BIOCHEMICAL PHARMACOLOGY 75 (2008) 847–856

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WRC-213, an L-methionine-conjugated mitoxantrone derivative, displays anticancer activity with reduced cardiotoxicity and drug resistance: Identification of topoisomerase II inhibition and apoptotic machinery in prostate cancers

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ARTICLE INFO

Article history:
Received 13 August 2007
Accepted 2 October 2007

Keywords:
Topoisomerase II inhibitor
Cell-cycle arrest
Mitochondria
Cardiotoxicity
Drug resistance

ABSTRACT

Anthracyclines and anthracenediones are well-known cancer chemotherapeutic agents but their uses are limited with cardiotoxicity and drug resistance. Several L- and D-form amino acids were introduced into the anthraquinone skeleton and numerous derivatives were synthesized for the evaluation of anticancer activity. The screening tests showed that WRC-213, an L-methionine conjugation, was the most effective derivative to inhibit proliferative effect of human androgen-independent prostate cancer PC-3 cells (IC_{50} = 50 nM). In an extension evaluation, WRC-213 displayed a potent anti-proliferative activity in various cancer cell lines, including non-small cell lung cancer A549, androgen-independent prostate cancer DU145, colorectal cancer HT-29, breast cancer MCF-7 and hepatocellular carcinoma Hep3B and HepG2. It induced cell-cycle arrest at S and G2, but not mitotic phase, in PC-3 cells. The comet assay revealed that induction of DNA damage and inhibition of topoisomerase II were the primary insults. After the checkpoint arrest of the cell-cycle, WRC-213 induced the mitochondria-mediated intrinsic apoptotic pathway, including Mcl-1 cleavage, Bcl-2 down-regulation and activation of caspase-9/caspase-3 cascades. Survivin degradation and caspase-2 activation also contributed to WRC-213-induced apoptosis. Moreover, the assessment of cytotoxicity in H9c2 cardiomyocytes and drug resistance in NCI/ADR-RES cells demonstrated that WRC-213 showed much lower cardiotoxicity and P-glycoprotein-related resistance than those of mitoxantrone, etoposide and doxorubicin. In conclusion, it is suggested that WRC-213 is a potential topoisomerase II inhibitor with reduced cardiotoxicity and drug resistance. It inhibits topoisomerase II activity and induces chromosomal DNA strand breaks, leading to S and G2 arrest of the cell-cycle and activation of mitochondria-mediated apoptotic pathways.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.10.012

1. Introduction

The nuclear enzymes topoisomerase I and II are essential for the determination of the number of supercoils in DNA that delicately regulates the DNA structure as well as numerous cellular functions including transcription, translation and mitosis [1,2]. There are many lines of evidence supporting that topoisomerase I and II are promising targets for active anticancer drugs [3,4]. To date, several topoisomerase inhibitors are clinically active against numerous types of tumors. Camptothecin and its derivatives interact with topoisomerase I. In contrast, several agents with complicated structures interact with topoisomerase II. These agents are actinomycin D, etoposide, doxorubicin, elliptinium acetate and olivacine derivatives [1,2,5]. Although topoisomerase I and II share the same target, they utilize very different enzymatic mechanisms. Accordingly, the inhibitors topoisomerase I and II trigger distinct signaling pathways in cancer cells. Topoisomerase II cleaves DNA double strands, leading to a transition state complex of the enzyme and DNA of altered linking number. A variety of topoisomerase II inhibitors stabilize the intermediate complex being ternary complexes that cause DNA damage and trigger the cell death mechanism [6].

Etoposide (a podophyllotoxin congener), doxorubicin (an anthracycline antibiotic) and mitoxantrone (an anthraquinone) belong to the class of topoisomerase II inhibitors and are commonly used chemotherapeutic drugs against solid tumors, leukemias and lymphomas [3–7]. Although topoisomerase II inhibitors induce apoptotic cell death, the kinetics of the death program show that the apoptosis is not an early event after the DNA damage [8–10]. More specific signaling pathways are indicated including ATM activation, cell-cycle arrest, inhibition of cyclin-dependent kinase-1 (Cdk1) [11,12], activation of p38 mitogen-activated protein kinase (MAPK) [12,13], generation of reactive oxygen species (ROS) [13,14] and activation of nuclear factor-κB (NF-κB) [15]. Although several mechanisms contribute to the anti-tumor activity of toposiomerase II inhibitors for the treatment of human malignancies, they always lead to the occurrence of multidrug resistance and undesired toxic effects. There are several lines of evidence that the multidrug resistance may result from several cellular events, namely, (i) the decrease of cellular level of the inhibitors by P-glycoprotein, (ii) the decrease of topoisomerase II expression, (iii) the reduction of topoisomerase II activity by deactivation or mutations of the topoisomerase II gene and (iv) the increased detoxification by glutathione-dependent enzymes [6,16,17]. Different topoisomerase II inhibitors may elicit various toxic effects at different levels. The toxicity that has been widely reported includes myelosuppression, cardiac toxicity and secondary leukemia [18,19]. In particular, the acute or chronic cardiac toxicity may limit the clinical use of topoisomerase II inhibitors.

