear translocation. Consequently, the combined administration suppresses anti-apoptotic proteins transcriptionally regulated by NF-κB. *In vivo*, 2-SeCD and TRAIL are well tolerated in mice, and their combination significantly inhibits the growth of MDA-MB-468 xenografts and promotes apoptosis. Up-regulation of DR5 and down-regulation of NF-κB by dual treatment were also observed in tumour tissues. Overall, 2-SeCD sensitises resistant breast cancer cells to TRAIL-based apoptosis *in vitro* and *in vivo*. These findings provide strong evidence for the therapeutic potential of this combination against breast cancers.

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1. Introduction

Tumour necrosis factor (TNF) α-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines. TRAIL exerts cytotoxic effects on malignant cells without any harm to normal cells.[1] To date, five members of the human TNF receptor (TNFR) superfamily have been identified that can bind TRAIL. The death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) contain two cysteine-rich extracellular TRAIL-binding domains and a cytoplasmic death domain that are required for transmitting a cytotoxic signal.[2,3] The decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) also possess comparable affinities for binding to TRAIL, but they do not transmit apoptogenic signals due to non-functional death domains.[4,5] Finally, TRAIL binds the receptor osteoprotegerin (TNFR11B), which is a soluble protein incapable of signalling.[6]

Following TRAIL engagement with either DR4 or DR5, the ligated death receptors cluster and microaggregate within the cell membrane, thereby initiating formation of the death-inducing signalling complex (DISC).[7] The functional death-inducing signalling complex is composed minimally of death receptors (DR4 and DR5), adapter protein fas-associated death domain (FADD), and caspase 8 or 10.[8] Active caspases 8 and 10 cleave and directly activate downstream effector caspases (3, 6, and 7), which ultimately cut vital cellular substrates and result in apoptosis.[9] Meanwhile, the TRAIL receptor signalling pathway also leads to the activation of the nuclear factor-κB (NF-κB), which operates as a negative regulator of DISC formation.[10,11] Once stimulated by TRAIL, the subunit of NF-κB could translocate to the nucleus and up-regulate anti-apoptotic genes, such as cellular FLICE-like inhibitory proteins (c-FLIP) and cellular inhibitors of apoptosis (c-IAPs), which eventually thwart the activation of caspases.[12,13]

As a promising therapy agent for the treatment of malignancies, TRAIL has been shown to induce apoptosis in breast carcinomas.[14] Unfortunately, the majority of cell lines are insensitive to TRAIL-induced apoptosis, and the mechanism of resistance has been attributed to the dysfunction of different steps in the apoptosis pathways, as well as the elevation of survival signals.[15] A combination therapy was, therefore, envisioned, based on the recent evidence that the pretreatment of tumour cells with a chemotherapeutic agent could sensitise them to TRAIL.[15,16] However, the combinations should be taken very carefully due to potential toxicity to normal tissues.[17,18] In this respect, it will be important to find a safe and effective agent to overcome TRAIL resistance in breast cancer therapy.

Seleno-cyclodextrin (2-selenium-bridged β-cyclodextrin, or 2-SeCD, Fig. 1A), a synthetic seleno-organic compound with unique glutathione peroxidase (GPx)-like activity, has exhibited anti-oxidant and anti-inflammatory properties with very low toxicity.[19,20] It has been extensively demonstrated as a promising substitute for ebselen, a well known GPx mimic,[21] with enhanced enzymatic activity and water solubility.[22–24] In recent years, 2-SeCD and other related GPx mimics have been shown to exhibit antitumour effects against breast carcinoma, glioblastoma and acute myeloid leukaemia.[25–28] Importantly, 2-SeCD and its tellurium (Te) derivative have been evidenced as selective inhibitors of thioredoxin reductase,[25,29,30] which is over-expressed in cancer cells and has become a target for cancer therapy.[31] Our recent study revealed that a 2-SeCD analogue was able to suppress the translocation of NF-κB that was stimulated by TNFα.[32] Considering that NF-κB could be a target for TRAIL-sensitisation, we hypothesised that a combination of 2-SeCD and TRAIL could be a promising strategy in the treatment of refractory breast tumours.

In this study, the effects of 2-SeCD in combination with TRAIL have been investigated in breast cancer cell lines. We report that 2-SeCD is able to sensitise cancer cells to TRAIL-mediated apoptosis via DR5 induction and NF-κB inhibition. Importantly, using the MDA-MB-468 xenograft model, we further demonstrate that this combination is well tolerated in mice and reduces tumour burden. For the first time, we present clear evidence that 2-SeCD, a GPx mimic, can overcome TRAIL resistance *in vitro* and *in vivo*; therefore, this combination provides a powerful therapeutic option for human breast cancer treatment.

2. Materials and methods

2.1. Cell lines and reagents

Human MDA-MB-468 and T47D breast carcinoma cells (American Type Culture Collection) were grown in DMEM (Gibco) supplemented with 4.5 g/L of glucose, 4 mmol/L of L-glutamine, 100 units/mL of penicillin/streptomycin and 10% FCS (Invitrogen). Human mammary epithelial cells (HMECs; Cambrex) were grown as described in the manufacturer's instructions. All cells were grown at 37 °C in a humidified incubator with 5% CO_2 atmosphere. 2-SeCD was prepared as described in our previous work.[22] Soluble Recombinant Human TRAIL was purchased from R&D Systems. The pan-caspase inhibitor z-VAD-fmk and the caspase-8 inhibitor Z-IETD-FMK were purchased from Alexis Corporation.

2.2. Transient transfections

Cells were transfected with the RelA/p65 expression vector pCMV4-p65 (Panomics) or the empty vector pCMV4-neo using Lipofectamine™ 2000 Reagent (Invitrogen) in Optimem serum-free medium. After overnight transfection, Optimem was replaced with fresh complete medium, and the cells were allowed to recover for 8 h. The resulting cells were used immediately in experiments evaluating the effects of 2-SeCD and/or TRAIL.

2.3. Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT, Chemicon) colorimetric method. Following the treatment of cells (5×10^3/well) with various concentrations of 2-SeCD and/or TRAIL in 96-well plates for 24 h, a 20-μL/well MTT (5 mg/mL) solution was added. After 4-h incubation at 37 °C, the formazan crystals were solubilised with 150 μL/well dimethylsulphoxide for 10 min. The absorbance values of the solution in each well

