Cytotoxic and antiproliferative activities of monensic acid and its metal(II) complexes against drug sensitive and multidrug resistant human tumor cell lines

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***Abstract* —** In the present study for the first time the anticancer activity of the polyeter ionophorous antibiotic Monensic acid (MonH) and its complexes with Ca(II), Co(II) and Mn(II) ions was evaluated against the drug sensitive human squamous cell carcinoma cell line A431 and its multidrug resistant clones that express MDR1, MRP1 or ABCG2 gene. For comparative purposes the non-tumor human cell line Lep3 was also included in the experiments. The investigations were carried out using MTT test and colony-forming method. The results obtained reveal that applied at concentrations of 0.5-25 μg/mL for 24-72 h the compounds investigated decrease the viability and proliferation of the treated cells in a time- and concentration-dependent manner. The investigated metal(II) complexes (especially those of Mn(II) and Co(II)) has been found to express higher cytotoxic and cytostatic activities as compared to the non-coordinated MonH.

***Keywords* —** Drug sensitive human squamous cell carcinoma A431, Non-tumor human cell line Lep3, Monensin biometal(II) complexes, Multidrug resistant A431 clones

I. Introduction

THE polyether ionophore Monensin (Scheme 1) is a natural antibiotic, applied in veterinary medicine as coccidiostatic and antibacterial agent [1]-[6]. This compound isolated from *Streptomyces cinnamonensis* complexes with alkali ions and transfers them across cell membranes causing disturbances in the metal homeostasis of microorganisms and parasites leading to their death [7]-[10]. Recent data have revealed that Monensin expresses also antitumor activity against cell lines established from various malignancies including leukemia, lymphoma, myeloma, renal cell carcinoma and cancers of the colon, breast and cervix [11]-[15].

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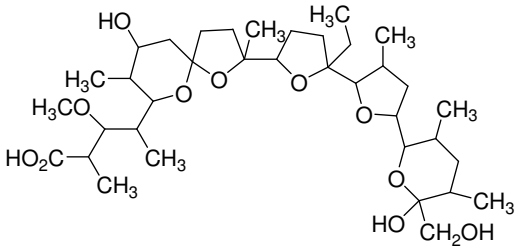


Scheme 1. Structure of Monensic acid A

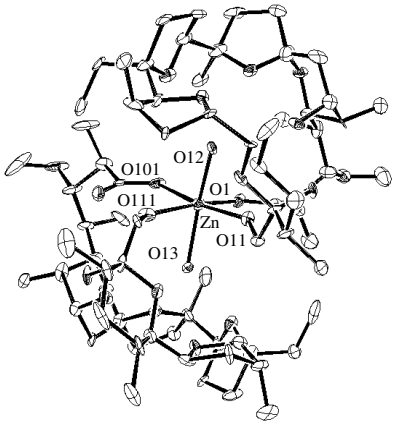
On the other hand, some studies have shown that the biological activity of Monensin is influenced by the presence of metal(II) ions as Mg(II), Pb(II), etc., but up to 2008 no isolation and characterization of metal(II) containing Monensin species was reported in the literature [16]-[18].

We have extensively studied the ability of Monensic acid (MonH) and its sodium complex (MonNa) to coordinate metal cations of valence higher than +1 and characterized series of complexes containing various compositions and different structures [19]-[24]. The complexes of biometal(II) ions such as Mg(II), Ca(II), Mn(II), Co(II), Ni(II) and Zn(II) consists of a similar stricture, with a molar ratio of M : Mon : H_2O = 1 : 2 : 2 (Scheme 2). The isostructural metal(II) compounds possess generally enhanced activity against Gram-positive aerobic and anaerobic bacteria in comparison to Monensic acid [20], [23]-[25]. We have proposed that the activity of Monensin biometal(II) complexes depends on the nature of metal(II) cations, as well as on the bacteria strain tested.

It is of further interest to evaluate whether the presence of the biometal(II) ion will influence the cytotoxic and cytostatic properties of Monensic acid.

It has been found in our investigations that Co(II), Ca(II), Mn(II) and Mg(II) complexes of Monensic acid significantly decrease the viability and proliferation of cultured human and animal tumor cell lines being more active as compared to the non-coordinated MonH [26]-[28]. The high cytotoxic and cytostatic activities of these compounds have also prompted us to examine their antitumor effect against multidrug resistant human cancer cells. Multidrug resistance is well known to be a major reason for failure of cancer chemotherapy since multiple

chemotherapeutic drugs of different classes are used to treat most malignancies.



Scheme 2. ORTEP structure of biometal(II) complexes of Monensic acid $[M(Mon)_2(H_2O)_2]$, M = Zn [23]

In the present paper we report the results on evaluation of the effects which Monensic acid and its complexes with ions of Ca(II), Co(II) and Mn(II) render on viability and proliferation of drug sensitive and multidrug resistant human skin carcinoma cell lines.