Several cellular events or mechanisms have been identified to involve in the cardiac toxicity including ROS generation, stimulation of sarcoplasmic reticulum calcium release and impairment of iron and calcium homeostasis [20]. The research work on the development of new derivatives or adoption of specific delivery systems has been focused to reduce the related toxicity [21]. In this study, more than 100 anthracenedione derivatives have been synthesized and screened for the determination of the anticancer activity and relative cardiotoxicity. We found that (*S*,*S*)1,4-bis[2-(4-methylsulfanyl-butyrylamino)ethylamino]-5,8-dihydroxyanthracene-9,10-dione, which was designated WRC-213, exhibited potent anti-proliferative activity against numerous cancer cell lines. The anticancer mechanisms of WRC-213 have been delineated including the determination of topoisomerase activities, the regulators of cell-cycle progression, mitochondria-related signaling pathways and caspase activation cascades. Moreover, the comparison of cardiotoxicity and drug resistance between WRC-213 and other topoisomerase II inhibitors has been determined to evaluate the potential for further development.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). The following antibodies were used: caspase-8, caspase-9 and $p21^{Cip1/Waf1}$ (Cell Signaling, Beverly, MA, USA), Bcl-2, Bcl-xL, Mcl-1, Bax, survivin, cyclin A, B_1 and E, cyclin-dependent kinase 1 (Cdk1) and Cdk2 (Santa Cruz, Delaware Ave, USA), caspase-3 (Imgenex, San Diego, CA, USA), α-tubulin (Serotec Products, Beverly, MA, USA), HRP-conjugated anti-mouse and anti-rabbit IgGs (Calbiochem-Novabiochem, San Diego, CA, USA). Antibody to MPM-2 was from Upstate Biotechnology (Lake Placid, NY, USA). Sulforhodamine B (SRB) and all of the other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). WRC-213, (*S*,*S*)1,4-bis[2-(4-methylsulfanyl-butyrylamino)ethylamino]-5,8-dihydroxyanthracene-9,10-dione (Fig. 1A), was synthesized and provided by our colleagues (Dr. Ling-Wei Hsin). The purity is more than 98% by the examination of HPLC and NMR.

2.2. Cell cultures

NCI/ADR-RES cell line was from NCI, USA. The other cell lines were from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI1640 medium with 10% FBS (v/v) and penicillin (100 units/ml)/streptomycin (100 g/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. Sulforhodamine B assay

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of compound addition (T_0). After additional incubation of vehicle (0.1% DMSO) or the indicated compound for 48 h, cells were fixed with 10% TCA and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (*C*) and cell growth in the presence of compound (Tx), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $[(Tx - T_0)/(C - T_0)] \times 100$ for concentrations for which

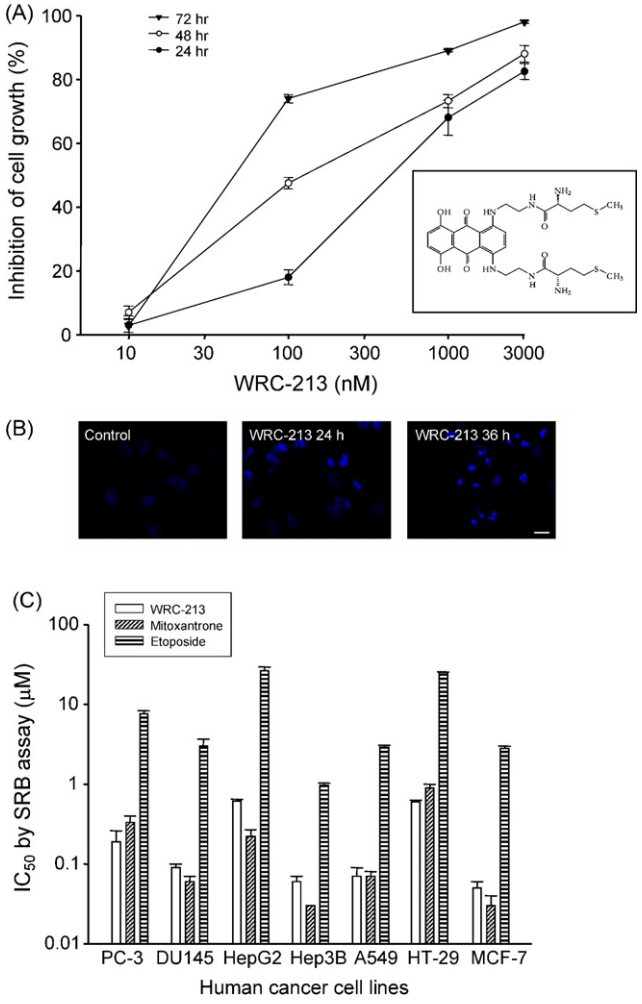

Fig. 1 – Identification of WRC-213-induced anti-proliferative effect and apoptosis. (A) The chemical structure of WRC-213. PC-3 cells were treated with WRC-213 at the indicated concentration for 24, 48 or 72 h. Then, the cells were fixed and stained with SRB. After a series of washing, bound SRB was subsequently solubilized and the absorbance was read at a wavelength of 515 nm. Data are expressed as mean ± S.E.M. of four determinations (each in triplicate). (B) PC-3 cells were treated without or with WRC-213 (3 μM) for 24 or 36 h. The apoptosis was detected by Hoechst 33342 staining. Scale bar, 20 μm. (C) Different cell lines were treated with WRC-213 at various concentrations for 48 h. Then, the cell proliferation was examined using SRB assay. Data are expressed as mean ± S.E.M. of three to five determinations (each in triplicate).

$Tx \geq T_0$. Growth inhibition of 50% (IC_{50}) is determined at the drug concentration which results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. *In situ labeling of apoptotic cells*

In situ detection of apoptotic cells was performed using Hoechst 33342 apoptosis detection methods. After the treatment, cells were washed twice with PBS, stained with Hoechst 33342 (1 μg/ml) for 15 min at 37 °C and fixed for 15 min with 4% paraformaldehyde. The cell apoptosis was examined under a confocal laser microscopic system (Leica TCS SP2).

2.5. *FACScan flow cytometric analysis*

After the treatment of cells with vehicle (0.1% DMSO) or compound for the indicated concentrations and times, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 ml of phosphate–citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH 7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml propidium iodide solution containing Triton X-100 (0.1%, v/v), RNase (100 μg/ml) and propidium iodide (80 μg/ml). DNA content was analyzed with the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA, USA).