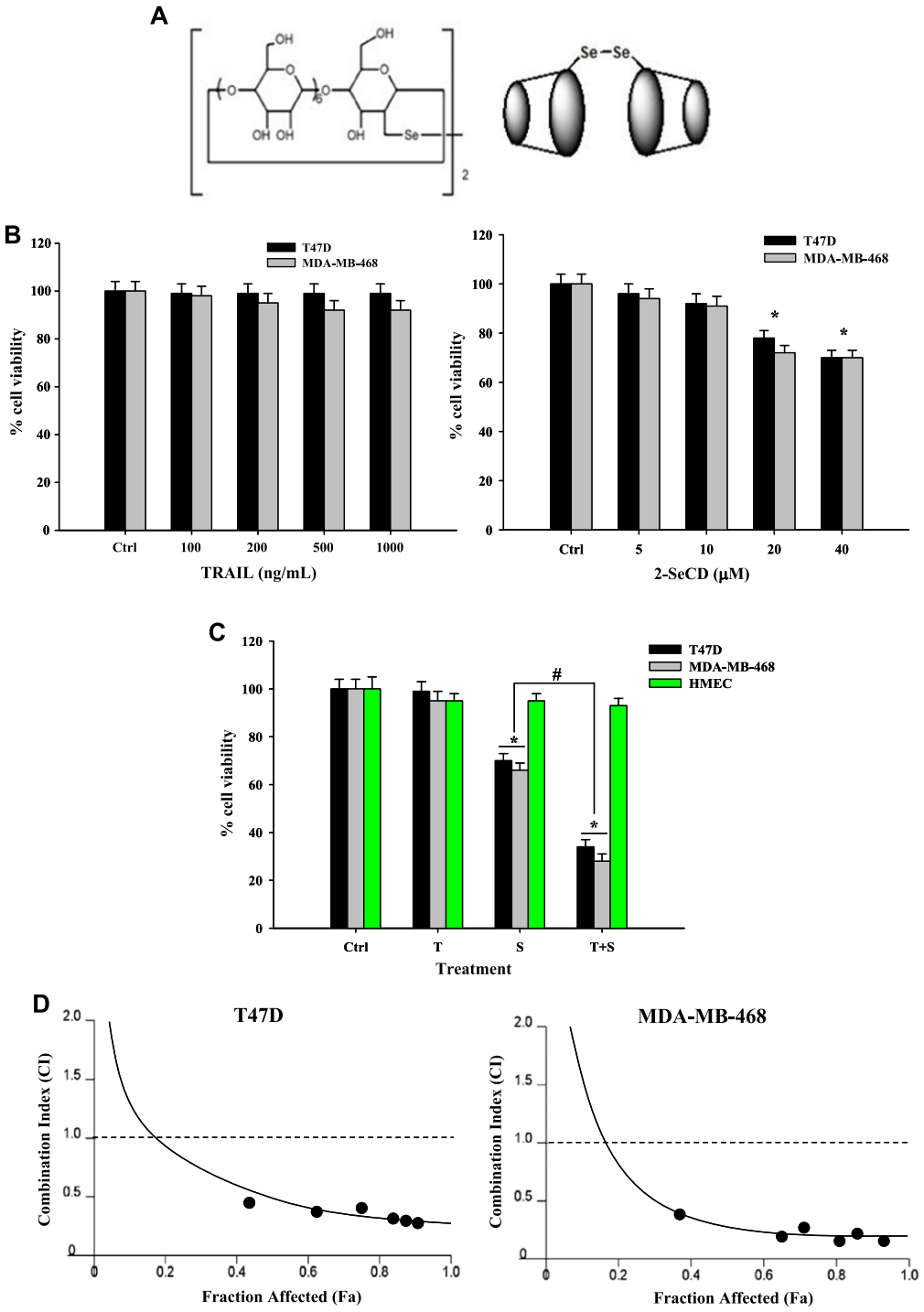


Fig. 1 – 2-Selenium-bridged β-cyclodextrin (2-SeCD) sensitises human breast cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity *in vitro*. (A) The structural formula of 2-SeCD. (B) 2-SeCD sensitises cancer cell lines to TRAIL-induced cytotoxicity. MDA-MB-468 and T47D cells were treated with different concentrations of TRAIL (0–1000 ng/mL, left) or 2-SeCD (0–40 μM, right) for 24 h; and then, cell viability was determined by MTT assay. (C) 2-SeCD sensitises resistant breast cancer cells, but not untransformed human mammary epithelial cells (HMECs), to TRAIL-induced cytotoxicity. Breast cancer cells and HEMC cells were treated simultaneously with 2-SeCD (20 μM) and/or TRAIL (200 ng/mL) for 24 h. Columns, mean percentage of viable cells relative to control group ($n = 3$); bars, standard deviation (SD). $^{*}P < 0.05$ versus control group. $^{\#}P < 0.05$ versus 2-SeCD alone group. T and S denote TRAIL and 2-SeCD, respectively. (D) Synergistic induction of cell death by 2-SeCD and TRAIL. For combination experiments, six doses of 2-SeCD and TRAIL were used from serial dilutions covering the IC_{50} (fractional affected 0.5) values. The combination index (CI) analysis was conducted according to the median-effect plot analysis of Chou and Talalay. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism of the two agents, respectively. Cell viability was determined by MTT assay.

were measured at 570 nm using an ELx808 microplate reader (BioTek Instruments).

2.4. Apoptosis assay

For caspase activity assay, cells were exposed to Caspase-Glo 3/7 and Caspase-Glo 8 assay kits (Promega) for 30 min at room temperature, and the luminescence was quantified in a plate-reading luminometer (Thermo Labsystems). Annexin V assays were done using the Annexin V-FITC apoptosis detection kit (Clontech) per the manufacturer's instructions. As a standard, 10,000 cells per treatment condition were analysed by FACS Calibur (Becton Dickinson) using a Cell Quest Software.

2.5. Cell cycle analysis

After vehicle or agent treatment, cells were washed twice in phosphate buffered saline (PBS) and then incubated overnight in 70% ethanol at –20 °C. The fixed cells were then incubated with 50 μg/mL propidium iodide containing 0.25 mg/mL RNase A at 37 °C for 30 min. Cell distribution was analysed with a Becton Dickinson FACS Calibur flow cytometer using the Cell Quest Software. For each experiment, 10^4 events per sample were recorded.

2.6. Flow cytometry of death receptors

Cells were analysed for the surface expression of DR4 and DR5 by indirect staining with primary goat anti-human DR4 and DR5 (R&D Systems), followed by FITC-conjugated rabbit anti-goat IgG (Sigma). Briefly, cells (1×10^6) were stained with 200 μL PBS containing saturating amounts of anti-DR4 or anti-DR5 antibody on ice for 30 min. After incubation, cells were washed twice and reacted with FITC-conjugated rabbit anti-goat IgG on ice for 30 min. After washing with PBS, the expression of these death receptors was analysed by FACS Calibur (Becton Dickinson).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative real-time PCR was performed to determine the mRNA expression levels of death receptors and NF-κB target gene expression level. Total RNA was isolated from cells treated with vehicle or various concentrations of 2-SeCD using the RNeasy Mini Kit (Qiagen). A reverse transcription reaction with 1 μg total RNA in 100 μL was carried out according to the TaqMan Reverse Transcription Kit (Applied Biosystems) instructions. For quantitative PCR, 1 μL gene primers with SYBR Green (Applied Biosystems) was applied to a 20-μL reaction volume. Primer sequences for each of the genes analysed are as follows: DR4 forward primer: 5′-GCTCAGGTTGTTTGTTGCATCGGC-3′, reverse primer: 5′-GCCAGTTTTGTTGGAGGCGTTCCG-3′; DR5 forward primer: 5′-GAGACAACAAAACGGCGCCGAGGT-3′, reverse primer: 5′-CAGCAACTGTGAGACTACGGCTAC-3′; cIAP-1 forward primer: 5′-CAGCCTGAGCAGCTTGCAA-3′, reverse primer: 5′-CAAGCCACCATCACAACAAAA-3′; XIAP forward primer: 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′, reverse primer: 5′-CCGCACGGTATCTCCTTCA-3′; survivin forward primer: 5′-TGCCTGGCAGCCCTTTC-3′, reverse primer: 5′-CCTCCAAGAAGGGCCAGTTC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, all purchased from Clontech. Temperature cycling and real-time fluorescence measurement were done using ABI prism 7300 Sequence Detection System (Applied Biosystems). The relative expression level for each target gene mRNA was calculated using the following formula: $[2 - (C_T \text{ target} - C_T \text{ GAPDH})] \times 100\%$, where C_T is the threshold cycle.

2.8. Small interfering RNA

The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and had the following sequences: DR5, ACAAUACCGACCUUGACCAUCCC; and green fluorescent protein (GFP), AAGACCCGCGCCGSGGUGAAG. For transfection, cells were seeded in 10-cm dishes at 30% confluency, and siRNA duplexes (200 nM) were introduced into the cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommendations.

2.9. Total, cytosolic and nuclear proteins extraction

Cells were collected after 24 h of treatment and pretreated with a 25-μL protease inhibitor cocktail (Pierce). An NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) was used to extract nuclear and cytoplasmic contents. Samples were concentrated with PEG 8000, and protein concentrations were estimated using a Micro BCA kit (Pierce).

2.10. Western blot analysis

Samples (30 μg protein) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose paper (Immobilon-NC, Millipore) soaked in a Tris (20 mM), glycine (150 mM) and methanol (20%) buffer at 55 V for 4 h. After washing, the blots were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal anti-cyclin A, anti-cyclin B1, anti-p21, anti-p27, anti-RelA (p65), anti-IκBα, PARP, anti-histone H4 (diluted 1:1000 v/v, Santa Cruz); anti-caspase-3, anti-caspase-8, anti-TRAIL-R2, anti-TRAIL-R1, anti-cIAP1, anti-cIAP2, anti-XIAP, anti-survivin (1:2000 v/v, Stressgen) and mouse monoclonal anti-β-actin and anti-α-tubulin (1:2000 v/v, Sigma). Following incubation, the secondary antibodies (diluted 1:2000 v/v, Sigma) were added for 1 h at room temperature. Proteins were visualised using an enhanced chemiluminescence system (Santa Cruz) and captured on X-ray films.

2.11. Luciferase assay

Cells were transiently cotransfected with an NF-κB reporter construct (pNF-κB) or a control reporter plasmid (pControl) (Panomics), together with a β-galactosidase reporter vector (Promega), which was used to normalise NF-κB reporter gene activity, using Lipofectamine™ 2000 (Invitrogen). After 16-h incubation, the cells were treated with TRAIL in the presence or absence of 2-SeCD for 24 h. Cell lysates were prepared using Reporter Lysis Buffer (Promega). For luciferase and β-galactosidase assays, the samples were measured by a luminometer using the Bright-Glo luciferase assay kit and β-galactosidase enzyme assay kit (Promega), respectively.

2.12. *In vivo tumour growth model*

MDA-MB-468 cells (5×10^6) resuspended in 0.1 mL serum-free DMEM were subcutaneously (s.c.) injected into the right axilla of 6-week-old female Balb/c nu/nu mice (National Academy of Medical Sciences). When the average size of tumours reached approximately 100 mm^3 (about 14 d), animals were randomly separated into four groups (n = 8/group) to receive treatment of an intraperitoneal (i.p.) injection of vehicle control (100 μL of 0.9% NaCl), 2-SeCD (60 mg/kg/2 d – dose of 0.05LD50, based on preliminary experiments, 1.2 mg/2 d for a 20-g mouse in a maximal volume of 100 μL 0.9% NaCl), TRAIL (10 mg/kg/2 d) and the combination of 2-SeCD plus TRAIL (6 h after 2-SeCD treatment). The volume of the tumours and the weight of the mice were measured every 3–4 d. Tumour size was measured with a calliper and calculated by the following formula: (long axis × short $axis^2$)/2. On day 52 after inoculation, treatments were terminated when the tumours in the control group (vehicle only) reached an average of 2.0 cm in the largest (L) dimension.

2.13. *Tissue and blood analysis*

Mice were killed under anaesthesia using avertin; blood samples were subsequently collected for the serum chemistry and toxicology studies. Tumour tissues were then immediately removed, fixed in paraformaldehyde at room temperature for 48 h, and then embedded in paraffin. For immunohistochemistry, dewaxed tissue sections (5.0 μm) were fixed and incubated with primary antibodies: PCNA, Ki-67, p65-NFκB and TRAIL-R2/DR5 (Santa Cruz). The detection of nuclei with fragmented DNA by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) was accomplished using an in situ cell death detection kit (POD; Roche) according to the instructions of the manufacturer.

2.14. *Statistical analysis*

The mean and SD were calculated for each experimental group. Differences between groups were analysed by one- or two-way ANOVA followed by Bonferroni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software version 5.0). Significant differences among groups were calculated at $P < 0.05$. The interaction between 2-TeCD and TRAIL was analysed by isobologram analysis using the CalcuSyn Software Program (Biosoft, Ferguson, MO) to determine whether the combinations were additive or synergistic. This program is based on the Chou–Talalay method,[33] which calculates a combination index (CI) to indicate additive or synergistic effects. In this method, CI values <1 indicate synergy (the smaller the value, the greater the degree of synergy), values >1 indicate antagonism and values equal to1 indicate additive effects.

