Biochemical Action of New Complexes of Ruthenium with Quinolones as Potential Antitumor Agents

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Abstract. *Background: The aim of the present study paper was to identify the role of reactive oxygen species (ROS) in apoptosis signaling mechanisms. We used for this purpose two ruthenium complex compounds based on that overproduce these reactive species by their metabolism thus manifesting their antitumor activity too. Materials and Methods: In vivo studies were performed in Walker 256 carcinoma-bearing Wistar rats treated with two ruthenium (III) (Ru(III)) complexes with -fluoroquinolones norfloxacin and ofloxacin. The treatment started 7 days after tumor grafting. We assayed the dynamics of apoptosis by flow-cytometry and the biochemical oxidative status parameters. The biological samples used were serum and whole-tumor tissues; the results were compared to the untreated control group. Results: The results showed an increase of apoptosis from 14.79% to 59.72% 14.79% to 59.72% in tumor cells treated with the most active combination, ruthenium complex with norfloxacin. We also noted an increase of the oxidative status and ROS production during treatment. Conclusion: The newly-synthesized complexes are less toxic and their activity is based on the induction of oxidative stress.*

Metal-based anticancer drugs were imposed in clinical practice starting with the success of cis-(PtCl2(NH3)2), often referred to as cisplatin, in the treatment of several types of cancers, including ovarian, cervical, bladder, head and neck, melanoma and lymphomas (1).

Cisplatin, carboplatin, oxaliplatin and other platinum compounds induce damage to tumors by apoptosis (2). It is generally accepted that the anticancer activity of platinum coordination complexes arises from their ability to damage DNA: the chloride ligands are hydrolyzed within the cell to generate a bis-(aqua) species that binds irreversibly to DNA, usually at two adjacent guanine bases, inducing distortion of the double helix, affecting both replication and transcription processes, thus leading to cell death (3, 4). Additionally, the apoptosis of tumor cells may be mediated by the activation of various signal transduction pathways, including calcium signaling, death receptor signaling and the activation of mitochondrial pathways. Therefore, it was concluded that platinum DNA damage may exert its cytotoxicity through an intra-cellular signaling pathway, rather than the disruption of DNA replication (5).

Recent evidence suggests that cisplatin also induces reactive oxygen species (ROS) that trigger cell death (6, 7). The tumor cell has the ability to produce ROS but, at the same time, several anti-oxidant defense systems are activated by different mechanisms. One of these is the production of proteins with anti-oxidant activity, such as metallothioneins, molecules containing thiol groups (-SH) that maintain the intracellular redox homeostasis. The thiol groups may lead to the formation of thiyl radicals that can interact with molecular oxygen, thus generating ROS (8).

In spite of their therapeutic value, the efficacy of cisplatin and its derivatives, carboplatin and oxaliplatin, is limited by the rather strong toxic side-effects, including nausea and vomiting, myelosuppresion, immunosuppression, nephrotoxicity, neurotoxicity and hearing loss (9, 10). Another drawback of platinum chemotherapy is the high level of resistance developed by tumor cells during treatment (11). The thiols groups derived from metallothioneins, glutathione (GSH), *etc.* appear to have a role in this resistance, so that elevated levels of GSH, and enhanced metallothioneins expression are linked to these phenomena. GSH may affect platinum complex cytotoxicity in one of the two following ways: (i) by sequestering the complex prior to reaction with its biological

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target (DNA) and/or (ii) by quenching and removing platinum-DNA monoadducts, thereby inhibiting formation of toxic lesions (12).

In order to overcome the main limitations of therapy with platinum drugs, new chemical entities were developed. Due to their high affinity for nitrogen and sulfur donor ligands of the Ru(II) and Ru(III) ions, similar to that of Pt(II), ruthenium-based compounds have attracted interest for treating tumors with a selective cytotoxicity to cancer cells. The coordination complexes indazolium (*trans*-tetrachlorobis (1*H*-indazole) ruthenate (III)) or KP1019 (13-15), and imidazolium (*trans*-tetrachloro(1H-imidazole)(S-dimethylsulfoxide)ruthenate(III), or NAMI-A (16-18) (both now in phase II clinical trials) were proved to be the most promising candidates as an alternative to platinum drugs. The two complexes present different types of anticancer activity: KP1019 is active against primary cancers through induction of apoptosis (18-19), whereas NAMI-A is active against secondary tumor cells (*i.e.* the metastases), although it shows no direct cytotoxic effects (16-17).

