ANTICANCER EFFECTS OF CO-ADMINISTRATION OF DAUNORUBICIN AND RESVERATROL IN MOLT-4, U266 B1 AND RAJI CELL LINES

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Abstract

Resveratrol (RES) is a naturally occurring compound with strong anti-oxidant effects that combats cancer *via* several mechanisms. It has been found to inhibit P-glycoprotein and protect cells from chemotherapeutics adverse effects. Daunorubicin (DAN) is mostly used in the treatment of leukaemia and some solid tumour such as glioma. In the current study, we evaluated the effect of co-administration of RES and DAN (RES/DAN) in MOLT-4, U266 B1 and Raji cell lines. MTT assay was used to investigate cell viability at different concentrations of RES, DAN and RES/DAN. Also, to elucidate the mechanism of cell death, flow cytometry study of Annexin V/PI staining was used. Our results from MTT assay showed that RES and DAN induce cell death. IC50 for RES (20 µM for MOLT-4, 73 µM for U266 B1 and 47 µM for Raji cell lines) and IC50 for DAN (0.5 µM for MOLT-4, 0.5 µM for U266 B1 and 0.6 µM for Raji cell lines) were calculated. Flow cytometry study using Annexin V/Pi showed induction of apoptosis following RES, DAN and RES/DAN. The effects of RES/DAN were significantly more marked as compared to DAN and RES (p < 0.001). Taken together, RES and DAN showed synergistic effects in induction of apoptosis in leukaemia cell lines.

Rezumat

Resveratrolul (RES) este un compus natural cu efecte puternice antioxidante care combate cancerul prin mai multe mecanisme. S-a constatat că inhibă glicoproteina P şi protejează celulele de efectele adverse ale agenţilor chimioterapici. Daunorubicina (DAN) este utilizată în principal în tratamentul leucemiei şi al unor tumori solide, cum ar fi gliomul. În această lucrare, am evaluat efectul administrării concomitente a RES şi DAN (RES/DAN) pe liniile celulare MOLT-4, U266 B1 şi Raji. Testul MTT a fost utilizat pentru a investiga viabilitatea celulară la diferite concentraţii ale SRE, DAN şi RES/DAN. De asemenea, pentru a elucida mecanismul morţii celulare, utilizând citometria în flux. Rezultatele noastre din testul MTT au arătat că RES şi DAN induc moartea celulelor. A fost calculat CI50 pentru RES (20 µM pentru MOLT-4, 73 µM pentru U266 B1 şi 47 µM pentru liniile celulare Raji) şi CI50 pentru DAN (0,5 µM pentru MOLT-4, 0,5 µM pentru U266 B1 şi 0,6 uM pentru liniile celulare Raji). S-a utilizat citometria în flux cu annexină V/Pi drept colorant, pentru a arăta inducerea apoptozei de către SRE, DAN şi RES/DAN. Efectele RES/DAN au fost semnificativ mai mare în comparaţie cu DAN şi RES (p < 0.001). Împreună, RES şi DAN au arătat efecte sinergice în inducerea apoptozei în liniile celulare leucemice.

Keywords: MOLT-4, Raji, U266 B1, resveratrol, daunorubicin, apoptosis, MTT, annexin V/PI

Introduction

Resveratrol (RES) (3, 5, 4′-trihydroxytrans-stilbene) is a polyphenol and phytoalexin compound which is derived from many fruits like grapes (especially skin of grapes), berries, and peanuts [23, 40]. RES has been widely investigated for its anti-oxidant, anti-inflammatory and wound healing activities, its inhibitory effects on platelet aggregation and cancer cell growth and also, its potential in induction of apoptosis [23]. Furthermore, *in vivo* and *in vitro* studies confirmed RES chemopreventive effects on

cancerous cell lines [9, 20, 23]. It is supplied in a capsule form as a nutritional supplement from Japanese and Chinese knotweed plant *Polygonum cuspidatum*, red wine or red grapes extract [12].