3. Results

3.1. *2-SeCD sensitises resistant human breast cancer cells to TRAIL-induced cytotoxicity*

We examined T47D and MDA-MB-468 cells for TRAIL sensitivity using different concentrations (100, 200, 500 and 1000 ng/mL) of recombinant human soluble TRAIL. Cells were treated for 24 h with TRAIL and then assessed for cell viability using MTT assay. Consistent with the previous studies,[15] less than 10% reduction of viability was observed in cells treated with TRAIL, even when 1000 ng/mL was added (Fig. 1B, left). Thus, we choose 200 ng/mL of TRAIL for the experiments further on.

To investigate whether 2-SeCD could exhibit anti-proliferative effects, cancer cells were incubated with the increasing concentrations of 2-SeCD (5, 10, 20 and 40 μM) for 24 h and then also subjected to the MTT assay. Although treatment with 5 and 10 μM 2-SeCD had no effect on cell growth rates, ~25% reduction in viability was observed in cells treated with 20 μM 2-SeCD. However, in cells treated with 40 μM concentrations, the viability was similar to that observed at 20 μM (Fig. 1B, right). On the basis of these results, we chose 20 μM 2-SeCD for the following characterisation of cell death.

We next evaluated whether 2-SeCD could cooperate with TRAIL to induce growth suppression of breast cancer cells, while sparing normal cells. As shown in Fig. 1C, approximately 70% decrease of cell viability in MDA-MB-468 and T47D cells was observed with a combined treatment of 20 μM 2-SeCD and 200 ng/mL TRAIL. Thus, a significant ~3.0-fold reduction was observed when compared to 2-SeCD individually. In addition, the combination treatment induced minimal cytotoxic effects in untransformed HMECs cells, indicating 2-SeCD did not abrogate the potential tumour selectivity of TRAIL. Besides, HMECs cells did not exhibit growth arrest by treatment with 2-SeCD alone, which is consistent with our previous findings that 2-SeCD is not toxic to cells derived from normal tissues. Notably, exposure to the combination of 2-SeCD and TRAIL exerted synergistic effects in T47D and MDA-MB-468 cells, as determined by the median dose-effect isobologram analysis described by Chou and Talalay.[33] For 2-SeCD and TRAIL, the CI values ranged from 0.25 to 0.5 in each cell type (Fig. 1D). Taken together, these results suggest that 2-SeCD synergistically sensitises resistant breast cancer cells, not untransformed HMECs, to TRAIL-induced cytotoxicity *in vitro*.

3.2. *The sensitisation of TRAIL-Induced cytotoxicity by 2-SeCD is associated with apoptosis*

TRAIL-induced cancer cell death is mainly apoptotic. We next examined whether cell death caused by TRAIL plus 2-SeCD was associated with apoptosis. Breast cancer cells were cultured with 2-SeCD and/or TRAIL for 24 h, and then subjected to Annexin V/propidium iodide staining, which could directly differentiate cell necrosis and cell apoptosis. Using the accepted criterion that apoptotic cells are Annexin V-positive/propidium iodide-negative, we found that treatment with TRAIL alone was not able to induce apoptosis in either MDA-MB-468 or T47D cells. However, a significant increase of apoptotic cells was observed in both of the cancer cell lines, upon exposure to 2-SeCD. Notably, more apoptotic cells were obtained when exposed to a combination of the two agents (Fig. 2A). These results support our previous MTT analysis, confirming that 2-SeCD is essential for the sensitisation of cancer cells to TRAIL-induced apoptosis. The minimal percentages of Annexin V-positive/propidium iodide-positive

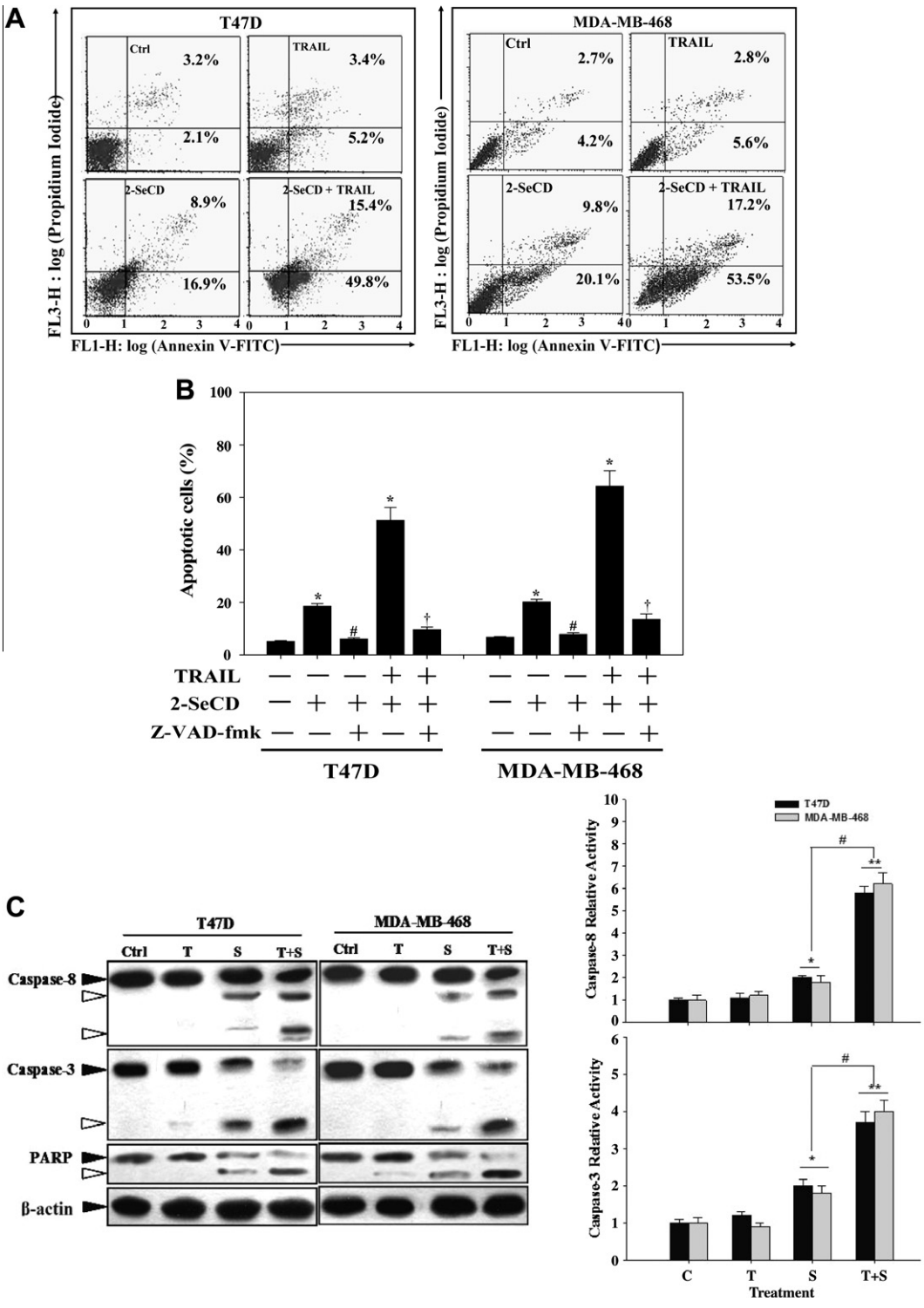


Fig. 2 – The sensitisation of TRAIL-induced cytotoxicity by 2-SeCD is associated with apoptosis. (A) Cells were treated with 2-SeCD (20 μM) and/or TRAIL (200 ng/mL) for 24 h and then assayed for apoptosis by Annexin V (*x*-axis)/propidium iodide (*y*-axis) staining. (B) Apoptosis was mostly blocked by the caspase inhibitor. Cells were incubated with 2-SeCD (20 μM) in the presence (+) or absence (–) of TRAIL (200 ng/mL) for 24 h after 1 h pretreatment with (+)/without (–) z-VAD-fmk (100 μM). Columns, mean percentage of apoptotic cells; bars, SD. $^{*}P < 0.05$ versus 2-SeCD alone group. $^{\#}P < 0.05$ versus 2-SeCD + TRAIL group. (C) Caspase-8, caspase-3 and PARP were analysed by immunoblotting (left) and activity assay (right). Columns, mean fold change of three independent experiments to control-only; *bar*, SD. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus control group. $^{\#}P < 0.05$ versus 2-SeCD alone group.

signals were due to secondary necrosis of cells that originally succumbed to apoptosis. Finally, the apoptosis induced by 2-SeCD alone or in combination with TRAIL were completely blocked when cells were pre-incubated for 1 h with 100 μM z-VAD-fmk (Fig. 2B), a broad-spectrum caspase inhibitor, suggesting a caspase-dependent mechanism.

We next analysed the caspase-8 cleavage, a proximal event in the TRAIL-induced caspase cascade. As shown in Fig. 2C left, TRAIL alone failed to induce detectable caspase-8 processing when compared to control. In contrast, cleaved caspase-8 was clearly detected in both of the cancer cells when treated with 2-SeCD alone. Moreover, the cleaved caspase-8 was dramatically promoted by the combination treatment of 2-SeCD and TRAIL. The immunoblotting results were further substantiated by a caspase activity assay (Fig. 2C, right). Compared to the agent-alone group, the combination group significantly increased caspase-8 activity in both the MDA-MB-468 and T47D cells. Collectively, these data indicate that treatment of resistant cancer cells with the combination of 2-SeCD and TRAIL significantly elevates caspase-8 activity.

Next, the essential apoptosis executor caspase-3 and the caspase-3 substrate PARP were also analysed to substantiate the above results. Caspase-3 and PARP cleavages were barely observed in both of the TRAIL-resistant cells when cultured with TRAIL alone. By comparison, the cleavages were clearly detected in both of the MDA-MB-468 and T47D cells when treated with 2-SeCD, and these were all enhanced when cells were cultured with the combination of 2-SeCD and TRAIL (Fig. 2C). The caspase-3 and PARP cleavages were abrogated when cells were incubated with Z-IETD-FMK (the caspase-8 inhibitor, data not shown), confirming the involvement of caspase-8 activity in the 2-SeCD enhanced sensitisation.