2.6. *Comet assay*

After the treatment, the cells (2 × 105, 30 min) were pelleted and resuspended in ice-cold PBS. The resuspended cells were mixed with 1.5% low melting point agarose. This mixture was loaded onto a fully frosted slide that had been pre-coated with 0.7% agarose and a coverslip was then applied to the slide. The slides were submerged in pre-chilled lysis solution (1% Triton X-100, 2.5 M NaCl and 10 mM EDTA, pH 10.5) for 1 h at 4 °C. After soaking with pre-chilled unwinding and electrophoresis buffer (0.3N NaOH and 1 mM EDTA) for 20 min, the slides were subjected to electrophoresis for 15 min at 0.5 V/cm (20 mA). After electrophoresis, slides were stained with 1× Sybr Gold (Molecular Probes) and nuclei images were visualized and captured at 400× magnifications with an Axioplan 2 fluorescence microscope (Zeiss) equipped with a CCD camera (Optronics). Over hundreds of cells were scored to calculate the overall percentage of comet tail-positive cells.

2.7. *Western blotting*

After the treatment, the cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μl ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% Triton X-100). For Western blot analysis, the amount of proteins (40 μg) were separated by electrophoresis in a 10 or 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4 °C in PBS/5% non-fat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 h and immuno-reacted with the indicated antibody for 2 h at room temperature. After four washings with PBS/0.1% Tween 20, the anti-mouse or anti-rabbit IgG (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 h and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham).

2.8. *Mitochondrial MTT reduction activity assay*

Cells were incubated in the absence or presence of the compound for the indicated concentrations and times, and

then the mitochondrial MTT reduction activity in PC-3 cells was assessed. MTT was dissolved in PBS at a concentration of 5 mg/ml and filtered. From the stock solution, 10 μl per 100 μl of medium was added to each well, and plates were gently shaken and incubated at 37 °C for 1 h. After the loading of MTT, the medium was replaced with 100 μl acidified β-isopropanol and was left for 5–10 min at room temperature for color development, and then the 96-well plate was read by enzyme-linked immunosorbent assay reader (570 nm) to get the absorbance density values.

2.9. Data analysis

Data are presented as mean ± S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by Bonferroni *t*-test and *P*-values less than 0.05 were considered significant.

3. Results

3.1. Effect of WRC-213 on the inhibition of proliferation in several cancer cell lines

The effect of WRC-213 on cell proliferation was examined in human androgen-independent prostate cancer PC-3. The data demonstrated that WRC-213 displayed a time- and concentration-dependent inhibition of cell proliferation. The IC_{50} values were 0.44, 0.12 and 0.05 μM for 24-, 48- and 72-h