The mechanism of action of KP1019 seems to involve two major pathways: (i) the complex might interact with DNA causing crosslinks like cisplatin does (15, 20) or induce strand breaks, and (ii) based on the similarity between ruthenium and iron (both belonging to the iron-triad), the complex is transferred into the cell *via* the transferrin pathway (for which it has a strong affinity), and needs a reductive activation after the cellular intake (21). It can be assumed that Ru(III) may substitute Fe(III), which induces Fenton-type redox processes and intracellular radicals. This may well result in cellular damage that induces apoptosis. Therefore, the induction of oxidative stress seems to be an essential component of the cytotoxic effect of Ru(III) complexes (22).

The mechanism of action of NAMI-A is still largely unknown. NAMI-A demonstrates some similarities with KP1019, but also important differences. Thus, it is capable of interacting with DNA in cell-free medium; however, similarly to KP1019, nuclear DNA does not seem to be its primary target. *In vitro* and *in vivo* studies showed that it also binds strongly to the plasma proteins albumin and transferring (23, 24); however, the association with plasma proteins causes a drastic decrease of the drug's capacity to reach the cellular targets. The results of some studies sustain the hypothesis that the mechanism underlying the anti-metastatic activities of NAMI-A does not involve DNA binding as the most significant process. Instead, they interfere with type-IV collagenolytic activity and reduce the metastatic potential of the tumors (25).

In the present study, the antitumor activity of two new complexes of Ru(III) with the anti-bacterial quinolones norfloxacin and ofloxacin (Figure 1), and their possible mechanisms of action were investigated. Previously, we found that these complexes interact *in vitro* with calf thymus DNA (26) and human serum transferring (27). Herein, we wanted to test whether ROS play an important role in the signaling pathway involved in the apoptotic transformations, when these complexes were used as antitumor treatment.

Until now, some organoruthenium (II) complexes with quinolones have been explored as potential anticancer agents. The cytotoxicity of complexes with the general formula ((Cym)RuCl(L)) (cym=η^6-*p*-cymene; L=ofloxacinate, cinoxacinate, nalidixicate, thionalidixicate anions) was investigated against human cancer cell lines (28-30), and their interaction of DNA was studied (28, 30).

Materials and Methods

Compounds and treatment. The ruthenium complexes were prepared according to a previously reported procedure (35): a solution prepared by dissolving the ligand (0.6387 g norfloxacin or 0.7227 g ofloxacin) and the metal salt (0.2074 g $RuCl_3$) in a 2:1 molar ratio in dimethyl sulfoxide (30 ml) was heated under reflux for 6 h. Once the solution turned into dark-brown, after cooling, a 2-M solution of NaCl (~30 ml) was added in order to obtain the solid product. The brown residue was filtered-off and washed several times with distilled water and air-dried.

The dose used for each compound may be considered as the maximum tolerated and equitoxic dose corresponding to the LD_{50} obtained with the same treatment schedule and route of administration in separate tests with healthy Wistar rats.

Animals. Wistar albino rats weighing 150-180 g (2-months-old) were fed on standard diet and water *ad libitum*. The animals were housed in standard boxes. All animal experiments were carried out at the animal care facility of the Bucharest Institute of Oncology. The experimental protocol was approved by the institutional animal ethics committee constituted for this purpose. The *in vivo* experimental models were elaborated on legally based decisions about principles of good laboratory practice and ethics.

Tumor implantation. Approximately 10^6 tumor cells of Walker 256 carcinoma were implanted intramuscularly in the right flank of the rats. Two weeks after implantation, the tumor grew locally early in the course of diseases without invading the surrounding organs. The tumoral volume was calculated using the formula $V=0.52\times a\times b^2$, where a and b represent the maximum and the minimum tumor diameters, respectively.

