Reversal of Multidrug Resistance in Murine Lymphoma Cells by Amphiphilic Dihydropyridine Antioxidant Derivative

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Abstract. *Background: Multidrug resistance, the principal mechanism by which cancer cells develop resistance to chemotherapy drugs, is a major factor in the failure of many forms of chemotherapies. Aim: The aim of the study was to investigate the effect of K-2-11 on the reversal of multidrug resistance. Materials and Methods: The effects of amphiphilic dihydropyridine derivative K-2-11 were tested on MDR1-expressing mouse lymphoma cells and their parental control. The effects of K-2-11 with and without doxorubicin were studied by determination of cell viability, cell proliferation and production of reactive oxygen species. Results: K-2-11 caused complete reversal of multidrug resistance of the MDR cells, being much more efficient than the positive control verapamil. Accordingly, the cytotoxic effects of doxorubicin were enhanced by K-2-11, both in the MDR and in parental cell line, while K-2-11 alone did not affect cell viability. K-2-11 also acted as an antioxidant, reducing the cellular generation of reactive oxygen species. Conclusion: Our results indicate the high potential of K-2-11 as a novel antioxidant with potent MDR-blocking ability that should be studied further for development in adjuvant anticancer treatments.*

Besides late diagnosis, resistance to multiple chemotherapies can be considered as major source of failure in cancer treatment. There may be several mechanisms involved in the development of multidrug resistance (MDR) and the best characterized involves the membrane localized P-glycoprotein (P-gp). MDR of cancer cells is associated with a large number of chemotherapeutic medicaments due to P-gp overexpression. This protein accomplishes drug resistance through its action as a drug efflux-pump by reducing the intracellular concentration of anticancer drugs (1). P-glycoprotein belongs to the group of the most important efflux pumps and causes resistance to various classes of cytostatic compounds such as the vinca alkaloids, anthracycline derivatives, podophyllotoxins and fluorouracil derivatives (2). Expression of MDR1 (multi-drug resistance protein 1) protein, which belongs to the P-gp family, is up-regulated during stressful stimuli such as chemotherapy. The enhancement of MDR1 expression by reactive oxygen species (ROS) in tumor cells is of particular interest because many anticancer treatments (*e.g.* radiotherapy and doxorubicin chemotherapy) rely on the cytotoxicity of ROS. Moreover, cancer cells themselves produce more ROS than normal cells, mainly due to alterations in metabolic pathways and an inadequate tumor vascular network. Cellular antioxidant mechanisms that in normal conditions scavenge ROS, under such excessive oxidative stress, are unable to prevent ROS impact on vital cellular functions (3). DNA damage and changes in signal transduction pathways lead to mutations resulting in malignant transformation, while ROS generated during the inflammation that accompanies carcinogenesis have been shown to facilitate tumor promotion (4). Hence, oxidative stress plays a role in tumor progression. Thereby, MDR inhibitors with antioxidant potential would be useful in treating cancer patients whose tumors are resistant to multiple chemotherapies. Such chemicals might prevent ROS increase in tumor cells and

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concomitant increase in P-gp that leads to MDR, thus, facilitating cancer treatment.

Many heterocyclic compounds are studied as potential MDR reversal compounds, but few of them also have antioxidant properties (5, 6). K-2-11 is an amphiphilic dihydropyridine (DHP) derivative not yet described in the literature. Dihydropyridine is a molecule based upon pyridine, and the parent of a class of molecules that have been semi-saturated with two substituents replacing one double bond. They are particularly well known in pharmacology as L-type calcium channel blockers. The DHP structure is regarded as a privileged one (7), meaning it ensures affinity to various receptors while its substituents determine binding specificity. We introduced specific ionogenic substituents into the DHP system to improve water solubility, resulting in the K-2-11 molecule. In the present work, the impact of K-2-11 was studied on MDR reversal in mouse lymphoma cells transfected with human *MDR1* gene, also in the presence of the frequently used anticancer drug doxorubicin.

Materials and Methods

Cell cultures. L5178Y mouse T-cell lymphoma cells (ECACC cat. no. 87111908; U.S. FDA, Silver Spring, MD, USA) were transfected with PHA mdr1/A retrovirus, as described previously (8, 9). The MDR1-expressing cell line was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the uniform expression of the MDR phenotype. The parental (PAR) mouse T-cell lymphoma cells and the human MDR1-transfected subline (MDR) were cultured at 37˚C in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics in a 5% CO_2 atmosphere.