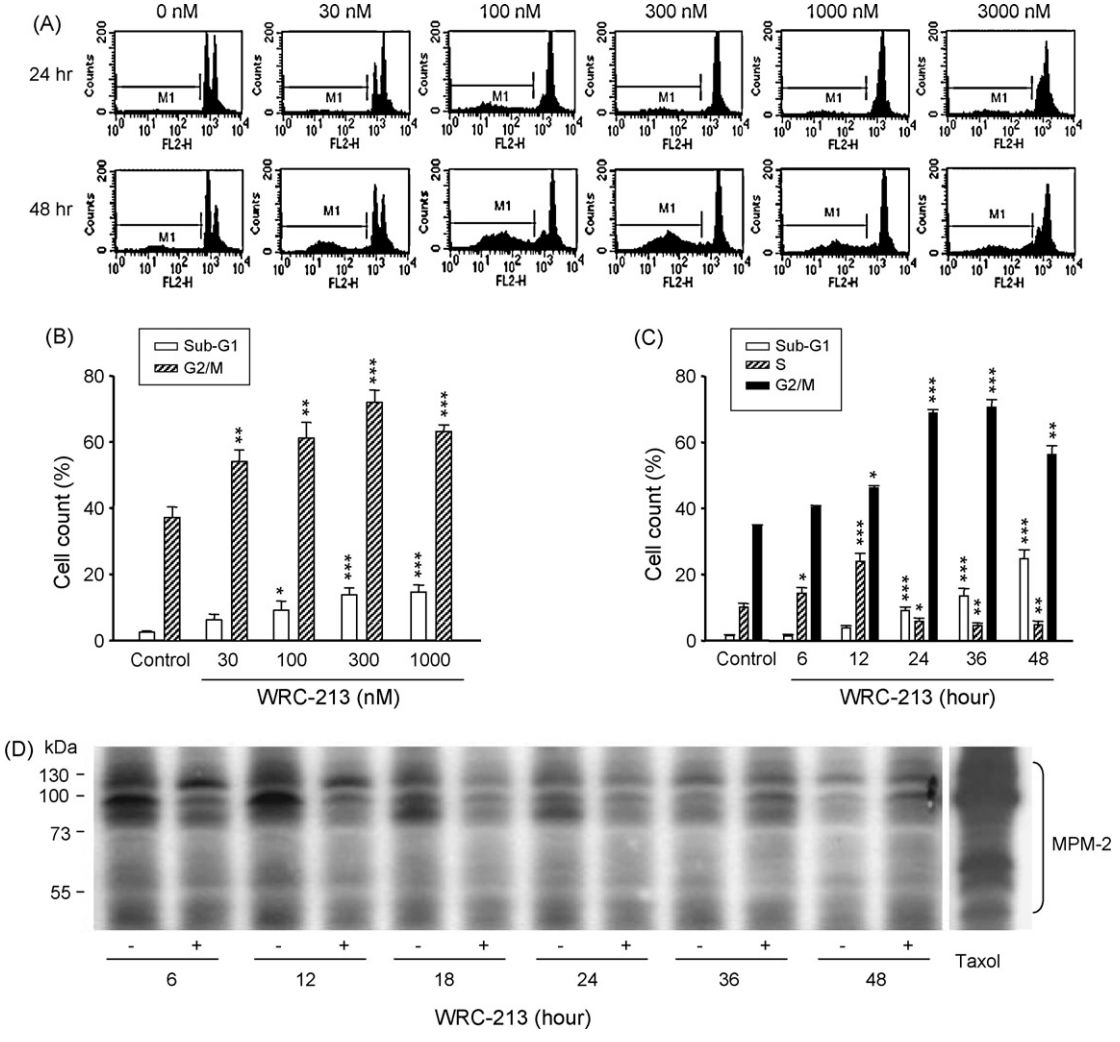

Fig. 2 – Examination of cell-cycle progression and detection of mitotic MPM-2 phosphoepitopes. PC-3 cells were treated with WRC-213 at the indicated concentration for 24 (A and B) or 48 h (A) or PC-3 cells were treated with 0.3 μM WRC-213 for various times (C). Then, the cells were fixed and stained with propidium iodide to analyze DNA content by FACScan flow cytometer. Data are representative of three independent experiments (A) or the data are expressed as mean ± S.E.M. of three determinations (B and C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the respective control. (D) PC-3 cells were incubated in the absence or presence of WRC-213 (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of MPM-2 phosphoepitopes. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. Data are representative of three independent experiments.

treatment, respectively (Fig. 1A). WRC-213 also induced apoptotic cell death by detection of condensed DNA with Hoechst 33342 (Fig. 1B). The anti-proliferative activity of WRC-213 was further evaluated against a panel of human cancer cell lines including non-small cell lung cancer (A549), androgen-independent prostate cancer (DU145), colorectal cancer (HT-29), breast cancer (MCF-7) and hepatocellular carcinoma (Hep3B and HepG2). The IC_{50} values varied from 50 nM to 0.6 μM, while did not show significant tissue specificity (Fig. 1C). WRC-213 showed equipotent activity with mitoxantrone, but was much more potent (about 50 times) than etoposide (Fig. 1C).

3.2. *Effect of WRC-213 on cell-cycle progression in PC-3 cells*

The FACScan flow cytometric analysis showed that WRC-213 induced a concentration- and time-dependent increase of S- and G2/M-phase population and a subsequent increase of sub-G1 population in PC-3 cells (Fig. 2A–C). Using monoclonal antibody MPM-2 to recognize mitotic phosphoproteins, the data showed that the MPM-2 expression was dramatically elevated in cells after a 24-h exposure to taxol (0.1 μM, the reference agent of mitotic arrest) (Fig. 2D). In contrast, WRC-213 inhibited the expression of MPM-2 up to a 24-h treatment (Fig. 2D). The data indicate that WRC-213 induces cell-cycle arrest at S and G2 other than mitotic phase in PC-3 cells. Notably, after a long-term treatment of 36 and 48 h, some of the cells entered into mitotic phase as a result of increased levels of MPM-2 phosphoepitopes (Fig. 2D).

3.3. *Determination of signals involving in cell-cycle arrest in PC-3 cells*

DNA damage frequently induces S- and/or G2-arrest of the cell-cycle in p53-defective tumor cells [6]. In this study, the comet assay demonstrated that after a 30-min exposure to WRC-213, DNA damage was induced in a concentration-dependent manner (Fig. 3A). ICRF-193, a specific catalytic inhibitor of topoisomerase II, significantly antagonized both etoposide- and WRC-213-mediated chromosomal DNA breaks (Fig. 3B), suggesting that topoisomerase II cleavable complexes contributed to WRC-213-induced effect.

It has been suggested that topoisomerase II can form complexes with various cell-cycle regulators including p53, 14-3-3 ε and Cdk1 kinase [7,8]. Accordingly, the expressions of cell-cycle regulators were examined. The data showed that WRC-213 induced an early and sustained elevation of cyclin E and cyclin A but not M phase-specific cyclin B1 expression (Fig. 4). The protein level of Cdk2 other than Cdk1 was correspondingly increased; whereas, the expression of $p21^{Cip1/Waf1}$ was inhibited by WRC-213 (Fig. 4). Collectively, these data confirmed that WRC-213 induced the cell-cycle arrest at S- and G2-phase but not M-phase. However, it was noted that the levels of cyclin B1 were increased when cells were exposed to WRC-213 for 36 h (Fig. 4). In accordance with the increased levels of MPM-2 phosphoepitopes at the same time (Fig. 2D), the data suggested that some of the cells were in mitotic phase.