3.3. *2-SeCD induced MDA-MB-468 and T47D breast cancer cells to apoptosis by a mechanism involving G_2/M phase arrest*

The loss of regulatory control of the cell cycle is a hallmark of cancer, leading to unrestrained tumour cell proliferation.[34] As selenium compounds could interfere with cell cycle regulators,[35] we examined whether the apoptosis induced by 2-SeCD was mediated by specific cell cycle arrest. Cells were treated with TRAIL and/or 2-SeCD for 24 h, and then the cell-cycle distributions were investigated using flow cytometric analysis. Fig. 3A reveals that 2-SeCD treatment (20 μM) not only increases apoptotic cells at sub-G_0 fractions (20.2%), but also causes a significant arrest of cells in the G_2/M phase (38.3%), whereas TRAIL alone has no such effect on the cell cycle distribution. It is notable that 2-SeCD plus TRAIL further increases the number of cells at the sub-G_0 and G_2/M phase, compared to either 2-SeCD alone or TRAIL alone treatment. These data demonstrate the synergistic effect between TRAIL and 2-SeCD is associated with enhanced apoptosis as well as G_2/M phase arrest.

To investigate possible molecular mechanisms involved in the 2-SeCD-mediated cell cycle arrest, key molecules regulating the G_2/M boundary of the cell cycle phase[36] were examined in both MDA-MB-468 and T47D cell lines (Fig. 3B). After 0–48 h of incubation with 2-SeCD, protein levels of cyclin A, cyclin B1, p21 and p27 were examined by Western blotting. Although the levels of cyclin A and p27 were basically unchanged, 2-SeCD significantly downregulated the expression of cyclin B1, known to be essential for the progression of DNA synthesis and G_2/M transition, in a time- and dose-dependent manner. In addition, p21, an inhibitor of Cdk–cyclin B1 complex, was also significantly amplified after treatment with 2-SeCD. As expected, TRAIL alone did not lead to significant changes in key regulators of G_2/M progression (data not shown). Our data, therefore, imply that G_2/M cell-cycle arrest could be attributed to the down-regulation of cyclin B1 and the up-regulation of p21 in breast cancer cells by 2-SeCD treatment.

3.4. *Up-regulation of TRAIL receptor DR5 is responsible for sensitisation of breast cancer cells to TRAIL-induced apoptosis by 2-SeCD*

The activation of caspase-8 in the combined treatment suggests that 2-SeCD targets an early step in the TRAIL-induced apoptosis pathway. Therefore, we evaluated the effects of 2-SeCD on the expression of various proteins involved in TRAIL-initiated DISC components, including FADD, death associated protein 3 (DAP3), DcR1, DcR2 and critical death receptors (DR4/DR5). Although 2-SeCD had minimal effects on the expression levels of the vast majority of these proteins (Fig. 4 and data not shown), it notably increased DR5 protein levels in resistant breast cancer cells.

As shown in Fig. 4A 2-SeCD increased DR5 expression in the absence or presence of TRAIL in a dose-dependent manner. Note that the DR5 antibody used here recognised two DR5 splice variants, with approximate molecular weights of 43-kDa [$DR5_{S(Short)}$] and 48-kDa [$DR5_{L(Long)}$]. In contrast, 2-SeCD did not detectably alter expression levels of DR4, even at the highest concentration (80 μM). Additionally, flow cytometric analysis of death receptors, considered as a more sensitive method, was performed in both of the cell lines. Compared to increased expression of DR5, DR4 levels were not affected by 2-SeCD (Fig. 4B). Moreover, both cell surface and total protein expression levels of DR5 were increased in response to co-treatment with 2-SeCD plus TRAIL.

To determine whether 2-SeCD could regulate the gene expression of DR4 and DR5, we treated MDA-MB-468 and T47D cells with varying concentrations of 2-SeCD for 24 h, and thereafter, we analysed the expression of death receptor transcripts by means of real-time PCR. 2-SeCD treatment increased DR5 mRNA levels in both of the cell lines in a dose-dependent manner (Fig. 4C). However, 2-SeCD has no significant effects on the expression of DR4 mRNA, which are in agreement with the above results.

To clarify the functional significance of DR5 in the 2-SeCD-mediated sensitisation of TRAIL-induced apoptosis, we assessed the effects of DR5 knock-down by transfecting a short-interfering RNAs (siRNA) into cells. Through the detection of caspase activation and the quantification of apoptotic cells, we found that siRNA-mediated suppression of DR5 effectively blocks 2-SeCD-stimulated TRAIL-induced apoptosis (Fig. 5). Taken together, these data confirm that 2-SeCD-mediated DR5 up-regulation is essential for TRAIL sensitivity in resistant breast cancer cells.

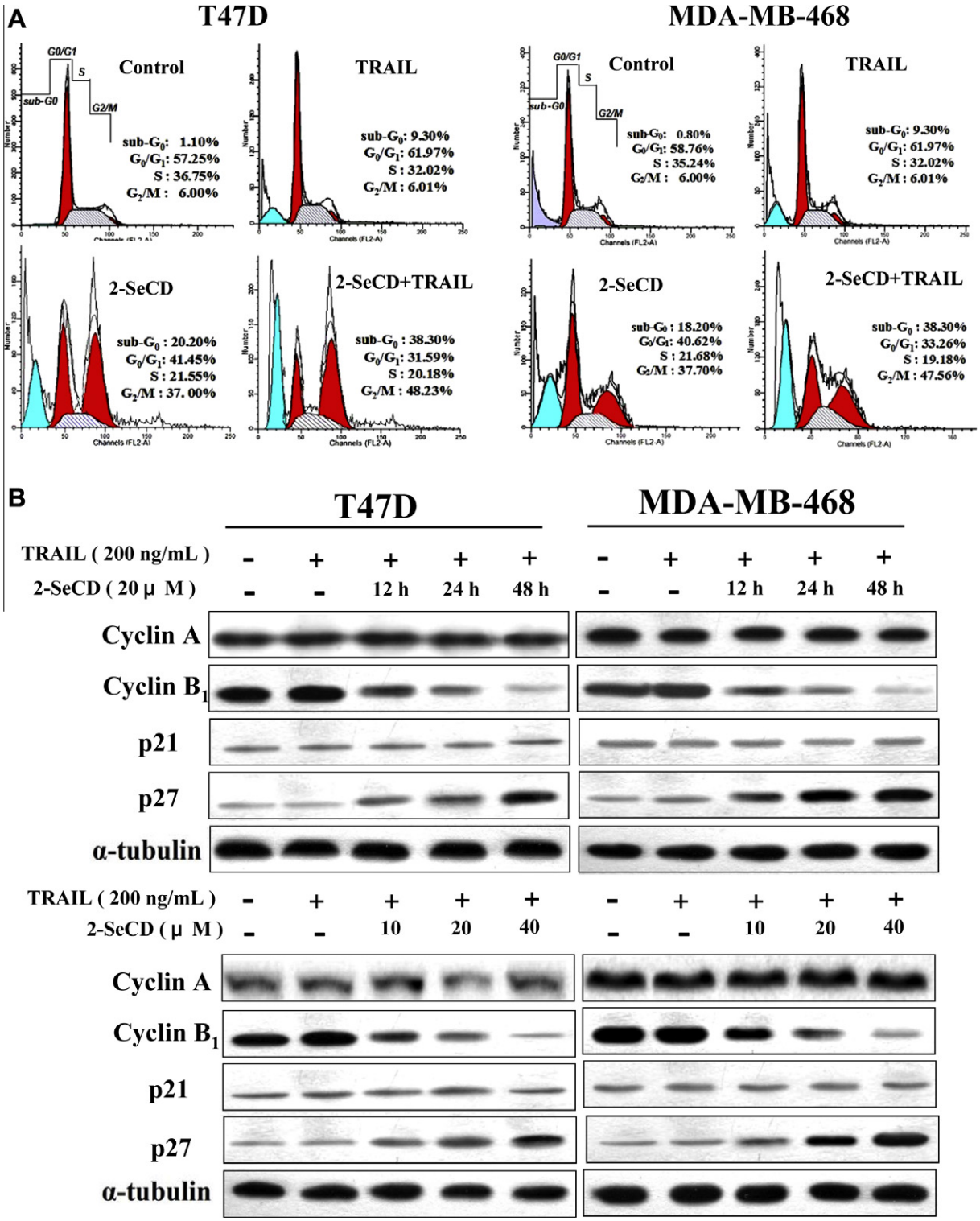


Fig. 3 – Effects of 2-SeCD on cell cycle arrest and cell cycle-associated regulators. (A) 2-SeCD enhances the accumulation of cells at G_2/M phase. Cells treated with TRAIL (200 ng/mL) and/or 2-SeCD (0–40 μM) were determined by flow-cytometric analysis. Insets indicated percentage of cells in sub-G_0, G_0/G_1, S and G_2/M phase of the cell cycle. Apoptotic cells were measured by quantifying the sub-G_0 peak in cell cycle pattern. (B) The expression levels of protein in response to treatment with control or 2-SeCD (20 μM) for 0–48 h. Cell lysates were then prepared immediately and analysed by Western blotting for cyclin A, cyclin B1, p21 and p27. Blots were re-probed for α-tubulin to establish equivalent loading.