Experimental design. The rats were divided into three groups, with each group consisting of 10 animals. Two groups were treated with RDQ-1 and RDQ-2 and the third represented the control group. The compounds were administered orally, daily in 1 ml suspension to both treated groups, containing 75 mg active compound/body weight in physiological saline solution. Treatment was conducted over a three week period, managing drug users being done five days a week At the end of each from three weeks the animals were killed by cardiac puncture. Blood samples were drawn into tubes and allowed to clot at room temperature for 30 min. After centrifugation, the serum was removed. Tests on all blood samples were performed in days 5, 10, and 15, following the treatment for all experimental lots.

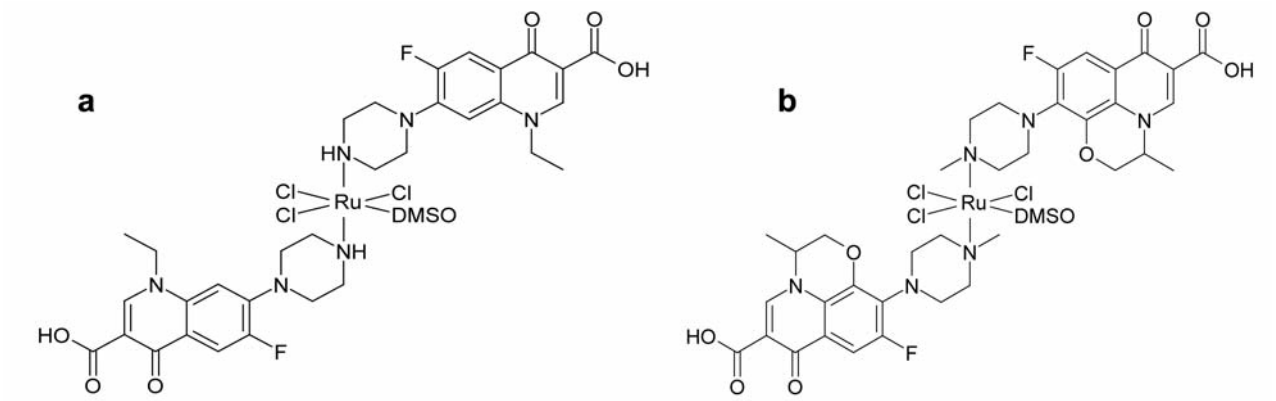

Figure 1. *Proposed structures of the complexes studied: (a) $Ru(nf)_2Cl_3(DMSO)\ H_2O$ abbreviated RDQ-1; (b) $Ru(of)_2Cl_3(DMSO)\ H_2O$ abbreviated RDQ-2.*

Oxidative stress assessment. Using serum as the biological sample, lipid peroxidation measurement was performent by the malonyaldehyde assay (36-38). Total thiol groups were assayed as described by Schosinski *et al*. (39), using Ellman reagent (40). Total antioxidant activity was measured by the ability of the biological sample to reduce iron, a method involving the Fe^{III}–TPTZ-Fe^{II}–TPTZ reaction (40). All biochemical determinations were carried-out in triplicate and are expressed as mean values.

Apoptosis measurement by flow-cytometry. Tissue samples from untreated and treated tumor-bearing rats and controls were minced into approximately 1-mm^3 pieces and suspended in a wash solution containing 0.115 M phosphate-buffered saline (PBS), pH 7.4, and 2% fetal bovine serum. Cell suspensions were then passed through a wire mesh (pore size 30 μm) and rinsed several times with the wash solution. The suspensions containing nuclei were centrifuged at 300 × *g* for 5 min. The nuclear pellets were dispersed in 2-4 ml of cold 70% ethanol and stored at 4˚C for 1 h, until processed.

The isolated nuclei were washed with PBS and adjusted at a final concentration of 1×10^6 cells/ml, the pellet was treated afterwards with 500 μl PI-Hypotonic Lysis Buffer (0.1% Triton X-100 and 50 μg/ml PI in 0.1% sodium citrate). The tubes were placed in the dark at 4˚C overnight, before flow cytometry analysis. Results (10,000 events) were acquired by a FACSCalibur™ flow cytometer using the CellQuest software. Analysis was performed using the WinMDI program.

The histogram graphics represent the DNA quantitative distribution, which is expressed as a function between the fluorescence 2-height (FL2-H) fluorescence detector measurement and the total cell number.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA). All results for the applied statistical tests (ANOVA, *t*-test) had a 90% confidence interval (CI). Results were considered statistically significant at $p<0.05$.