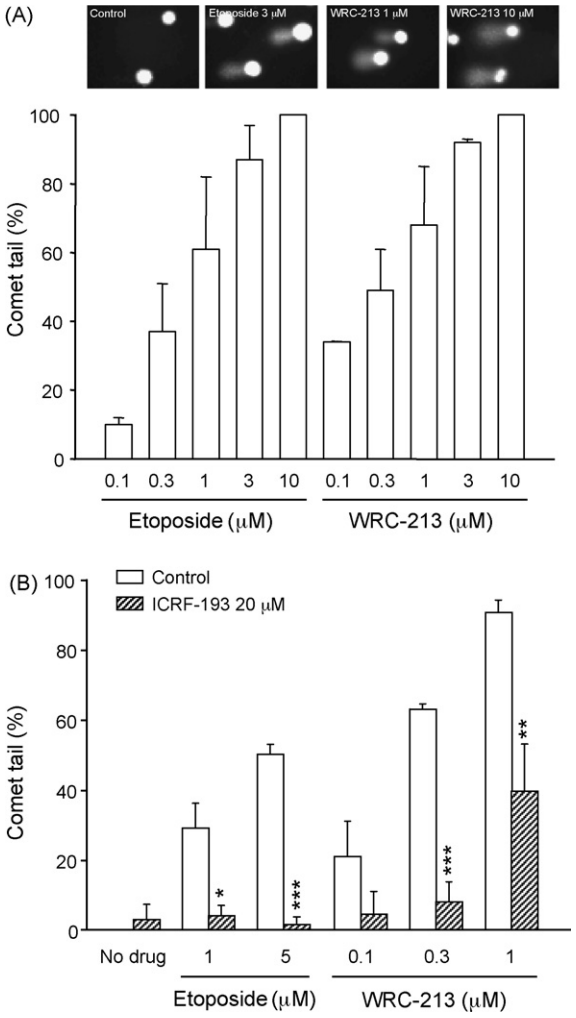

Fig. 3 – Effect of WRC-213 on DNA damage and competition of topoisomerase II inhibition. (A) PC-3 cells were treated with etoposide or WRC-213 at various concentrations for 30 min. The cells were then processed immediately for analysis by comet assay as described in Section 2. Data are expressed as mean ± S.E.M. of three determinations. (B) Cells were pretreated with or without ICRF-193 (20 μM, a topoisomerase II specific catalytic inhibitor) for 30 min. Various concentrations of etoposide and WRC-213 were added for additional 30 min. The cells were then processed immediately for analysis by comet assay. Data are expressed as mean ± S.E.M. of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the respective ICRF-free control.

3.4. *Examination of extrinsic and intrinsic apoptosis pathways*

Both death receptor-mediated extrinsic and mitochondria-dependent intrinsic apoptosis pathways are involved in DNA damage-mediated apoptosis [9]. Several mitochondria-related Bcl-2 family protein members were detected. The data showed that WRC-213 induced Mcl-1 cleavage and the formation of cleaved product (24 kDa); Bcl-2 expression was also down-

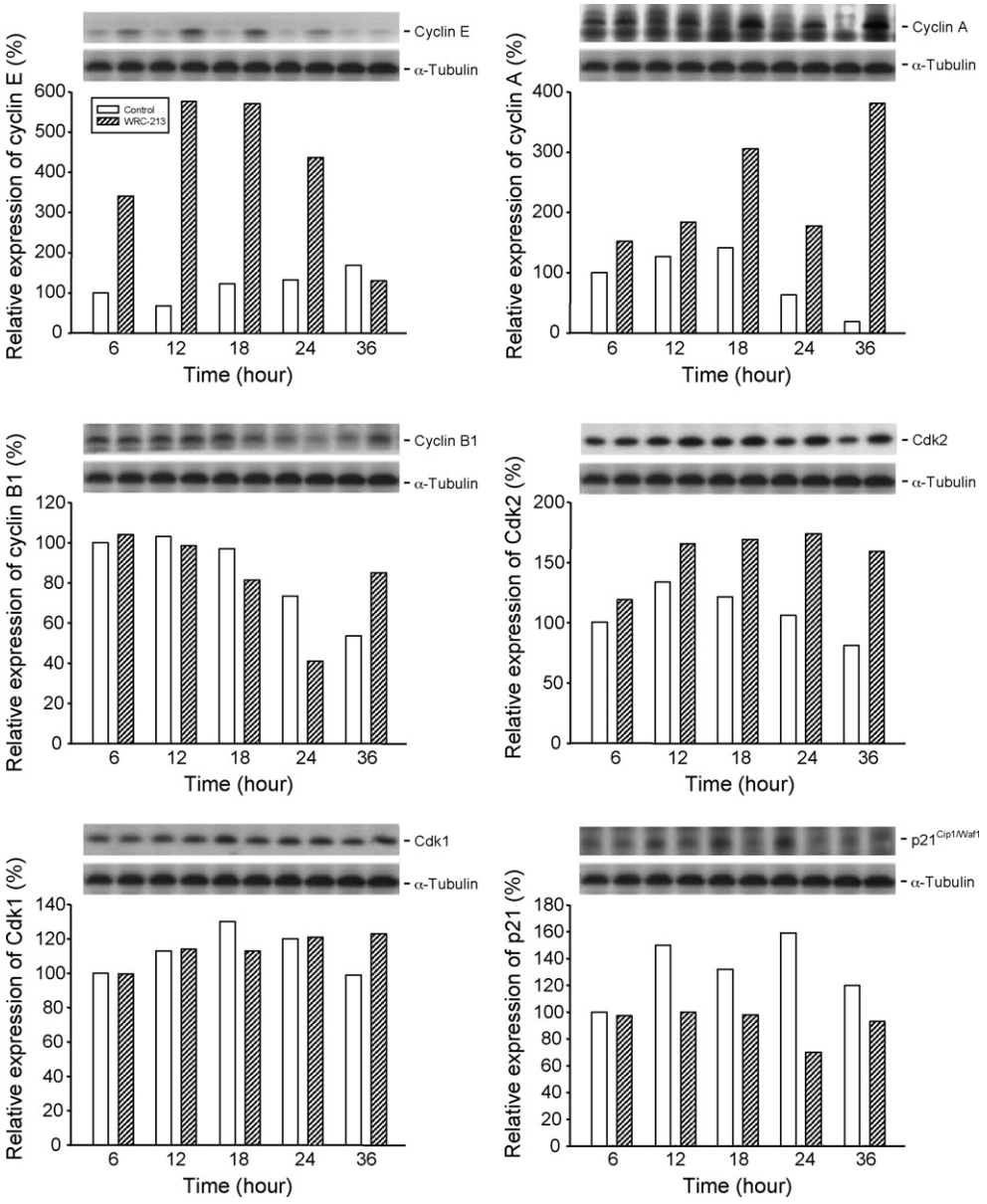

Fig. 4 – Effect of WRC-213 on the expression of cell-cycle regulators. PC-3 cells were incubated in the absence or presence of WRC-213 (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of the indicated protein expressions. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10 or 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. Data are representative of two to three independent experiments.

regulated (Fig. 5A). However, the levels of death receptors (Fas, DR4 and DR5) and their ligands (FasL and TRAIL) were not modified by WRC-213 (data not shown). Caspase-8 and -9 are major initiator caspases in extrinsic and intrinsic pathways, respectively. WRC-213 induced the activation of caspase-9 other than caspase-8 (Fig. 5B). The caspase-3 activation and cleavage of downstream substrate, PARP, were also induced by WRC-213 (Fig. 5B). These data suggest that mitochondria-mediated pathway plays a central role in WRC-213-mediated effect.