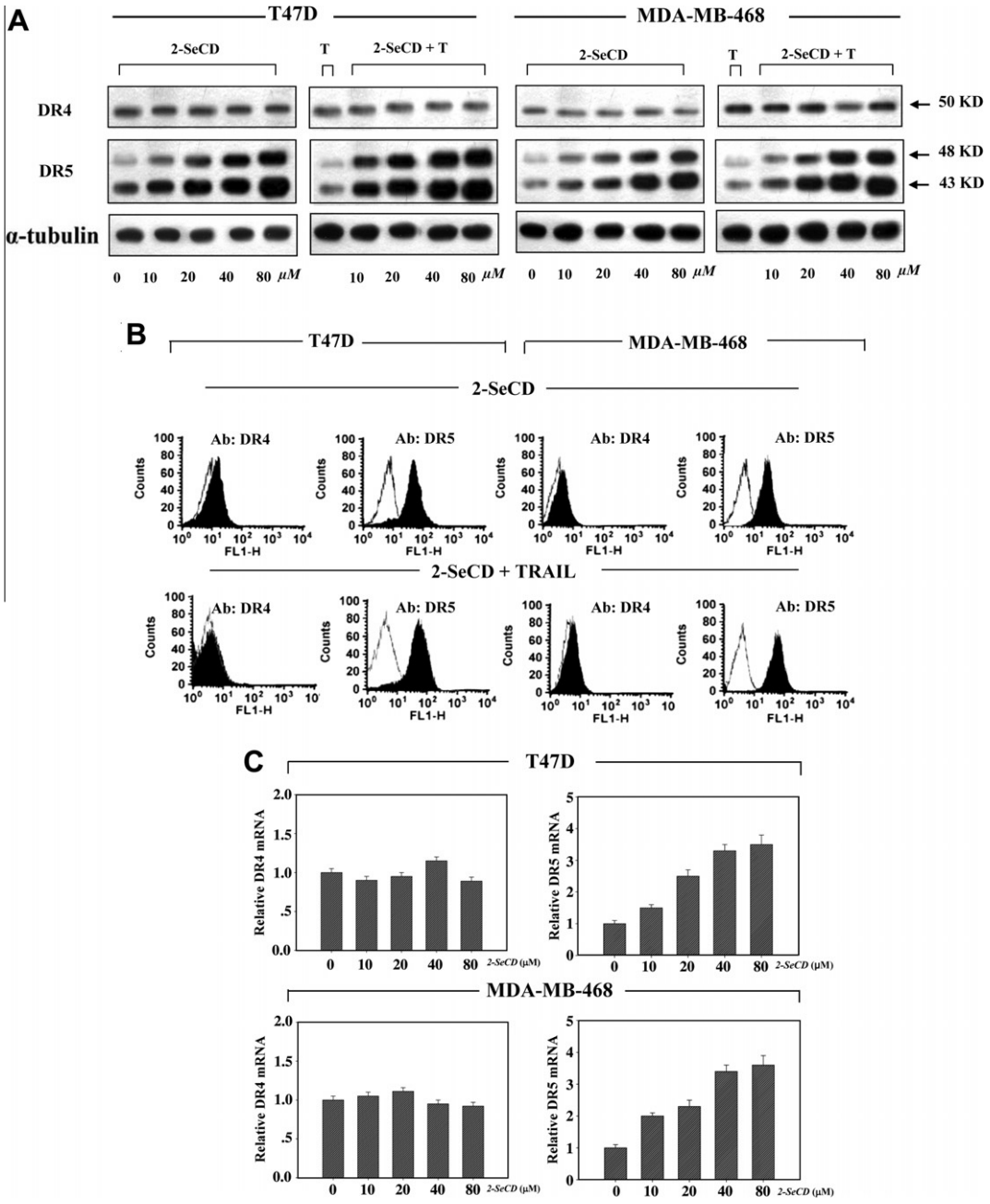


Fig. 4 – 2-SeCD increases DR5 protein and mRNA levels in breast cancer cell lines. (A) 2-SeCD treatment increases the DR5 protein levels, but not DR4. Immunoblotting was used to quantify total DR4 and DR5 protein levels in whole cell lysates following 24 h of 2-SeCD treatment, using cell lysates normalised for total protein content. (B) 2-SeCD alone or 2-SeCD plus TRAIL increase the surface expression levels of DR5. MDA-MB-468 and T47D cells were incubated with or without 2-SeCD (40 μM) for 24 h, and the surface expression of DR5 and DR4 proteins was analysed by flow cytometry. *x*-axis, Fluorescence intensity; *y*-axis, relative number of cells. Black histograms, cells treated with 2-SeCD alone or 2-SeCD plus TRAIL; white histograms, control cells. (C) 2-SeCD treatment increases the DR5 mRNA levels. Real-time RT-PCR was used to quantify the DR4 and DR5 mRNA levels following 24 h of 2-SeCD treatment. Fold increase of gene expression was calculated by dividing the normalised gene expression activity by that of the untreated control.

3.5. *2-SeCD inhibits TRAIL-induced NF-κB activation*

Several studies have implicated that resistant breast cancer cells could rapidly activate NF-κB to protect themselves from TRAIL-induced death.[37] However, the activity of NF-κB is regulated by its interaction with IκBα proteins, which can block NF-κB from entering the nucleus.[38,39] We, therefore, sought to examine whether 2-SeCD might regulate the expression

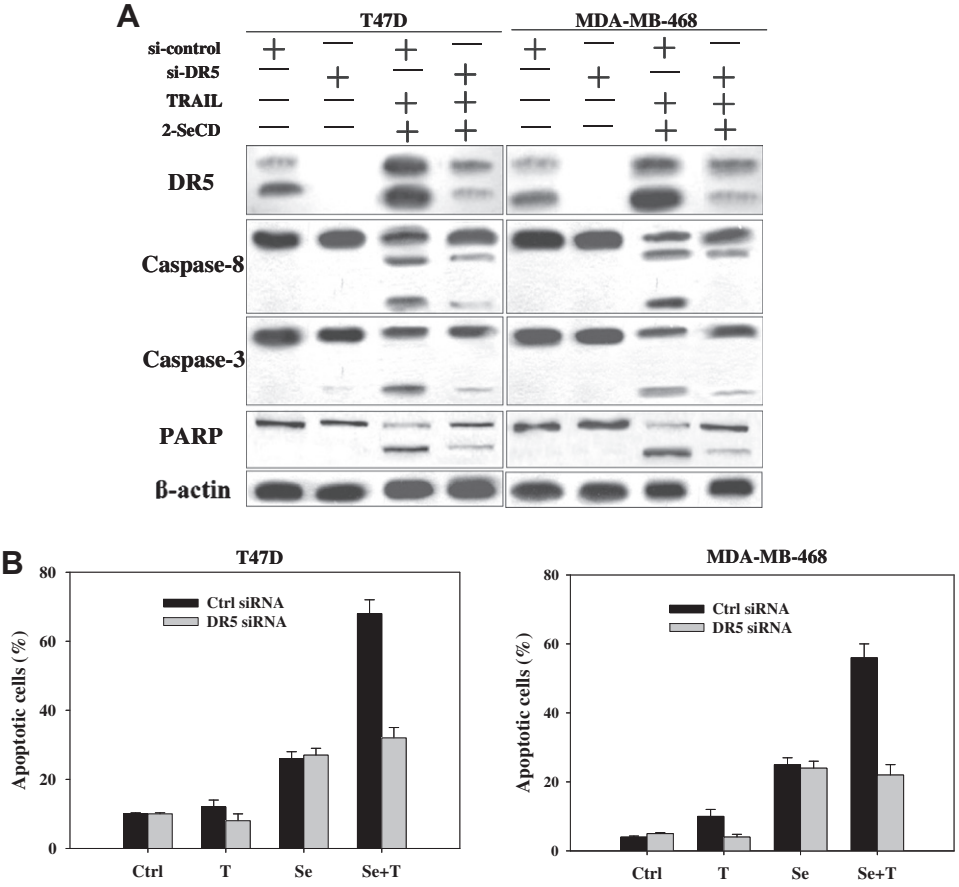


Fig. 5 – Suppression of DR5 expression by small interfering RNA (siRNA) reduces 2-SeCD-stimulated TRAIL-induced apoptosis in breast cancer cells. MDA-MB-468 and T47D cells were transfected with the control GFP siRNA or DR5 siRNA. Twenty four hours later, the cells were treated with TRAIL (200 ng/mL) and/or 2-SeCD (80 μM) for another 24-h treatment. (A) Cell lysates were then subjected to Western blot analysis with anti-caspase 8, -caspase 3, -PARP, respectively. Western blots of DR5 were done to confirm the knockdown of DR5 by siRNA transfection. (B) Apoptosis was also measured by Annexin V/propidium iodide method using flow cytometry. Columns, mean of three independent experiments; bars, SD.