K-2-11 compound. The tested compound, denoted as K-2-11, was synthesized at the Latvian Institute of Organic Synthesis (10). As a result of appropriate condensation and substitution reactions, the target compound was obtained comprising positively charged groups, mobile hydrogen atoms and lipophilic moieties. The K-2-11 compound samples used had >98% purity as revealed by the high performance liquid chromatography.

Assay for reversal of MDR in tumor cells. The MDR cells were diluted to a density of 2×10^6 cells/ml, resuspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots. K-2-11 was added at different concentrations, as detailed in Table I, and the samples were incubated for 10 minutes at room temperature. Rhodamine 123 (R123) (Sigma-Aldrich Corp., St. Louis, MO, USA) indicator was added to each sample to a final concentration of 10 μg/ml and the cells were incubated for a further 20 minutes at 37˚C, washed twice and resuspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured with a FACS Star Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil (EGIS Pharmaceuticals PLC, Budapest, Hungary) was used as a positive control in the R123 exclusion experiments at a final concentration of 5 μM, which is the optimal dose of verapamil (8). The percentage mean fluorescence intensity was calculated for the treated MDR cell line as compared with the untreated PAR and MDR cells. Fluorescent activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{MDR\,treated\,/\,MDR\,control}{PAR\;\;treated\,/\;PAR\;control}$$

Table I. *The effect of dihydropyridine derivative K-2-11 on accumulation of MDR1 indicator rhodamine 123 by mouse lymphoma cells.*

Sample	Concentration (μM)	FL-1	FAR
PAR	-	891.59	78.80
MDR	-	11.32	
Verapamil	5	89.15	7.88
K-2-11	0.5	89.51	7.91
	5	599.86	53.01
	50	869.41	76.84
DMSO control		11.26	0.99

FL-1: Mean fluorescence intensity of the cells; FAR: fluorescence activity ratio; PAR: control, wild-type cells not expressing MDR1; MDR: MDR1-expressing cells; DMSO: dimethyl sulfoxide.

Cell viability and proliferation assays. Both cell lines (MDR and PAR) were seeded at a density of 2×10^5 cells/well and treated with different concentrations of K-2-11 (1, 2.5, 5, 10, 25, 50, 100 μM) and doxorubicin (0.0156, 0.0312, 0.0625, 0.0125, 0.25, 0.5, 1 μg/ml) alone, or in combination. After 48 h incubation, non-radioactive cell proliferation assay (EZ4U kit, Biomedica Austria) was performed according to the manufacturer's instructions, as described elsewhere (11). For ^{3}H-thymidine incorporation assay, both cell lines were seeded at a density of 6×10^4 cells/well and treated with K-2-11 at concentration of 50 μM in combination with different concentrations of doxorubicin (0.0156, 0.0312, 0.0625 μg/ml). After 48 h, ^{3}H-thymidine (1 μCi/well; Amersham, USA) was added to each well and left for an additional 24 h (12), and radioactivity was measured by a β-scintillation counter.

Measurement of intracellular ROS production. After seeding at a density of 2×10^5 cells/well, MDR and PAR cells were incubated for 30 minutes with a nonfluorescent probe for intracellular ROS detection by 2',7'-dichlorofluorescein diacetate (DCFH-DA, Fluka). This cell-permeable dye remains nonfluorescent inside the cell until the acetate groups are removed by intracellular esterases and oxidized by intracellular ROS to the fluorescent compound 2',7'-dichlorofluorescein (DCF) which can be detected as a measure for the sensitive and rapid quantitation of intracellular ROS in response to oxidative stress (13). Following incubation with DCFH-DA, cells were treated as described for ^{3}H-thymidine assay, but in Hank's solution instead of culture medium, and after 45 minutes, fluorescence intensity was read with a Cary Eclipse Fluorescence Spectrophotometer (Varian) with excitation at 500 nm and emission detection at 530 nm.

Statistics. MTT assay, ROS measurement and ^{3}H-thymidine incorporation assay were carried out in quadruplicates. Statistical analyses were performed using Student's *t*-test. Values of $p<0.05$ were considered significant.