Results and Discussion

Treatment dynamics by tumoral volume measurement. Based on the hypothesis that our new-synthesized compounds have antitumor activity, the animal weight and the tumor diameters were measured. Animal weight gain is in principle due to the increasing tumor mass. Determining the dynamics by repeated measurements of this parameter, we can provide information about blocking the process of tumor proliferation and indirectly monitor the antitumor activity of the test compounds. Values are presented in Figure 2. Animal weight was recorded daily; the data represent the average of five consecutive weekly measurements. Growing weight is done by the increase of tumoral mass. The results show an increase of tumor volumes with an initial evolution in the first 10 days; the average tumor volume of these animals was lower than that of the control group as depicted in Figure 3. The lowest tumor growth was registered with RDQ-1 administration, which can be explained by the dosage protocol used, *i.e*. the concentration of the active compound used could not reach the anticipated biological activity. Since a dose level was used, when no toxic side-effects were observed, it was probably because the active compound concentration was lower than the biologically active one. However, we measure the diminishing of tumor growth, more efficient for the investigated compound RDQ-1.

Although an initial evolution of the tumor was recorded in the first 10 days, the average tumor volume of these animals was lower than that of the control group. This observation suggested that the investigated compounds exhibited an antitumor effect; the chosen dose was probably not the most efficient.

Lipid peroxidation measurements. From the data presented, we presumed that the primary target of oxidative attack were the double bonds in the structure of polyunsaturated fatty acids, essential components of lipids, glycolipids, structural lipoproteins. This attack can induce strand breaks and initiate chain reactions with production of new ROS that can migrate away from the place of production, initiating tumor cell devastating events, but also to the surrounding