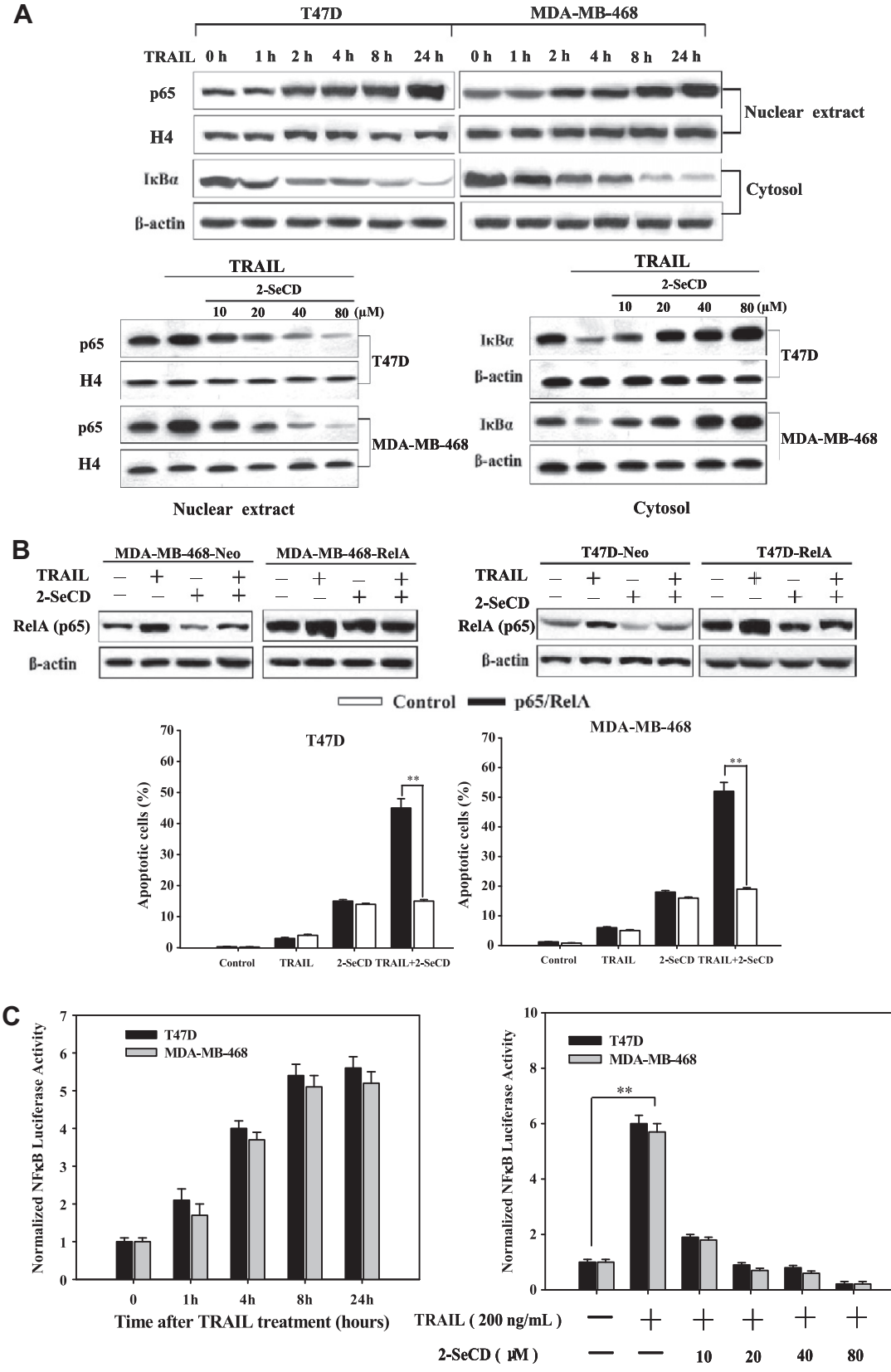
of NF-κB and its inhibitors to the sensitisation of breast cancer apoptosis induced by TRAIL. Fig. 6A reveals that the nuclear levels of p65 are elevated in TRAIL-treated cancer cells compared to the control. However, 2-SeCD treatments significantly attenuate p65 expression in a dose-dependent manner and almost abolished its nuclear translocation at 80 μM. Moreover, the findings of the cytoplasmic extracts indicated that 2-SeCD supplementation completely blocked TRAIL-induced degradation of IκBα, the NF-κB inhibitory subunit,[37] in both of the cell lines. Hence, the lack of IκBα degradation with escalating doses of 2-SeCD can be correlated with the minimal nuclear accumulation of p65.

To confirm that the increased sensitivity to TRAIL-mediated apoptosis was due to inhibition of p65, cells were transiently transfected with the pCMV4-p65 vector or the control vector (pCMV4-neo). Immunoblot analysis of whole cell extracts confirmed that MDA-MB-468-RelA cells and T47D-RelA cells expressed higher levels of RelA as compared to corresponding neo-cells (Fig. 6B). Then, we examined the effects of 2-SeCD in TRAIL-induced apoptosis. Consistent with the above results, 2-SeCD enhanced TRAIL-induced apoptosis in both of the cell lines; however, the sensitisation could be greatly reversed by ectopic expression of p65/RelA. This result indicates that 2-SeCD-mediated potentiation of TRAIL-induced apoptosis occurs partially through the inhibition of p65-mediated activation of NF-κB.

To further validate that 2-SeCD could inhibit the NF-κB pathway, we analysed the effects of 2-SeCD on NF-κB activation induced by TRAIL in cells using a luciferase-based NF-κB reporter assay. As shown in Fig. 6C, 24 h TRAIL treatment results in ~5-fold NF-κB activation compared to the control group. However, at this time point, 2-SeCD inhibited TRAIL-induced NF-κB activation in a dose-dependent manner, and it reduced the basal activity of NF-κB in both of the cell lines at 80 μM.

As discussed above, NF-κB translocation contributes to the TRAIL-resistance in cancer cells via its ability to up-regulate the IAPs family.[13] Hence, we evaluated the expression of mRNA and protein expression of these IAPs in cells treated with TRAIL in the absence and presence of 2-SeCD. As shown in Fig. 6D, TRAIL alone increased mRNA and protein expression levels of c-IAP1, c-IAP2, XIAP and survivin after 24-h incubation, whereas 2-SeCD co-treatment significantly

reduced the four IAP genes and protein expressions in both of the cell lines. This result indicates that 2-SeCD could suppress expression of anti-apoptotic molecules that are transcriptionally regulated by NF-κB.



3.6. 2-SeCD and TRAIL results in tumour growth inhibition and apoptosis in vivo

Based on the above findings, we next sought to evaluate whether the effect of 2-SeCD alone and in combination with TRAIL could inhibit tumour growth *in vivo*. As shown in Fig. 7A, TRAIL is ineffective in inhibiting the tumour growth; however, 2-SeCD clearly inhibits the growth of MDA-MB-468 xenografts in nude mice. More importantly, the combination of 2-SeCD and TRAIL leads to a significant inhibitory effect compared to either agent alone or vehicle control groups. Indeed, the inhibition of tumour growth begins as early as within the first 7 d and is maintained throughout the entire experimental period, representing a substantial antitumour effect between the two agents *in vivo*. Finally, these treatments were well tolerated in nude mice, based on the 100% survival rate and the minimal changes in body weight that were observed (Fig. 7A).

We next evaluated the effects of agent treatment on tumour cell proliferation and apoptosis (Fig. 7B). Very few apoptotic cells were found in the vascularised tumour tissues derived from control mice, indicating an environment able to support the establishment of xenografts. Scoring immunohistochemical examination of tumour tissues revealed that 2-SeCD alone inhibited cell proliferation [proliferating cell nuclear antigen (PCNA) and Ki-67 staining] and induced apoptosis (TUNEL staining) in xenograft tumours. On the contrary, TRAIL had no effect on either tumour cell proliferation or apoptosis compared with the control group. Importantly, the combination of 2-SeCD and TRAIL was more effective in inhibiting tumour cell proliferation and inducing apoptosis than the use of a single agent alone.

3.7. 2-SeCD in combination with TRAIL regulates the expression of p65-NF-κB and DR5 in vivo

We next sought to confirm the effects of 2-SeCD in combination with TRAIL on p65-NF-κB and DR5 expression levels *in vivo* (Fig. 7C). Xenograft sections from each of the treatment groups were immunostained with anti-p65 and anti-DR5 antibodies, respectively. TRAIL alone had no significant effect on the expressions of p65-NF-κB and DR5. By comparison, treatment of mice with a combination of 2-SeCD and TRAIL significantly led to more expression of DR5 and less expression of p65-NF-κB proteins than that seen in mice treated with vehicles or TRAIL, which were all consistent with our *in vitro* results.

4. Discussion

The advantage of the clinical application of TRAIL is that normal cells are resistant to its cytotoxic activity; and thus, it specifically targets cancer cells. In spite of this observation, TRAIL will probably not be viable as a single agent since the majority of tumour cells are resistant to TRAIL. A combination therapy (chemotherapy or radiation) is, therefore, essential for the use of TRAIL against refractory tumours. In this report, we found that 2-SeCD is capable of sensitising highly resistant breast cancer cells to TRAIL-mediated apoptosis, which is evidenced by the activation of caspase-3, caspase-8 and PARP. Furthermore, 2-SeCD acts synergistically with TRAIL to reduce the tumour burden in the MDA-MB-468 xenograft model. Importantly, we revealed the underlying mechanisms of apoptosis induction and TRAIL-sensitisation by 2-SeCD, which involve cell cycle arrest, DR5 induction and NF-κB suppression (summarised in Fig. 8).

As an organoselenium compound, 2-SeCD induced MDA-MB-468 and T47D breast cancer cells to apoptosis at least in part by a mechanism involving G_2/M phase arrest. Specifically, we have shown that cell cycle arrest is associated with the reduction of cyclin B1, as well as the induction of p21, which have been confirmed as the key regulators of G_2/M progression. These results are consistent with other studies showing that organic selenium compounds are involved in the control of G_2/M arrest in cancer cells.[40] As TRAIL has no such effects on cell cycle regulation, 2-SeCD could stimulate other apoptosis pathways in breast tumours; thus, providing a possible explanation for the observation that 2-SeCD, in the absence of TRAIL, also modestly induces apoptosis in breast tumours. Indeed, this finding is encouraging as the

Fig. 6 – 2-SeCD inhibits TRAIL-induced nuclear factor-κB (NF-κB) activation. (A) Effects of TRAIL combined with 2-SeCD on p65 and IκBα expression. Cells were treated with 200 ng/mL TRAIL for the indicated time (top). Then, cells were treated with 200 ng/mL TRAIL in combination with various concentrations of 2-SeCD (10–80 μM) for 24 h (bottom). Nuclear proteins and cytosolic fractions were extracted and assayed for p65 and IκBα by Western blotting. H4 and β-actin were used as internal controls in nuclear and cytosolic specimens, respectively. (B) RelA (p65) overexpression decreases 2-SeCD mediated sensitisation of breast cancer cells to TRAIL. MDA-MB-468 and T47D cells were transiently transfected with neo or p65/RelA vectors, respectively. The cells were then treated with 2-SeCD (80 μM) and/or TRAIL (200 ng/mL) for 24 h. After incubation, Western blots were performed to determine the overexpression of p65/RelA (top). The apoptotic cells (Annexin V-positive/propidium iodide-negative) in each treatment condition were evaluated by FACS (bottom). (C) 2-SeCD inhibits TRAIL-induced NF-κB activation in NF-κB luciferase reporter assay. MDA-MB-468 and T47D were transiently cotransfected with pNF-κB or pControl together with β-galactosidase plasmid, and then treated with TRAIL for the indicated time (left). Transfected cells were treated with 200 ng/mL TRAIL in the presence of 2-SeCD (10–80 μM) for 24 h (right). Data are expressed as fold increase relative to untreated cells. Columns, mean of three independent experiments; bars, SD. $^{**}P < 0.01$ versus untreated cells. (D) Treatment with 2-SeCD decreases gene and protein expression levels of NF-κB targets inhibitors of apoptosis (IAPs) in breast cancer cells. Cells were treated with 200 ng/mL TRAIL in combination with various concentration of 2-SeCD (10–80 μM) for 24 h. After incubation, mRNA and protein levels of IAPs (IAP-1, XIAP and survivin) were analysed using real-time PCR (top) and Western blot (bottom), respectively.