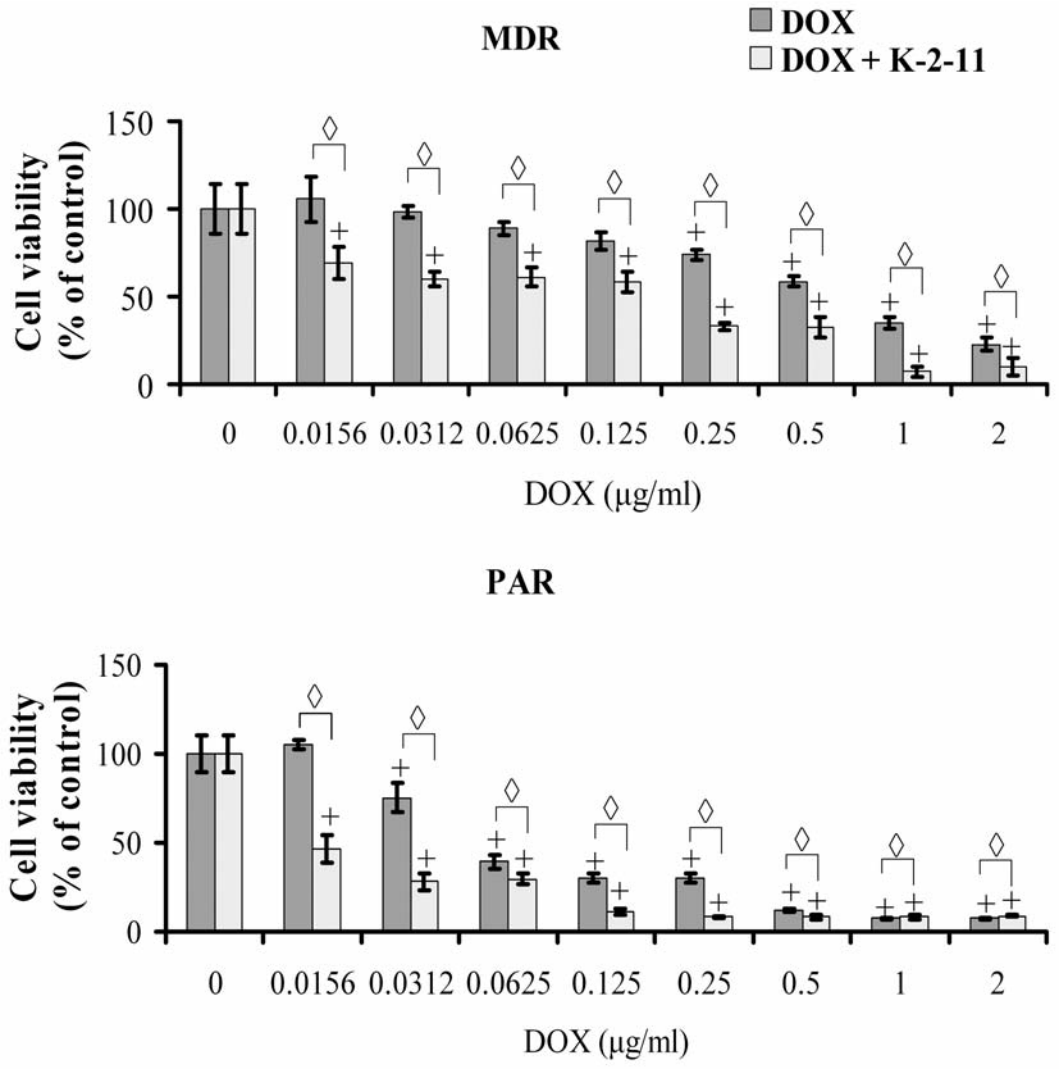

Figure 1. *Influence of doxorubicin (DOX) and K-2-11 alone, or in combination, on cell viability in cell proliferation assay. Control cells without DOX (0) are presented in the 1st column. Results were expressed as mean values as a percentage of control ±S.D. Significantly different compared to: +control (untreated cells), ◊ doxorubicin-treated group at p<0.05.*

Results

MDR reversal by K-2-11. The results obtained by comparison of concentration-dependent effects of K-2-11 and positive control verapamil are presented in Table I. When used at the lowest concentration (0.5 μM), K-2-11 achieved an MDR reversal effect equal to that of verapamil; when used at the same concentration as verapamil (5.0 μM) K-2-11 was six-fold more effective than verapamil ($p<0.05$). At the highest concentration used (50 μM), K-2-11 entirely reversed MDR. Since K-2-11 itself did not show any signs of toxicity of the cells, subsequent investigations used only a 50 μM dose.