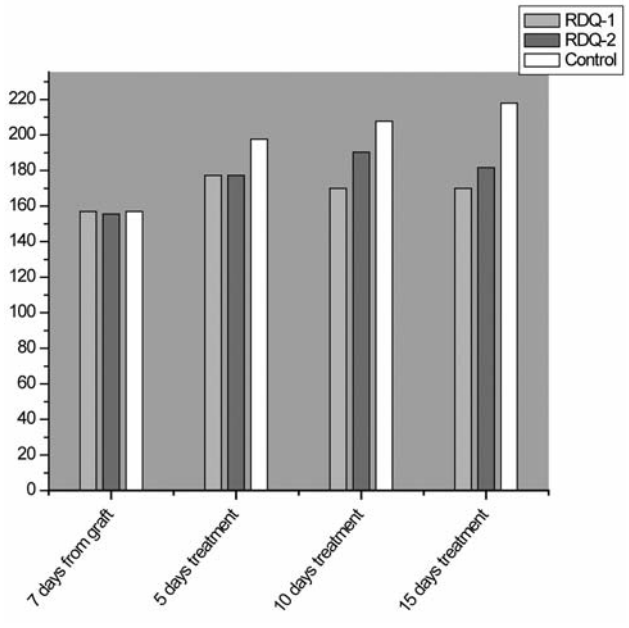

Figure 2. *Tumor-bearing animals weight expressed in grams.*

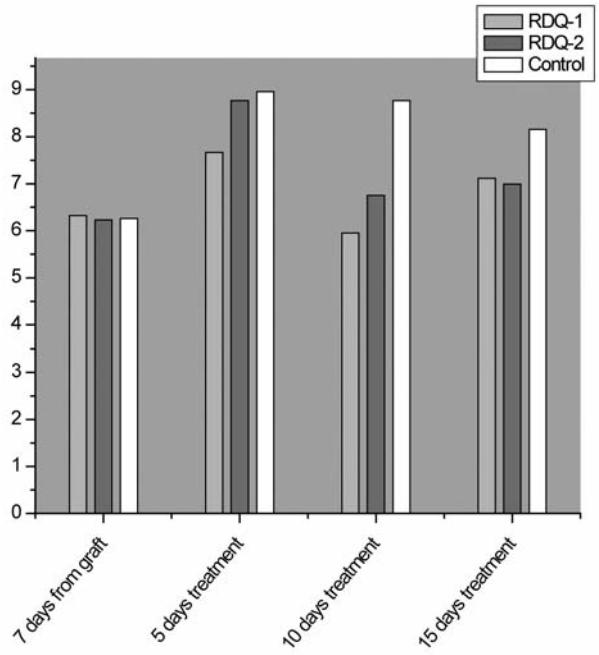

Figure 4. *MDA concentration (μmol/100 ml).*

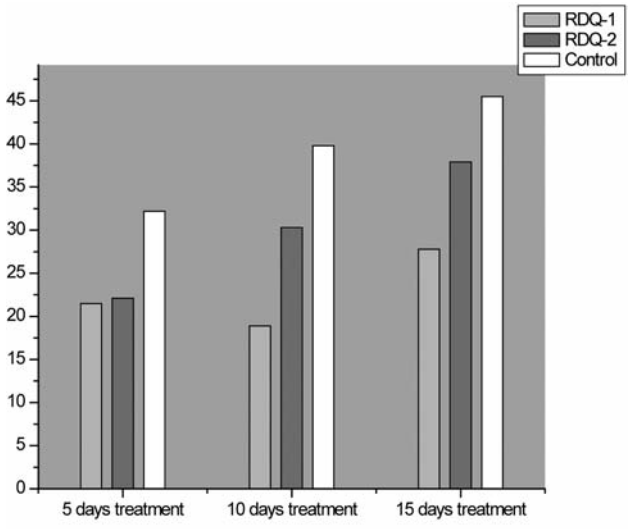

Figure 3. *Tumor volume expressed in mm^3.*

tissue cells. As a result, the index of lipid peroxidation may be an expression of cytotoxicity mediated by free radicals, produced, in our case, from the metabolism of the tested compounds. The results indicate an increase in the tested reaction the first phase of the treatment and the storage of the obtained values in a plateau, suggesting that the metabolism of RDQ-1 and RDQ-2 are not by ROS production. Growth in the first round is on ROS production by the tumor, knowing that the tumor tissue itself is producer of free radicals. The increase of lipoperoxidation is a common feature of all tumors due to cell proliferation, and also to the angiogenic transformation of the tumor tissues (31). We did not register significant changes between the two tested compounds. These results are shown in Figure 4, demonstrating that the dynamic management of the two ruthenium-based compounds do not induce significant lipid peroxidation compared to untreated control, thus confirming, once again, that the mechanism of metabolism of the two test compounds is not based on excess production of free radicals.

Total thiol groups determination. Action of the reactive oxygen metabolites in the structural protein induce their denaturation (32) Interactions frequently occur with protein thiol groups and new radical formation, which in turn can initiate oxidation or disulphide formation, such as sulphonic acid derivatives.

The concentration values of total thiol groups are shown in Figure 5. The decrease in the concentration of total thiol groups signifies that proteins are not the first molecules attacked by ROS. This finding also suggests, comparing the treated groups to the normal level, that there is a higher concentration of sulfur-containing proteins synthesized by