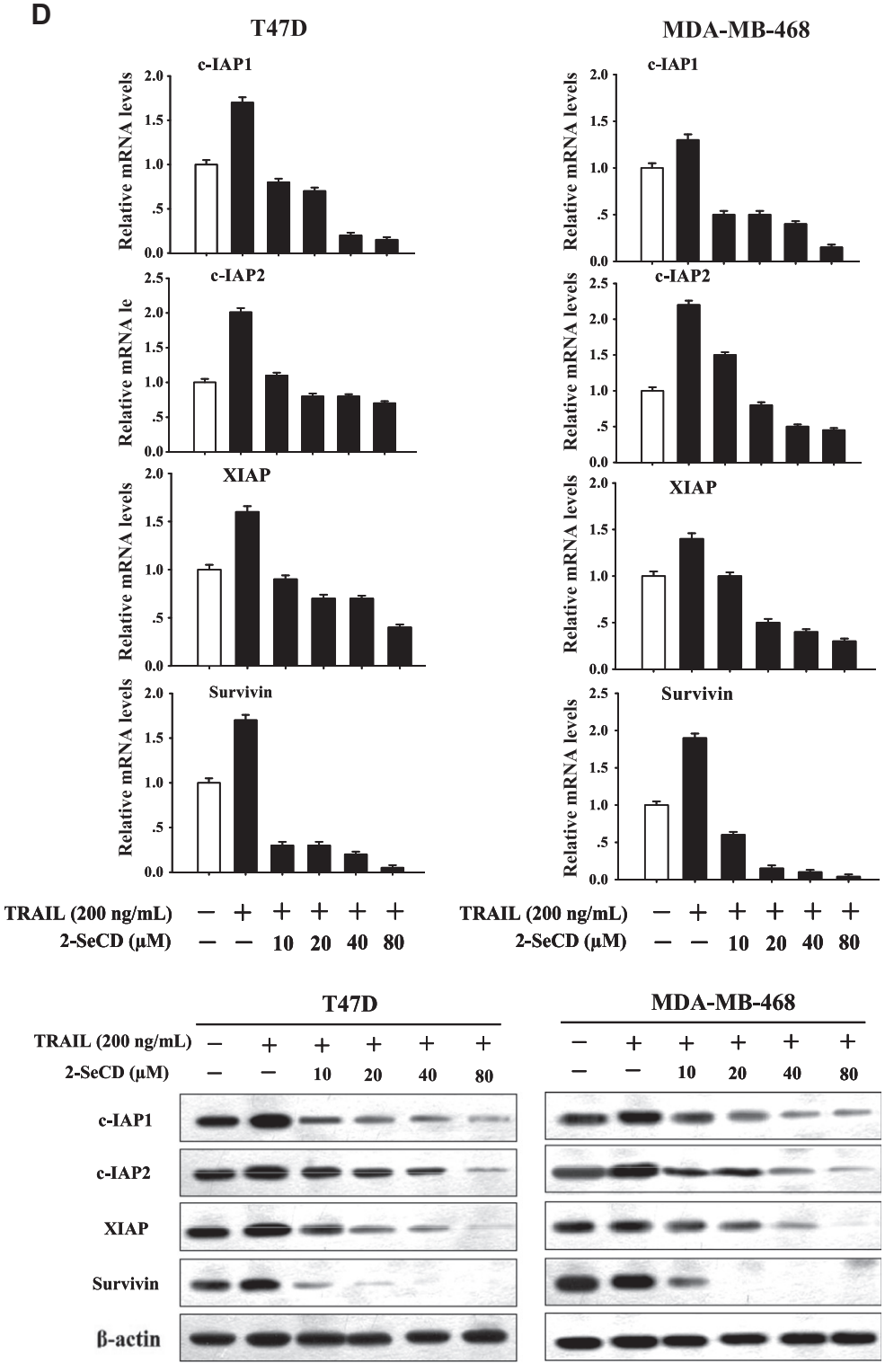


Fig. 6 *(continued)*

dual treatment might activate distinct apoptosis mechanisms, which can result in greater potency for the killing of tumour cells.

The sensitisation of tumour cells to TRAIL by 2-SeCD is associated with the down-regulation of anti-apoptotic protein NF-κB and up-regulation of death receptor DR5. We speculate that both of these factors affect TRAIL sensitivity in cancer cells. As a member of the Rel transcription factor family, NF-κB is normally retained in the cytoplasm by binding IκBα. Once triggered by different pathogenic stimuli (chemical agents or cytokine), IκBα starts to degrade, and NF-κB can translocate into the nucleus to up-regulate a number of genes involved in survival responses. Evidence suggests that NF-κB is a negative regulator of TRAIL-mediated cancer apoptosis

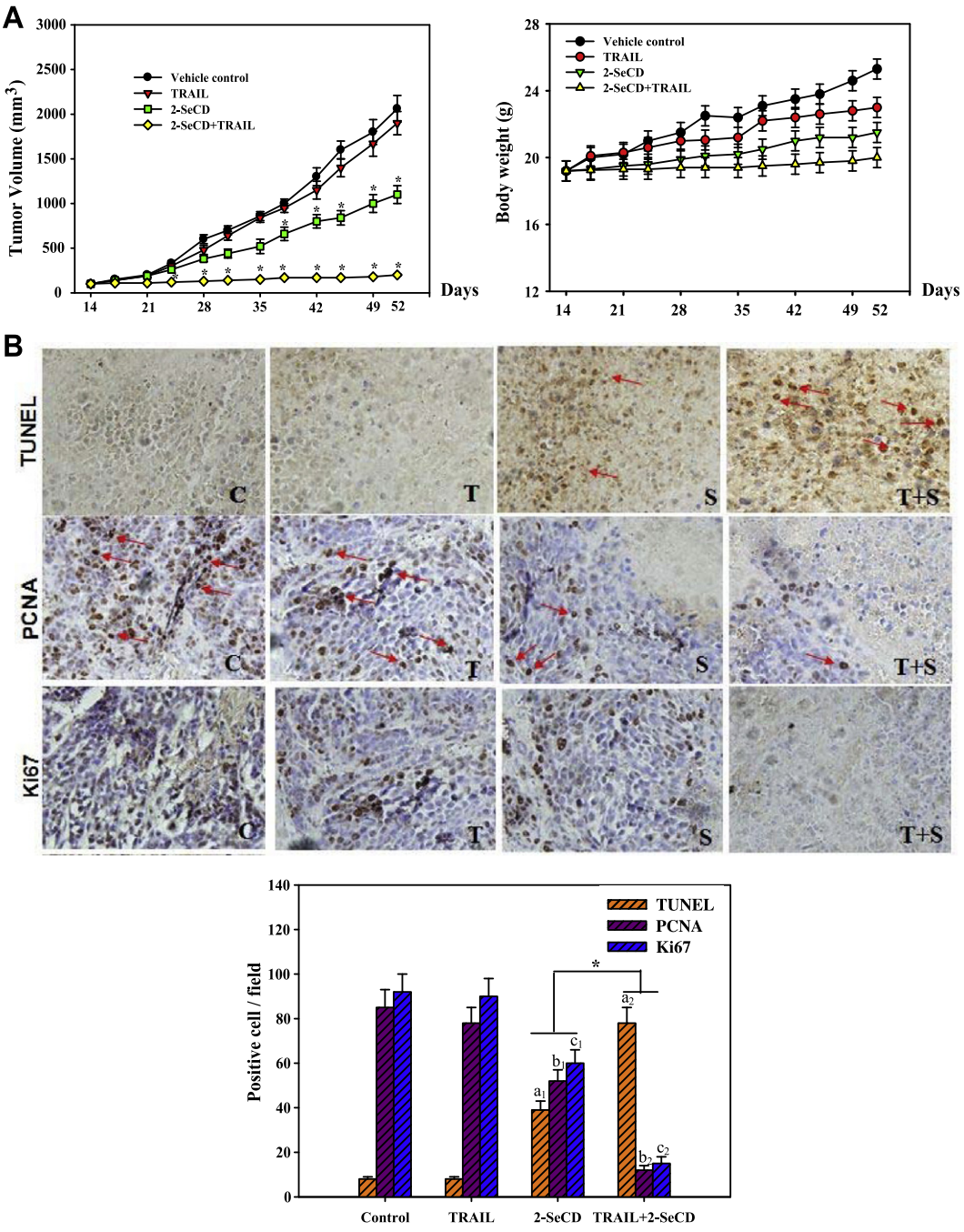


Fig. 7 – Effects of 2-SeCD and TRAIL on MDA-MB-468 xenografts in nude mice. (A) Tumour volumes and animal weight were recorded during the 38 d of treatment. Points, mean of eight mice in each group; bars, SD. *P < 0.05 versus control group. (B) Effects of 2-SeCD and/or TRAIL on apoptosis (terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining) and cell proliferation (proliferating cell nuclear antigen (PCNA) and Ki-67 staining) of tumour tissues derived from control and treated mice on day 52 by immunohistochemistry staining (top). C, T and S denote control, TRAIL and 2-SeCD, respectively. Quantification of TUNEL, PCNA and Ki-67 positive tumour cells (bottom). a_1, a_2; b_1, b_2 and c_1, c_2 are significantly different from their respective control groups. *P < 0.05 versus 2-SeCD group. (C) Effects of 2-SeCD and/or TRAIL on p65-NFκB and death receptors, and quantification of positive tumour cells. a_1, a_2 and b_1, b_2 are significantly different from their respective control groups; *P < 0.05 versus 2-SeCD group; #P < 0.05 versus TRAIL treated group. Tumour slides of different treatment groups were visualised under a microscope, and the positive cells (red arrows) were quantified. Original magnification 40×.