Effects of doxorubicin and K-2-11 on cell growth. Effects of doxorubicin and combination of doxorubicin and K-2-11 on viability of PAR and of MDR cells determined by the MTT assay are presented in Figure 1. PAR cells were more sensitive to doxorubicin than were MDR cells. Doxorubicin at 1 μg/ml caused 60% mortality of MDR cells, and the same effect on PAR cells was achieved with a 16-fold lower doxorubicin concentration (0.0625 μg/ml). However, cytotoxicity of doxorubicin towards the MDR cells was significantly enhanced in combination with K-2-11 ($p<0.05$), even with the lowest doxorubicin concentration (0.0156 μg/ml). Hence, while the lethal dose of doxorubicin that killed 50% of cells (LD_{50}) was found to be 0.5 μg/ml, in combination with K-2-11, the same level of cytotoxicity was achieved at 0.0312 μg/ml doxorubicin (16-fold lower concentration). A similar enhancement of cytotoxicity of

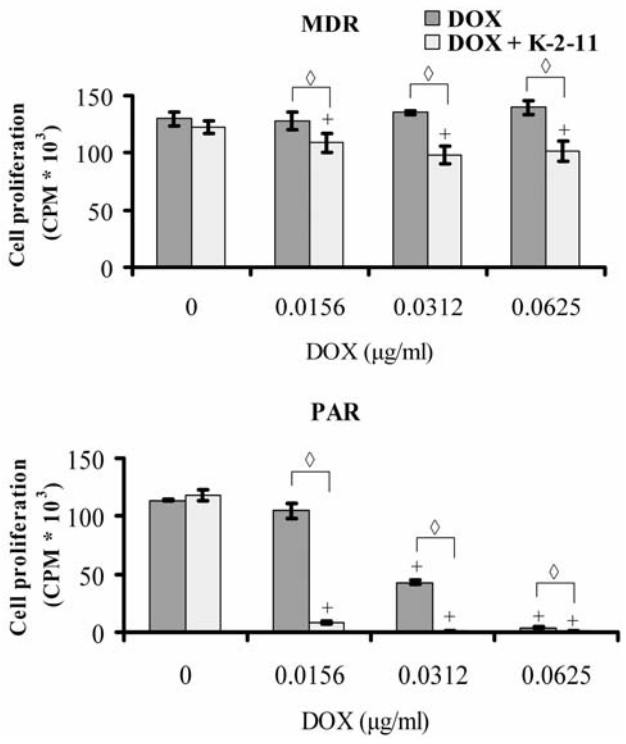

Figure 2. *Influence of doxorubicin (DOX) and K-2-11 alone, or in combination, on MDR and PAR cell proliferation in ^{3}H-thymidine assay. Control cells without DOX (0) are presented in the 1st column. Results were expressed as mean values of counts per minute (CPM) ±S.D. Significantly different compared to: $^+$control (untreated cells), ◊ doxorubicin-treated group at p<0.05.*