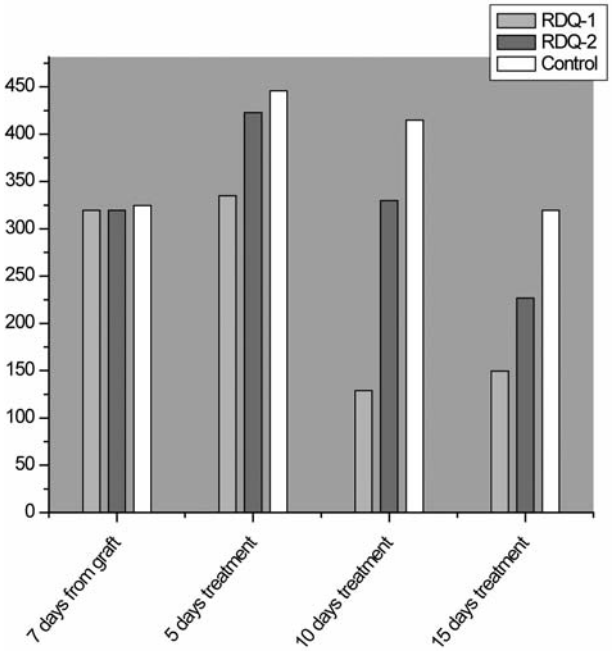

Figure 5. *Total thiol groups (μmol/l).*

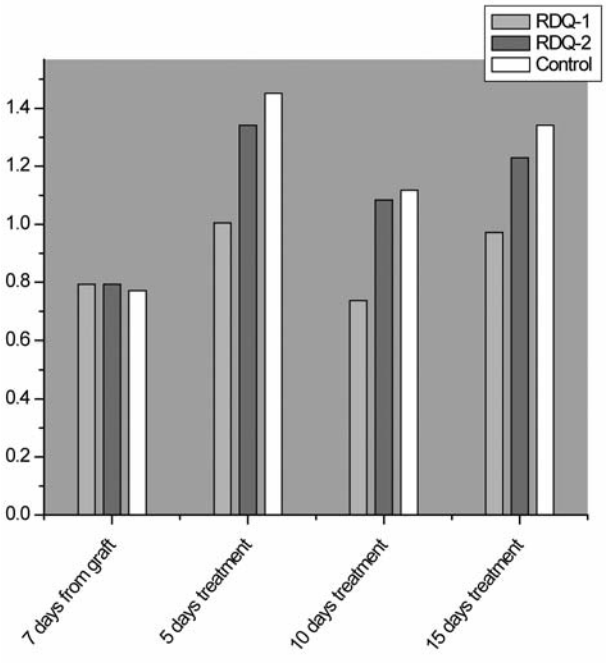

Figure 6. *FRAS as a total antioxidant activity measurement (μmol/ml).*

the tumor as a protective mechanism. Linking the two new synthesized compounds to the –SH groups derived from the protein oxidation may explain the lower levels registered in the dynamic of treatment. In addition, these levels can explain the possible resistance to the anti-tumor treatment of the chelated-heavy metal-based compounds.

The anti-oxidant activity. Figure 6 shows the total anti-oxidant activity monitored by the ability of the biological sample to reduce iron. The increase of the antioxidant activity is associated with the activation of natural protection systems as a result of oxidative stress after the above installed treatment. Recorded values suggest the production of ROS after metabolizing the ruthenium derivation compounds. Excess ROS production implicates their participation in cellular cytotoxicity associated with the treatment, thus marking the anti-tumor effect. This can be explained, at the molecular level, by the destruction of key macromolecules by oxidative reactions. In addition, proof of this mechanism is the activation of antioxidant systems at the cellular level.

The apoptosis measurements. Apoptosis is characterized by specific morphological modifications; one of them is the nuclear DNA cleavage by endonucleases activation at cell death time. As a result of the fragmentation, the apoptotic cells lose a part of DNA (33). The coloring with propidium iodide (fluorochrome, which is stoichiometrically intercalated between the bases of the double helix and emits red light after excitation with a laser beam) permits the detection of a cell population with a reduced DNA content (hypodiploid or sub-diploid) vis-à-vis a normal G0/G1 cell population with euploid (diploid) DNA content. The appearance of a sub-diploid DNA peak is a specific marker of apoptosis (34). As a result, the identification and quantification of apoptotic cells can be achieved by flow cytometry analysis.

In all tested cases, we observed that the suspension of tumor cells in hypotonic buffer facilitates the loss of fragmented DNA and produces a clear shift in the sub-diploid peak of apoptotic nuclei (Figure 7 a, b).

The tested compound RDQ1 induced a more pronounced apoptosis, compared to that of RDQ2 as it increased almost twice (59.72%), suggesting a more intense biological activity. The possible mechanisms involved are the same, mediated by ROS and DNA adducts.

Conclusion

In the present study, we attempted to delineate the relationship between the antitumor activity of two novel ruthenium (III) complexes and their capacity to induce ROS formation and apoptosis in tumor cells. Between the two new ruthenium derivative complexes with quinolones, we found that the active one was RDQ-1, where ruthenium is bound with norfloxacin as a ligand. Treatment with the studied