Survivin, a member of IAP family protein, renders many tumors resistant to various forms of chemotherapy [22]. WRC-213 induced an early down-regulation of survivin expression in PC-3 cells. It might impair the resistance machinery and facilitate apoptosis; however, the protein levels of survivin were increased after a 36-h treatment with WRC-213 (Fig. 5A). It is likely that the cells following a long-term exposure (36 h) to WRC-213 may undergo mitotic catastrophe (Section 4). Caspase-2 is a unique caspase with features of both initiator and effector caspases. Two forms of procaspase-2 are identified: procaspase-2L, whose overexpression triggers apoptosis and a truncated isoform, procaspase-2S, which prevents apoptosis [23]. After a 12-h treatment with WRC-213, the down-regulation of procaspase-2S was induced (Fig. 5C).

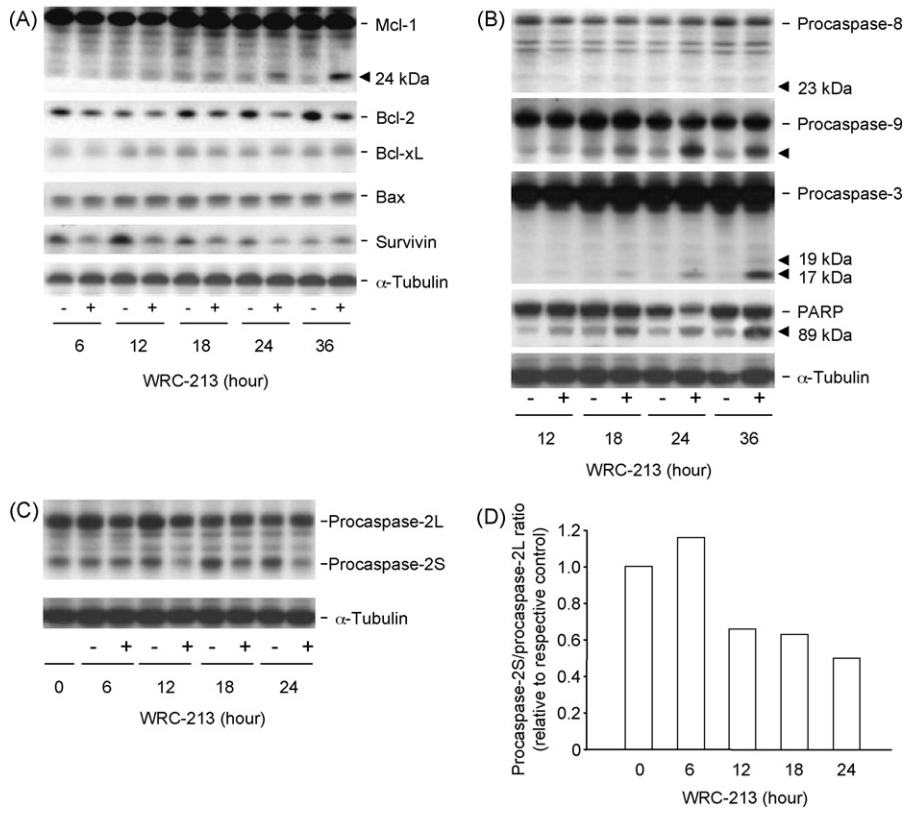

Fig. 5 – Effect of WRC-213 on the expression of Bcl-2 family member proteins and caspases. PC-3 cells were incubated in the absence or presence of WRC-213 (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of the indicated protein expressions. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10 or 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. Data are representative of three independent experiments.

The ratio of procaspase-2S to procaspase-2L was decreased in a time-dependent manner, indicating proapoptotic contribution of this protease (Fig. 5D).

3.5. *Effect of WRC-213 on cardiotoxicity and drug resistance*

WRC-213 and several topoisomerase II inhibitors also inhibited the proliferation of H9c2 cardiomyocytes (Fig. 6A). The anti-proliferative effect of WRC-213 was about eight-fold less than that in PC-3. In contrast, mitoxantrone and doxorubicin displayed higher activity in H9c2 with 2.2- and 4.5-fold potency, respectively (Fig. 6B). Furthermore, both mitoxantrone and doxorubicin exhibited acute cytotoxicity in H9c2 by mitochondrial MTT reduction assay; whereas, WRC-213 and etoposide had little acute toxic effect (Fig. 6C). These topoisomerase II inhibitors showed varied efficacies against P-glycoprotein (P-gp)-rich NCI/ADR-RES cells. The anti-proliferative effects were reduced in this P-gp-rich cell line (Table 1). The resistance factor (RF) was determined based on IC_{50} ratio in NCI/ADR-RES cells compared with cancer cell lines. The average RF of WRC-213 was 12 (a range from 2 to 24)

Table 1 – Comparison of drug resistance of WRC-213 and topoisomerase II inhibitors in several cancer cell lines