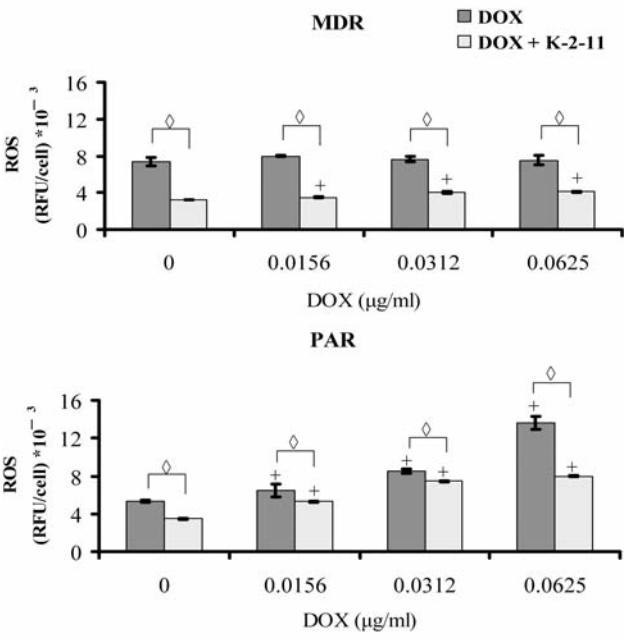

Figure 3. *Influence of doxorubicin (DOX) and K-2-11 alone, or in combination, on ROS production in MDR and PAR cell lines. Control cells without DOX (0) are presented in the first column. Results were expressed as mean values of relative fluorescence units (RFU) per cell number±S.D. Significantly different compared to: $^+$control (untreated cells), ◊ doxorubicin-treated group at p<0.05.*

doxorubicin in the presence of K-2-11 was also noticed for PAR cells, but was not as pronounced as in the case of MDR cells due to higher cytotoxicity of doxorubicin when used alone on PAR cells.

Accordingly, three concentrations of doxorubicin (0.0156, 0.0312, 0.0625 μg/ml) corresponding to the LD_{50} concentration range for both cell lines were chosen to be tested on these cells in the ^{3}H-thymidine incorporation assay, reflecting DNA synthesis and therefore the influence of K-2-11 and doxorubicin on the cell growth *in vitro*. This proliferation assay confirmed the more pronounced cytotoxicity of doxorubicin for PAR than for MDR cells, as well as the enhanced anticancer effects of doxorubicin for MDR cells when treated by doxorubicin in the presence of K-2-11 (Figure 2). While, K-2-11 alone did not influence the proliferation of MDR and PAR cells, doxorubicin used alone was not effective for the MDR cells. For the PAR cells, doxorubicin itself caused a significant decrease of the ^{3}H-thymidine incorporation ($p<0.05$) as shown in the cell proliferation assay when used at concentrations of 0.0625 μg/ml or 0.0312 μg/ml. While the same doses of doxorubicin were not effective for MDR cells ($p>0.1$) when doxorubicin was used alone, not only these doses, but even the 0.0156 μg/ml dose of doxorubicin reduced the proliferation of MDR cells when given in combination with K-2-11 ($p<0.05$). Interestingly, while the effect of doxorubicin on PAR cells was concentration dependent, the effect of the drug for the MDR cells did not show such a dose dependency.

Effect of doxorubicin and K-2-11 on ROS production. The doxorubicin and K-2-11 treatments analogous to those used in the ^{3}H-thymidine incorporation assay were also used to test for the generation of ROS by fluorescence in the MDR and PAR cells. These results were expressed as relative fluorescence units (RFU) per viable cell (Figure 3). K-2-11 significantly decreased spontaneous production of ROS in control cell cultures of both cell lines ($p<0.05$), indicating the antioxidative capacity of the substance. As expected, doxorubicin caused a significant increase of ROS in PAR cells ($p<0.05$), while it did not cause an increase in ROS in MDR cells, regardless of concentration ($p>0.05$). K-2-11 decreased ROS production in PAR cells treated by doxorubicin ($p<0.05$), with the most pronounced decrease in combination with the highest dose of doxorubicin. K-2-11 decreased ROS production in MDR cells in the presence of doxorubicin ($p<0.05$).