Carcinogenesis in human is a multi-step process and RES affects all steps of carcinogenesis including initiation, promotion and progression [24]. Likewise, RES could inhibit angiogenesis in cancer tissues and showed its anticancer effects via attenuation of angiogenesis in the colon cancer-xenografts in mice [23]. Also, RES suppressed proliferation and induced apoptosis in MOLT-4 acute lymphoblastic leukaemia cells via different pathways [11]. Furthermore, RES has been widely investigated in hematopoietic cell lines and posed anticancer effects [10, 16, 29, 48]. In addition to anticancer effects of RES it also has cardio-protective effects against ischemia/reperfusion (I/R) in animal models [50]. Interestingly, RES inhibits P-glycoprotein and multidrug resistance 1 (MDR 1) gen down-regulation which can potentiate its anticancer effects [4].

Daunorubicin (DAN) is a chemo-preventive drug that is successfully used in the treatment of many types of cancers especially leukaemia and some solid tumours. As it is mostly used in leukaemia, three different blood cell lines were used in this study. It has been widely used in cancer treatment alone or in combination with other chemotherapeutic agents. It poses many adverse effects including myelo-suppression and cumulative cardio-toxicity [47], bone marrow suppression, congestive heart failure (that can even be continued for months to years after the end of therapy) pericarditis-myocarditis, left ventricular dysfunction, and arrhythmias that confine its use [3, 8, 17, 22, 29, 49]. Based on the above-mentioned evidence, we evaluated the effects of RES administration and co-administration of RES and DAN (RES/DAN) on cancer cell lines.

Materials and Methods

Chemicals: Lymphoblastic leukaemia cell line (MOLT-4), human multiple myeloma cell line (U266B1) and Burkitt's lymphoma cell line (Raji cell) were obtained from Pasture Institute (Tehran, Iran). RPMI 1640 medium, Cell Proliferation Kit (MTT), fluorescent probe propidium iodide (PI) (Sigma Aldrich Chemicals Pvt Ltd, USA), Annexin V-FITC Apoptosis Detection Kit and fluorescent probe 2′, 7′-dichlorofluorescein di-acetate (DCFHDA) (Abcam, USA) and resveratrol (Sigma Aldrich Chemicals Pvt Ltd, USA) were purchased.

Cells and cultures: MOLT-4 is a human leukemic cell line (T cell line), derived from an individual suffering from T-lymphoblastic leukaemia relapsed after multidrug chemotherapy, multiple myeloma U266B1 cell line, human B cell line and Raji cells obtained from human Burkitt's lymphoma were incubated at 37°C in culture medium of RPMI 1640. All media were supplemented with 10-15 % fetal bovine serum (FBS) and 110 IU/mL of penicillin and 100 μg/mL of streptomycin.

Detection of cell viability by MTT assay: In all three cell lines, cell viability following treatment with different concentrations of RES (5, 10, 20, 40, 60, 100 and 150 μM) and DAN (0.1, 0.2, 0.5, 0.7 and 1 μM) were assessed using MTT assay in 96-well plates (1.5×10^4 cells/well). As the next step, 72 h after incubation at 37°C, after removing the medium, dimethyl sulfoxide was added to the medium to dissolve formazan crystals. Then, absorbance was read at 570 nm using Stat Fax 2100 ELISA reader (UK). For all three cell lines, MTT test was performed in triplicates.

Detection of necrosis and apoptosis by flow cytometry: Here, RES (20, 73 and 47 μM for MOLT-4, U266B1 and Raji cell lines) and DAN (0.5, 0.5, and 0.6 μM for MOLT-4, U266B1 and Raji cell lines) and RES/DAN were used. Incubation lasted up to 3 h for DAN, 20 h for RES and RES/DAN in 12-well plates (2×10^5 cells/well). After that, cells were collected, washed with PBS and re-suspended in culture media for further investigation. In order to determine apoptosis, cells were incubated at room temperature for 5 min with Annexin V (5 μL) and PI (5 μL) in dark. For all three cell lines this test was performed in triplicates. Annexin V /PI-treated cells were analysed using a Partec PAS flow cytometer (Partec GmbH, Germany).