Agent	NCI/ADR-RES		MCF-7		PC-3		A549		HT-29	
	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
WRC-213	1.2 ± 0.04	–	0.05 ± 0.01	24	0.19 ± 0.07	6	0.07 ± 0.02	17	0.6 ± 0.03	2
Mitoxantrone	5.9 ± 0.3	–	0.03 ± 0.01	197	0.33 ± 0.07	18	0.07 ± 0.01	84	0.9 ± 0.1	7
Etoposide	600.0 ± 0	–	2.8 ± 0.2	214	7.6 ± 0.7	79	2.9 ± 0.19	207	25.3 ± 0.2	24
Doxorubicin	7.2 ± 1.0	–	0.013 ± 0.005	554	0.27 ± 0.08	27	0.09 ± 0.02	78	0.4 ± 0.02	18

Different cell lines were treated with various concentrations of the indicated agent for 48 h. Then, the anti-proliferative effects of these agents were examined by SRB assays. The IC_{50} (μM) values were obtained and compared between NCI/ADR-RES and the other cell lines. Data are expressed as mean ± S.E.M. of three to five determinations (each in triplicate). The number indicates the resistance factor (RF), which was determined based on IC_{50} ratio in NCI/ADR-RES cells compared with the other cell lines.

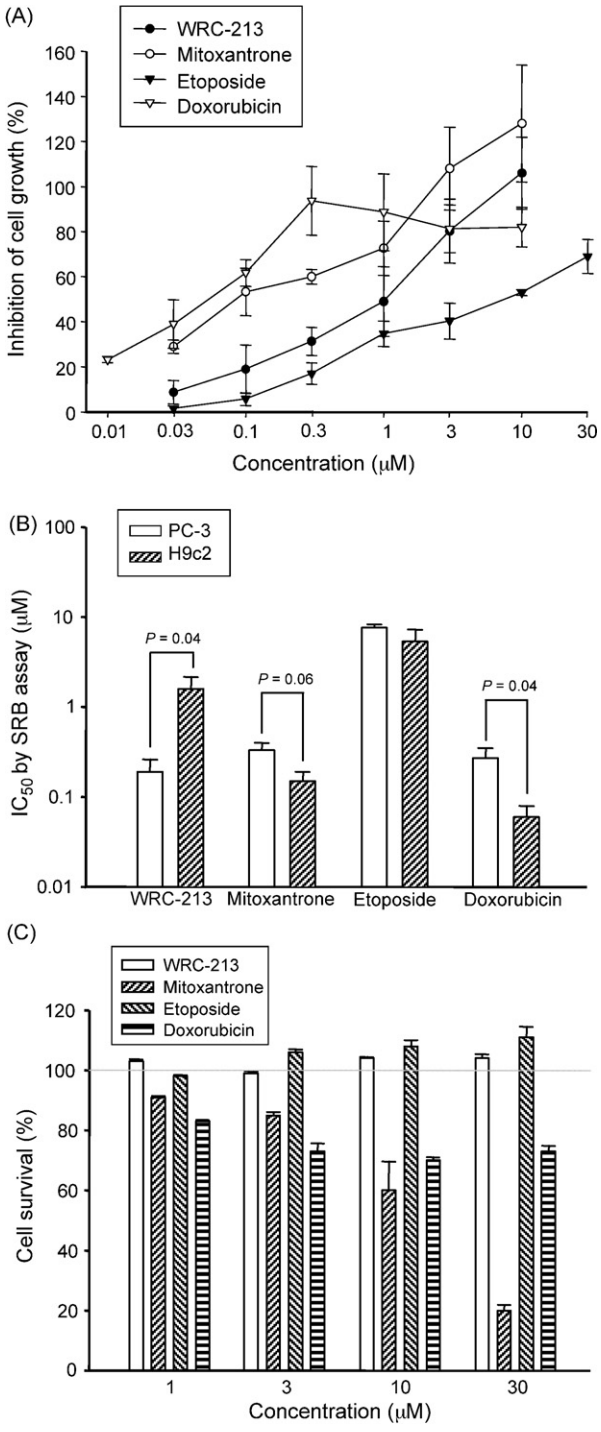

Fig. 6 – Effect of WRC-213 and several topoisomerase II inhibitors on the cardiotoxicity. (A) H9c2 cells were treated with the indicated agent at various concentrations for 48 h. Then, the anti-proliferative effects were examined by SRB assays. Data are expressed as mean ± S.E.M. of three determinations (each in triplicate). (B) H9c2 or PC-3 cells were treated with the indicated agent at various concentrations for 48 h. Then, the anti-proliferative effects of these agents were examined by SRB assays. The IC_{50} values were obtained and compared between these two cell lines. Data are expressed as mean ± S.E.M. of three to five determinations (each in triplicate). (C) H9c2 cells were treated with the indicated agent at various concentrations for 7 h. Then, a final concentration of 0.5 mg/ml MTT (dissolved in PBS) was added for another 2 h. Then, the medium was replaced with 100 μl acidified β-isopropanol for color development and the absorbance density values were obtained (570 nm).

which was much lower than mitoxantrone (RF 77, from 7 to 197), etoposide (RF 131, from 24 to 214) and doxorubicin (RF 169, from 18 to 554) (Table 1).

4. Discussion

Anthracyclines and anthracenediones are well-known cancer chemotherapeutic agents. However, their uses are limited with cardiotoxicity and drug resistance. Recently, the search for topoisomerase II inhibitors with reduced cardiotoxicity has been focused [24]. To achieve this purpose, the approach of structure modification to conjugate amino acid to known structure of topoisomerase II inhibitor has been done by our colleagues because of several rationales including (i) to improve permeability into cells, (ii) to increase metal ion-chelating activity for stabilizing DNA-topoisomerase II cleavable complex, (iii) to produce electrostatic interaction with DNA and (iv) to alter the lipophilic property of the compound [25,26]. Several L- and D-form amino acids, such as lysine, serine, tyrosine and methionine, were introduced into the anthraquinone skeleton and more than fifty derivatives were synthesized for the evaluation of anticancer activity. In a screening test by SRB assays we found that WRC-213, an L-methionine conjugation, was the most effective derivative to inhibit the proliferation of PC-3 cells (data not shown). In this study, the pharmacological property of WRC-213 was further characterized. In the extension of evaluation of anticancer activity, WRC-213 displayed a potent anti-proliferative activity in numerous cancer cell lines. Furthermore, it showed much lower cardiotoxicity and P-gp-related resistance than mitoxantrone, etoposide and doxorubicin. The data demonstrate the development potential of WRC-213, since P-gp confers multiple drug-resistance by effluxing numerous anti-tumor drugs [27,28].