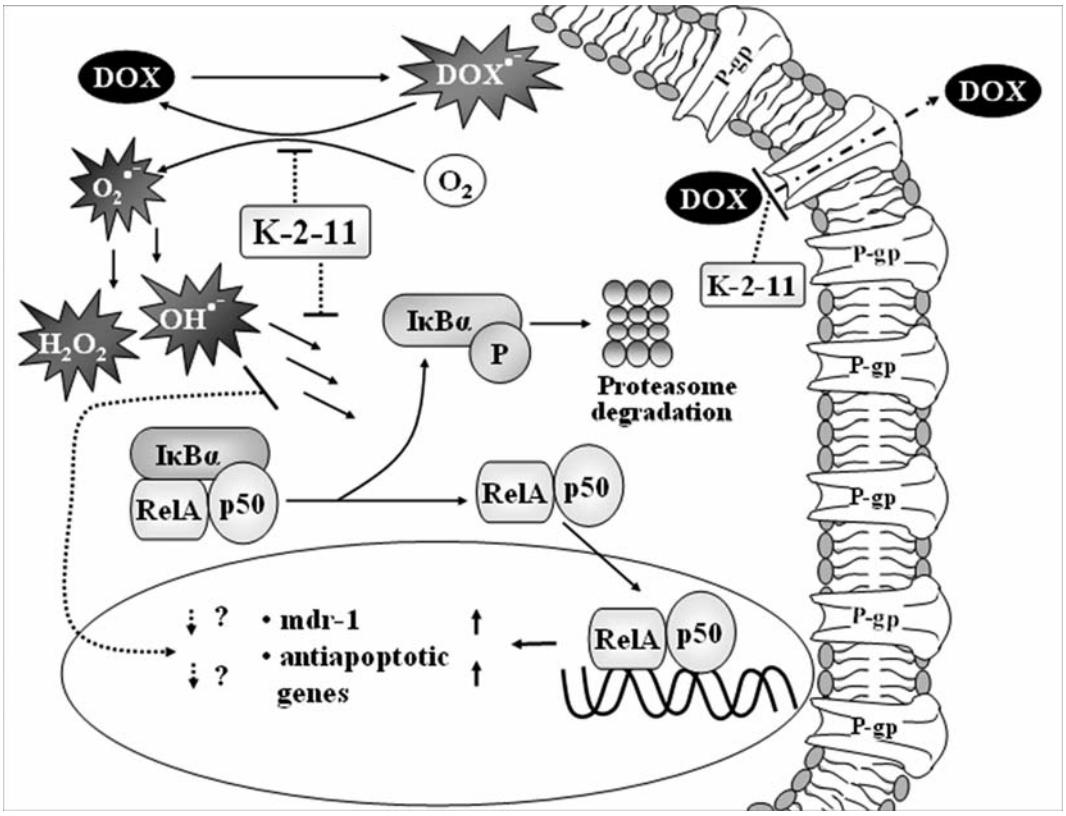

Figure 4. *Proposed mechanism of K-2-11 action on cancer cells. Doxorubicin (DOX) reduction generates doxorubicin semiquinone, a free radical capable of reducing molecular oxygen and generating superoxide, thereby initiating ROS production. ROS activates NF-κB through activation of kinases, causing IκBα phosphorylation and releasing RelA/p50 dimer that binds DNA, thus inducing overexpression of MDR1 and antiapoptosis genes. This cascade results in an increase of P-gp, leading to chemoresistance of cancer cells. When K-2-11 is added, P-gp activity is blocked, making cancer cells chemosensitive due to doxorubicin retention in cells. In addition, K-2-11 suppresses ROS increases, thus preventing NF-κB activation that could consequently lead to a normal expression of MDR1 and antiapoptosis genes, restoring chemosensitivity of cancer cells.*

Discussion

Malignant tumors are often treated by chemotherapy, in particular, in the case of disseminated, metastatic disease. However, cancer cells are often intrinsically resistant to anticancer compounds or exhibit treatment-induced acquired resistance, which complicates efforts to successfully eradicate or cause long-term cancer regression (14). In MDR cancer cells, various efflux-pump mechanisms are responsible for treatment failure. Therefore, the inhibition of the most common efflux mechanism, that of P-gp, can result in an effective anticancer chemotherapy. However, it must be taken into consideration that normal cells also contain ATP-binding cassette transporters (ABC transporters), the transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to detoxify xenobiotics, ROS and related toxic compounds such as 4-hydroxynonenal (HNE) and other products of lipid peroxidation. Thus, drugs with selective inhibition of the MDR efflux mechanism in cancer cells are needed to avoid toxic side-effects of novel compounds for MDR reversal (1). In the 1980s, calcium channel blockers were found to be inhibitors of MDR development (15). Even though DHPs were initially introduced as a class of calcium channel blockers widely used in cardiovascular diseases, their use as MDR reversal agents is still preliminary. Only recently has it been shown that DHP derivatives can be very good MDR reversal agents depending on their structure (16, 17). Our results showed that K-2-11 had a much better MDR reversal effect than the calcium antagonist verapamil has that is usually used as a positive control; therefore, we believe that K-2-11 might be an attractive substance for the study of MDR reversal.