Statistical analysis

Statistical analysis was performed using Student's t-test. Data are expressed as means ± SD (n = 3 independent experiments). P values less than 0.05 were considered significant.

Results and Discussion

Effects of RES on cell viability of MOLT-4, Raji, and U266B1: MTT assay was used to evaluate cell viability in all cell lines (MOLT-4, U266B1 and Raji). Result showed that RES induced cell death in all three cell lines (Figure 1).
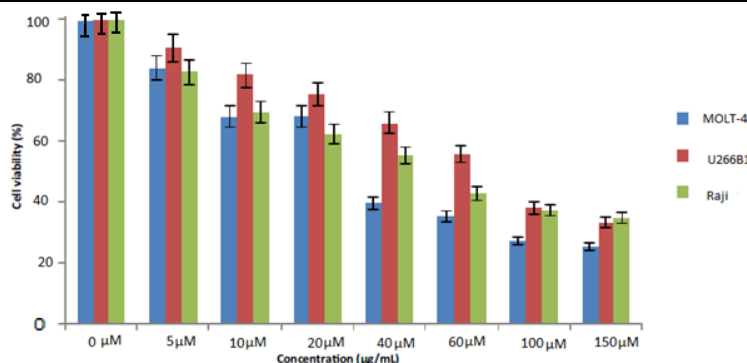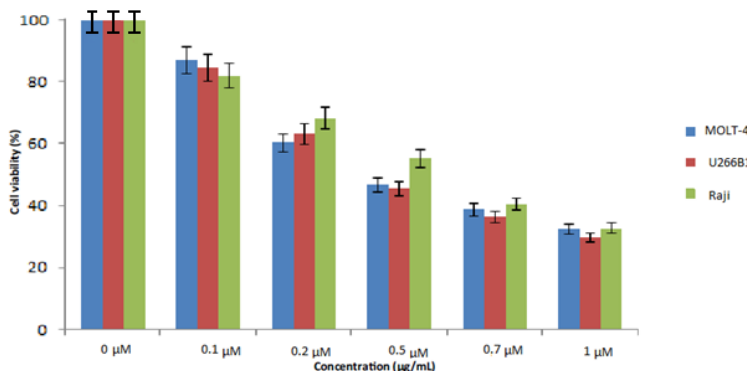
Figure 1.
Effects of different concentration of RES on MOLT-4, U266B1 and Raji cell lines proliferation

Effects of DAN on cell viability of MOLT-4, Raji, and U266B1: DAN induced cell death as evaluated by MTT assay. Based on previous studies, the time of incubation for DAN was shorter that for RES (3 and 20 h for DAN and RES, respectively) (Figure 2).
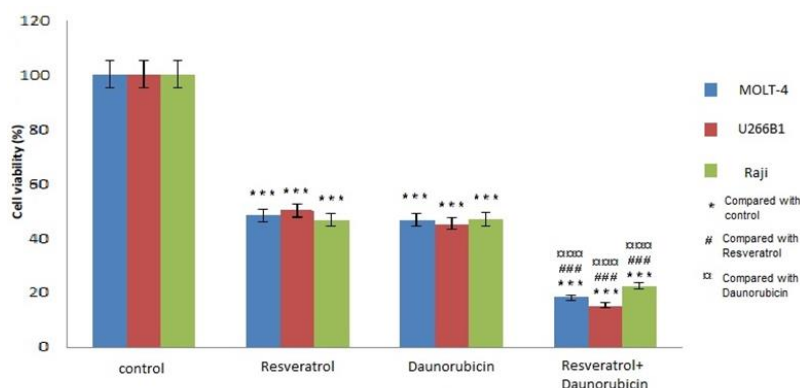

Figure 2.
Cytotoxic effects of different concentrations of DAN in cell lines of MOLT-4, U266B1 and Raji after 72 h incubation as evaluated by MTT assay

Effects of DAN + RES on cell viability of MOLT-4, Raji, and U266 B1: DAN, RES and DAN/RES significantly increased cell death in MTT assay as compared to control group (***p < 0.001). As shown in Figure 3, DAN/RES significantly increased cell death as compared to RES or DAN alone (***p < 0.001).