The signals for cell-cycle progression are frequently modified and changed, leading to uncontrolled growth in cancer cells. One of the efficient anticancer strategies is targeting on cell-cycle regulators. After the examination, we suggest that WRC-213 induces cell-cycle arrest at S- and G2-phase in PC-3 according to several lines of evidence, namely, (i) the direct assessment via flow cytometric analysis, (ii) elevation of cyclin E, cyclin A and Cdk2 protein levels that are central regulators in both S- and G2-phase and (iii) the lack of up-regulation of M phase-specific cyclin B1 expression. Numerous lines of evidence suggest that p53 plays an important role in DNA damage-mediated response. The resulting increase of $p21^{Cip1/Waf1}$ associated with reduced levels of cyclin A and cyclin B warrants the G1 checkpoint arrest [29,30]. However, DNA damage preferentially causes S and/or G2-arrest in p53-defective tumor cells, driving them into apoptosis [31]. In this study, WRC-213 induced S and G2

arrest associated with decreasing levels of $p21^{Cip1/Waf1}$ in p53-null PC-3. After comet assays, we confirmed that the inhibition of topoisomerase II contributed primarily to WRC-213-induced checkpoint arrest at S- and G2-phase.

WRC-213-induced G2 arrest and inhibition of MPM-2 phosphoepitopes were consistent with other studies that the phosphorylation of MPM-2 epitope was depressed in cells exposed to X-ray and etoposide [30]. However, it was observed that the G2 arrest was terminated with the detection of increased levels of MPM-2 phosphoepitopes and cyclin B1 expression after the exposure of PC-3 to WRC-213 for 36 h. It has been suggested that p53-defective tumor cells respond to DNA damage by arresting cell-cycle at G2-phase and, following release from G2 block, they may undergo mitotic catastrophe, a type of cell death thought to share some apoptotic pathways [31]. Mitotic catastrophe is controlled by several biochemical regulators, such as cyclin B1/Cdk1 complex, cell-cycle checkpoint proteins, survivin, caspases and Bcl-2 family of proteins [31,32]. In this study, the long-term (36 h) exposure of cells to WRC-213 showed various biochemical criteria, including up-regulation of cyclin B1 expression and MPM-2 phosphoepitopes, activation of caspases, elevation of survivin expression and alteration of Bcl-2 family of proteins. It is likely that the cells following a long-term exposure to WRC-213 share, at least, some characteristics with those of undergoing mitotic catastrophe.

Bcl-2 family of proteins and caspases involve in signal transduction pathways induced by many apoptotic stimuli. Our data suggest that WRC-213, following topoisomerase II inhibition and DNA damage, induces PC-3 apoptosis through mitochondria-mediated intrinsic pathway by several observations, including down-regulation of Bcl-2 expression, cleavage of Mcl-1 and activation of caspase-9 and -3 cascades but not caspase-8. It has been identified that Mcl-1 is a substrate for caspase-3. The Mcl-1 cleavage occurs after Asp^{127} and Asp^{157} and generates four products of 24, 19, 17 and 12 kDa [33]. Our data revealed that the onset of Mcl-1 cleavage and production of 24-kDa fragment correlated with caspase-3 activation, indicating the involvement of caspase-3 in Mcl-1 cleavage. Caspase-2, a unique caspase with features of initiator and effector caspase, appears to be necessary for the onset of apoptosis induced by certain stimuli. In this study, the down-regulation of procaspase-2S was apparent when exposed to WRC-213 for 12 h. Because procaspase-2S can prevent apoptosis [23], the decrease of procaspase-2S level may contribute to WRC-213-mediated apoptotic mechanism.

The data demonstrated in this study suggest that WRC-213 is an effective anticancer agent. One of the most important issues for this compound is the mechanistic study of the cytotoxic selectivity between H9c2 and cancer cells. The doubling times of the individual cell lines may play a role in the mechanism of this selectivity. The doubling times were determined in this study although they were varied by the inoculation density. The doubling times for the respective cell lines and the inoculation densities (cells/well in 96-well plate) in this study are as follows ($n = 3$): PC-3 (28.2 ± 1.1 h, 5000 cells), MCF-7 (26.7 ± 0.9 h, 10,000 cells), A549 (24.6 ± 0.8 h, 5000 cells), HT-29 (22.1 ± 0.8 h, 5000 cells) and H9c2 (43.6 ± 0.6 h, 5000 cells). The data showed that H9c2 cells divided more slowly than the cancer cells. The doubling time may, at least partly, contribute to the selectivity of WRC-213-mediated cytotoxicity between H9c2 and cancer cells. In summary, our data suggest that WRC-213 is a potential topoisomerase II inhibitor with reduced cardiotoxicity and drug resistance. It induces chromosomal DNA strand breaks, leading to S and G2 arrest of the cell-cycle and activation of mitochondria-mediated apoptotic pathways.

Acknowledgements

This work was supported by a research grant of the National Science Council of the Republic of China (NSC 95-2323-B-002-006 and NSC 96-2323-B-002-004). Facilities provided by grants from the Ministry of Education, Taiwan to the Center for Genomic Medicine in National Taiwan University (93-K001) are also acknowledged.

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