Doxorubicin is frequently used in the clinic to treat cancer patients (*e.g.* those with breast cancer, ovarian cancer, Kaposi's sarcoma) but is also a well known P-gp substrate. In this study, doxorubicin showed LD_{50} on MDR cells at concentration of 0.5 μg/ml, while on PAR cells considerably lower doxorubicin concentration (0.0156 μg/ml) accomplished the same cytotoxic

effect. This could easily be explained because MDR cells have high P-gp activity and can succeed in expelling the majority of doxorubicin, unlike PAR cells. This explanation is fortified by the results of combined doxorubicin and K-2-11 treatment, which showed that K-2-11 accomplished its mission as a potent MDR reversal agent making MDR cells more sensitive to doxorubicin.

Of additional relevance might also be the observed antioxidant bioactivity of K-2-11 manifesting in the reduction of endogenous ROS production both in PAR and MDR cells. The importance of this bioactivity lies in the concept of cancer as a persistent oxidative stress disorder (18, 19). The theory describes ongoing oxidative stress in cancer cells, which is not present in normal cells, causing oncogenic transformation, alterations in metabolic activity, and increased generation of ROS as a consequence. This oxidative stress is a part of tumor biology, because tumor is a rapidly growing formation of cells. Rapid growth is not accompanied by adequate growth of the blood supply, leading to glucose deprivation and hypoxia. Glucose deprivation rapidly induces cellular oxidative stress by depleting intracellular pyruvate, thereby preventing the decomposition of endogenous oxygen free radicals (20-22). Doxorubicin treatment induces oxidative stress and lipid peroxidation, thereby causing additional increase in ROS and lipid peroxidation in cancer cells resulting in tumor decay (23-26).

However, long-term administration of doxorubicin causes cumulative dose-dependent cardiomyopathy. The mechanism of this doxorubicin-induced cardiotoxicity is attributed to free radical generation, stimulation of lipid peroxidation, and subsequent alteration of cellular membrane integrity (27). Doxorubicin undergoes one-electron reduction through a metabolic activation caused by NADPH-cytochrome P-450 reductase or other flavin-containing enzymes in microsomes (28). This reduction generates doxorubicin semiquinone free radicals. In the presence of molecular oxygen, the semiquinone rapidly reduces oxygen to superoxide, thereby regenerating doxorubicin. Superoxide radical spontaneously converts to hydrogen peroxide or is rapidly converted by superoxide dismutase (29-31). Further increase in ROS in cancer cells due to doxorubicin treatment may have significant consequences, such as the stimulation of cellular proliferation, promotion of mutations, genetic instability, and alterations in cellular sensitivity to anticancer agents (22). ROS have also been shown to participate in MDR1 overexpression, especially when generated by insulin, epidermal growth factor, tumor necrosis factor-α, and doxorubicin *via* the nuclear factor kappa B (NF-κB) pathway, leading to increased expression of P-gp (32, 33). Finally, ROS production activates NF-κB in a tyrosine kinase-dependent mechanism (34). Consequently, activated NF-κB enhances the expression of antiapoptosis and *MDR1* gene, thereby, increasing MDR in cancer cells and circumventing apoptosis (35). It could be presumed that K-2-11 exerted dual activity, preventing efflux of doxorubicin possibly by interfering with P-gp, and also preventing an increase in ROS, thus probably disabling activation of NF-κB. Inhibition of NF-κB could result in normal expression of MDR1 and antiapoptosis genes, leading to normal P-gp levels and apoptosis regulation, making cancer cells chemosensitive (Figure 4). In support of this mechanism is the fact that inhibition of NF-κB increases cell responses to cytotoxic drugs such as chemotherapeutics (35). If doxorubicin treatment results in a higher ROS level, which consequently increases expression of P-gp and therefore drug resistance, potent antioxidants that also exert MDR reversal, such as K-2-11, could be ideal adjuvant agents in cancer therapy. K-2-11 was not only more potent than verapamil in MDR reversal, but its antioxidative properties were also pronounced in control (untreated) cells, as well as in cells treated with doxorubicin. This is an important finding taking into consideration that the earlier mentioned doxorubicin semiquinone can also react with hydrogen peroxide to yield hydroxyl radical (36). This highly toxic reactive species can induce lipid peroxidation, an autocatalytic and degenerative process affecting cell membranes and other lipid-containing structures, and is associated with numerous pathological implications including cancer. Thus observed reduction in ROS levels in both MDR and PAR cells potentiate the role of K-2-11 as a possible adjuvant agent in cancer therapy.

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