and a functionally important target for TRAIL-sensitising actions.[41,42] In this study, we clearly demonstrated that 2-SeCD inhibits the nuclear translocation of p65-NF-κB in a dose-dependent manner via stabilisation of its physiological

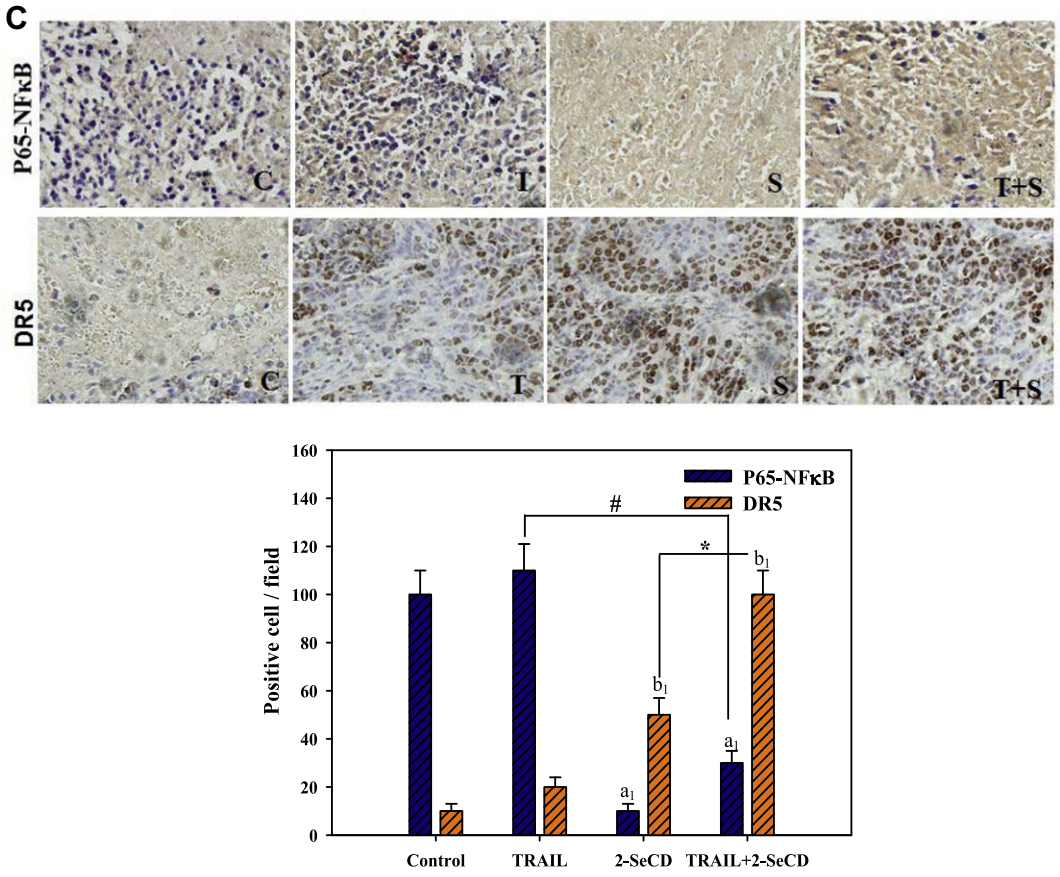


Fig. 7 *(continued)*

inhibitor, IκBα. This result is consistent with our earlier observations that 2-TeCD, a similar product to 2-SeCD, is able to suppress the translocation of NF-κB that was stimulated by TNFα.[32] Since traditional selenium compounds were not reported to target NF-κB activity in sensitising TRAIL-resistant cancers,[43,44] we hypothesise their unique GPx-like activity would be an attractive explanation. GPx and its mimics have been demonstrated to inhibit NF-κB translocation by increasing the IκBα half-life, and, therefore, they play significant roles in the prevention of cancer cell growth.[28,45,46] Furthermore, other evidence indicates that GPx over-expression could specifically impair NF-κB activation, p65 phosphorylation, and its nuclear translocation through a redox-regulated mechanism.[47–49] In addition, 2-SeCD might also down-regulate NF-κB by inhibiting the activity of thioredoxin reductase, which have been demonstrated to activate NF-κB through direct or indirect pathways.[50,51] Taken together, our findings indicate that the TRAIL-sensitising effects of 2-SeCD are mediated at least in part by NF-κB down-regulation, which is also strongly supported by critical *in vivo* data.

The death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, are selectively expressed in cancer cells and thus, offer an advantage for TRAIL-targeted therapy and prevention. In this study, it is clearly evident that 2-SeCD causes up-regulation of DR5 in a dose-dependent manner, thus creating a more TRAIL-sensitive environment. Further studies are still necessary to explore the intrinsic mechanisms; however, 2-SeCD-suppressed p65 translocation might provide a possible explanation. It has been recently discovered that p65 induction specifically inhibits the expression of death receptors.[52,53] Hence, 2-SeCD-up-regulated DR5 could be a consequence of p65 suppression. In this respect, the sensitisation of breast cancer cell to TRAIL by 2-SeCD can be explained by a unifying mechanism. Intriguingly, we observed that 2-SeCD does not promote DR4 production in the tested cancer cell lines. The molecular mechanism associated with the differential effects is not yet known; however, we cannot exclude the possibility that 2-SeCD may also affect the localisation, posttranslational modification, or other specific components of the DR4 signalling complex.

The potent pro-apoptotic effect of 2-SeCD to sensitise TRAIL against breast carcinoma cell lines *in vitro* prompted us to evaluate its *in vivo* antitumour effects in Balb/c nude mice bearing MDA-MB-468 tumour xenografts. Our results show that 2-SeCD significantly sensitises xenografts to undergo apoptosis by TRAIL. Tumour tissues derived from the combined treatment group showed that 2-SeCD and TRAIL synergistically inhibit proliferation (PCNA and Ki67 staining) and induce apoptosis (TUNEL staining). Moreover, the sensitising effects of 2-SeCD on TRAIL-induced apoptosis *in vivo* are also associated with DR5 up-regulation and NF-κB suppression, which are all consistent with the *in vitro* results. Of note, this report provides the first preclinical data concerning the *in vivo* efficacy and safety of 2-SeCD in combination with TRAIL. At doses resulting in significant suppression of tumour xenograft growth, the combination of 2-SeCD and

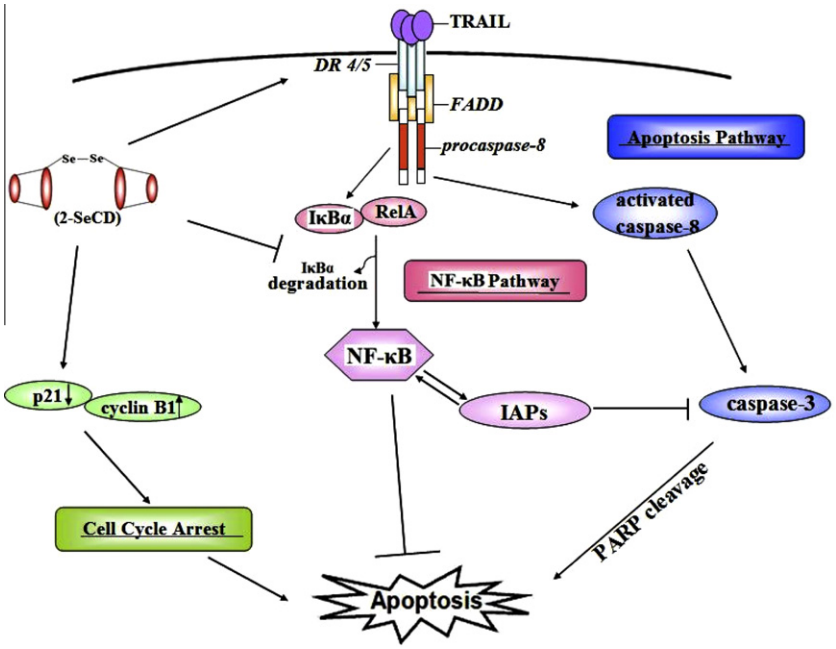


Fig. 8 – Working model of 2-SeCD in sensitising TRAIL-induced apoptosis by diverse mechanisms. Induction of DR5 expression by 2-SeCD contributes to cell sensitivity to TRAIL-based apoptosis and subsequent activation of initiator caspase-8, as well as effector caspase-3. In addition, 2-SeCD suppresses TRAIL-induced NF-κB activation by preventing IκBα degradation and RelA (p65) nuclear translocation. As a consequence, blockade of NF-κB – cellular inhibitors of apoptosis (cIAPs) signalling by 2-SeCD abolishes counteraction of pro-survival factors on TRAIL-mediated apoptosis. Moreover, G_2/M cell cycle arrest induced by 2-SeCD was associated with p21 induction and cyclin B1 suppression, which can result in greater potency for the killing of cancer cells by the combination treatment.

TRAIL is well tolerated in mice, without apparent toxicity to normal tissues.

In summary, we have highlighted a novel function of 2-SeCD: it sensitises human cancer cells to TRAIL-induced apoptosis via the up-regulation of DR5 and down-regulation of NF-κB. Our studies provide strong clinical evidence that 2-SeCD in combination with TRAIL can be used for the treatment of breast cancer.

Conflict of interest statement

None declared.

Acknowledgements

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