Figure 3.
The effects of RES, DAN and RES/DAN on proliferation in MOLT-4, U266B1 and Raji cell lines as evaluated by MTT assay (***p < 0.001, ###p < 0.001 and ¤¤¤p < 0.001 as compared to control, RES and DAN, respectively)

Based on the results, IC50 for DAN, RES and DAN+RES (Table I), were calculated and used for apoptosis evaluation.

IC50 values of RES and DAN for MOLT-4, U266B1 and Raji cell lines were calculated based on MTT assay results

Mean ± SEM

Cell line	DAN Concentration (μM)	RES Concentration (μM)
MOLT-4	0.5	20
U266B1	0.5	73
Raji	0.6	47

Induction of apoptosis in MOLT-4, U266B1 and Raji cell lines using Annexin V/PI: Our results showed that RES and DAN significantly induce apoptosis in MOLT-4, U266B1 and Raji cell lines ($p < 0.01$ and $p < 0.001$ as compared to control group that received no-treatment). RES/DAN caused significant apoptosis as compared to control group and RES or DAN alone ($p < 0.001$ for all cell lines as compared to control group) (Figure 4).
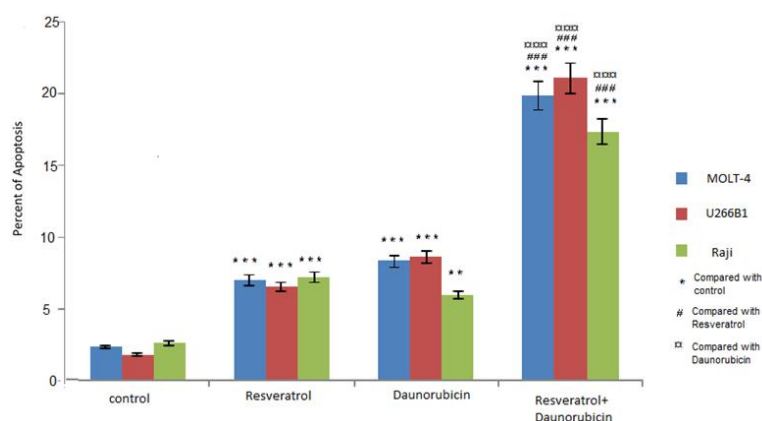
Figure 4.

Induction of apoptosis in MOLT-4, U266B1 and Raji cell lines treated with RES, DAN and RES/DAN following Annexin V/PI staining (***$p < 0.001$, ###$p < 0.001$ and ¤¤¤$p < 0.001$ as compared to control, RES and DAN, respectively)

Cancer is still the leading cause of death in the world and the use of anticancer drugs is confined because of their adverse effects and ineffectiveness due to drug resistance [31]. As a chemo-preventive agent, DAN is widely used in the treatment of leukaemia and some solid tumours such as glioma [47]. It has many adverse effects and also chemo-resistance for some tumours has been reported [8, 17, 21]. During recent years, natural products attracted researchers' attention as cytotoxic agents and chemo-sensitizers to be co-administered with chemo-preventive drugs [5, 21, 32, 35, 38, 44]. RES is a naturally occurring phytoalexin with diverse health benefits such as anti-oxidant, anti-aging, cardio-protective, anti-inflammatory, neuroprotective, anti-lipid peroxidation and anti-diabetes effects [4, 26, 30]. Furthermore, it posed anticancer effects especially against leukaemia [1, 37] and induced apoptosis in solid tumours such as breast, liver, prostate, colorectal, stomach, thyroid, melanoma, head and neck squamous cell carcinoma, ovarian carcinoma, cervical carcinoma and pancreas tumours as well as glioma [1, 13, 20]. RES, besides being able to empower anticancer effect and reduce resistance to chemotherapeutics,

has protective effects against cardiovascular adverse effects [2, 6, 43, 46].