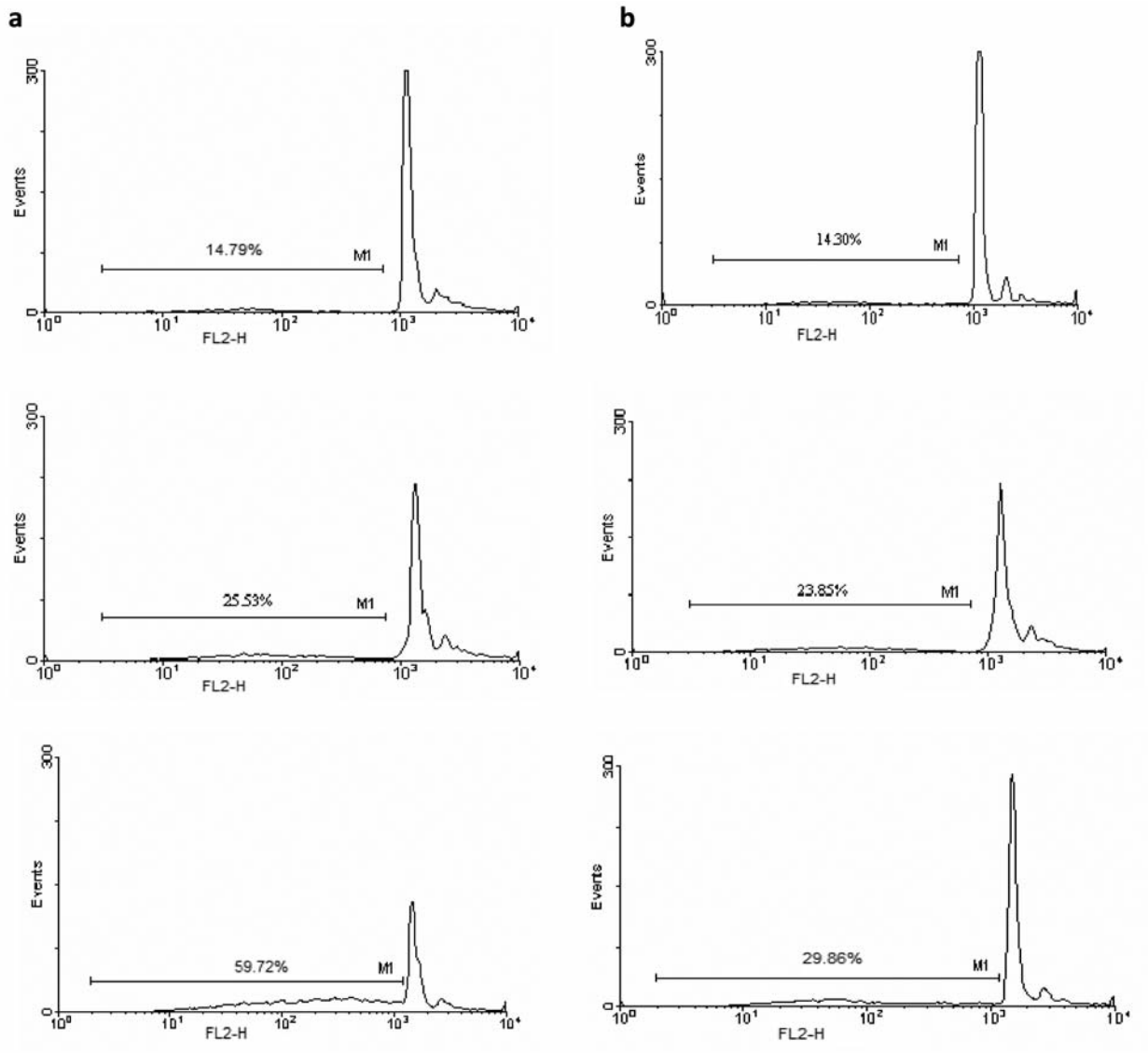

Figure 7. *DNA fluorescence histograms of (a) RDQ-1 and (b) RDQ-2 inducing apoptosis in rat tumors.*

compounds led to a marked increase of hypodiploid particle formation. The number of cells accumulated in this phase exceeded 59.72% for RDQ-1. These results demonstrated the ability of the studied compounds to induce apoptosis. The consequences of ROS production were cells cytotoxicity by direct attack due to structural lipid and protein degradation and also DNA-adduct formation. ROS also activated the apoptosis signaling pathways but one consequence is the activation of natural anti-oxidant defense system.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

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