Herein, cell viability and apoptosis induction of DAN and RES alone or their co-administration (RES/DAN) were investigated in MOLT-4, U266B1 and Raji cell lines. Our results showed that RES alone induces cell death and showed synergistic effects when co-administered with DAN.

Anticancer mechanisms of RES include inhibition of cell cycle, reduction of gene receptor of CDC6, CDK4, and cyclin D, activation of caspase 3, 7 and 9, activation of BAX, DNA fragmentation, release of cytochrome C oxidase, reducing protein kinase B receptors, inhibition of heat shock proteins, and activation of intrinsic and extrinsic pathways of apoptosis [42].

RES posed its anticancer effects in all three phases of carcinogenesis (initiation, promotion and progression) with mechanisms that were proposed previously [1, 7]. Its anti-oxidant effects play a beneficial role in inhibition of cancer promotion (Leone *et al.*, 2012). Cycloxygenase-2 (COX-2), prostaglandins, inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines production and activity are reduced following administration of

RES. Since these enzymes and mediators are involved in carcinogenesis especially in promotion and progression, RES could halt both promotion and progression steps [23-25].

Doxorubicin (DXR), another anthracycline was also co-administered with RES in order to elucidate the mechanism of RES synergistic effects [41]. Topoisomerase enzyme especially type 2 is expressed in cells with high proliferation rate like tumour cells [25]. Topoisomerase 2 is a target for anthracyclines in cancer cells and it has been shown that RES has toxic effects on it [25]. It can be one of the most plausible mechanisms of RES when co-administered with DAN.

In addition, RES potently inhibits cell proliferation, reduces reactive oxygen species (ROS) production and induces apoptosis through cell cycle arrest in G1 and G2/M phases in HepG2 cell line [34]. These data are almost consistent with our study. Here, we showed that RES can induce apoptosis and showed synergistic effects when co-administered with DAN.

Another mechanism of RES anticancer effects is related to intracellular signalling including cyclines, cyclin-dependent kinases and cycline-dependent kinase inhibitors [14]. These signalling pathways are involved in cell growth and proliferation [18]. Also, RES could decrease cancer cell proliferation by regulating cell cycle progression [28].

In addition to *in vitro* investigations, *in vivo* investigations showed that angiogenesis is an important factor for blood supply of implanted tumours [33]. Substances that attenuate angiogenesis in implanted tumour, pose anti-cancer effects. Past studies showed that RES retarded tumour growth and angiogenesis by means of inhibition of AKT (protein kinase B, PKB) and MAP (mitogen-activated protein kinase) as well as vascular epithelial growth factor (VEGF) [19, 27].

Tumour invasion and metastasis is the leading cause of neoplastic progression that is inhibited by RES. RES significantly attenuated signalling that leads to metastasis in ovarian cancer cell [36].

Several studies reported that RES induces cell death via activation of apoptosis in hematopoietic cell lines [10, 15, 16, 39, 45, 48].

In addition to all anticancer effects of RES, RES ability to suppress MDR 1 (multi-drug resistance 1) and inhibit P-glycoprotein that can reduce drug resistance especially in solid tumours makes it a promising candidate for being used as an anticancer drug [4]. Also, it should be considered that RES has cardio-protective effect that was shown when co-administered with DXR. This is a valuable property for reduction of DAN cardiac adverse effects as well as reduction of drug resistance [4].

Conclusions

Our results showed that RES, DAN and RES/DAN induce cell death in three blood cell lines of MOLT-4, U266B1 and Raji via induction of apoptosis.

Acknowledgements

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