II. Experimental part

A. Materials

The commercially available sodium Monensin (MonNa) was supplied from Biovet Ltd. (Bulgaria). Metal(II) salts and solvents were purchased from Riedel de Häen AG (Germany). Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO) and trypsin were obtained from AppliChem (Germany); purified agar (Difco) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich Chemie GmbH (Germany). Ethylendiaminotetraacetic acid (EDTA) and all other chemicals of the highest purity commercially available were purchased from local agents and distributors.

B. Preparation of Monensin metal(II) complexes

The synthesis of Monensic acid (MonH, $C_{36}H_{62}O_{11}$) and its biometal(II) complexes was performed as it was earlier described [20], [23]. The cytotoxic activity of MonH and $[M(Mon)_2(H_2O)_2]$ (M = Ca (**1**), Co (**2**), Mn (**3**)) was further studied in order to evaluate their properties. Monensic acid and complexes **1-3** are sparingly soluble in water. For that reason the compounds were applied as solutions in DMSO in the concentration range from 0.5 μg/mL to 25 μg/mL. The effect of DMSO administered at 0.05 – 2.5% was also evaluated.

C. Cell cultures and cultivation

The drug sensitive human skin derived epidermoid carcinoma cell line A431 and its multidrug resistant clones that express MDR1, MRP1 or ABCG2 gene were used as experimental models in the present research [29]. The cell line Lep3 established from 3-month-old human embryo was also included in our study for comparative investigations.

The cells were grown as monolayer cultures in D-MEM medium, supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained at 37 ºC in a humidified CO_2 incubator. For routine passages adherent cells were detached using a mixture of 0.05% trypsin – 0.02% EDTA. The experiments were performed during the exponential phase of cell growth.

D. MTT assay

The effect on cell viability was determined by measuring MTT (thiazolyl blue tetrazolium bromide) dye absorbance of living cells. Cells were seeded in 96-well flat-botommed microplates (Orange Scientific) at a concentration of 2×10^4 cells/well. At the 24th h cells from monolayers were washed and covered with media modified with different concentrations of the compound tested. Each concentration was applied into 4 to 6 wells. Samples of cells grown in non-modified medium served as a control. After 24, 48 and 72 h of incubation, the solutions were removed from the plates and MTT colorimetric assay of cell survival was performed as described by Mossman [30]. The method consisted of three hours incubation with MTT solution (5 mg MTT in 10 mL D-MEM) at 37 ºC under 5% carbon dioxide and 95% air, followed by extraction with a mixture of absolute ethanol and DMSO (1:1, vol/vol). Optical density was measured at wave length 540/615 nm using an automatic microplate reader (TECAN, Sunrise^{TM}, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. "Concentration – response" curves were prepared. The effective concentrations of the compounds causing a 50% reduction of cell viability (cytotoxic concentration 50, CC_{50}) and/or CC_{90} (causing a 90% reduction of cell viability) were estimated from these curves.

All data points represent an average of three independent assays.

E. Colony forming method

Tumor cells (10^3 cells/well) suspended in 0.45% purified agar in D-MEM medium containing different concentrations of the compounds examined (ranging from 0.5 to 25 μg/mL) were layered in 24 well microplates (Orange Scientific). The presence/absence of colonies was registered using an inverted microscope (Carl Zeiss, Germany) during 16 days period. Colony inhibitory concentrations (CIC) at which the compounds tested inhibit completely the ability of tumor cells to grow in semi-solid medium were determined.

F. Statistical analysis

The data are presented as mean ± standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.

III. RESULTS

For evaluation of antitumor properties of metal(II) derivatives of the polyether ionophore, complexes of Monensic acid with ions of Ca(II), Co(II) and Mn(II) were prepared. Coordination of the ligand undergoes formation of compounds with composition $[M(Mon)_2(H_2O)_2]$ (M = Ca (**1**), Co (**2**), Mn (**3**)). The structure of complexes is similar, with a metal(II) ion placed in an octahedral environment due to the coordination of two bidentate Monensin monoanions and two water molecules (Scheme 2) [20], [23], [24].

Applied at concentrations examined MonH and complexes **1-3** decrease the viability and proliferation of the treated cells in a time- and concentration-dependent manner. The activity of compounds against A431 cell line and its clones expressed as CC_{50} and CC_{90} values are presented in Tables 1-6. Examples of concentration-response curves are shown in Figs. 1-4. The influence of tested compounds in effective concentrations (CIC) on tumor cell growth inhibition is presented in Table 7.

The investigated metal(II) complexes (especially those of Mn(II) and Co(II)) have been found to express higher cytotoxic and cytostatic activities against drug sensitive A431 cell line and its multidrug resistant clones. According to their sensitivity to MonH and its complexes, the cell lines used as experimental models in our investigations are graded in hierarchic orders as it is presented in Table 8.

The influence of DMSO (administered at concentrations of 0.05 – 2.5%, corresponding to those in the solutions of the tested compounds) has been found to be weaker than those of Monensin and complexes **1-3** (especially in the range of 0.05-1% that corresponds to 0.5-10 μg/mL of the compound).

Table 1. Cytotoxicity assay using cell line A431 (CC_{50}, μM)

Compound	24 h	48 h	72 h
Monensic acid	31.9	4.8	<
$[Ca(Mon)_2(H_2O)_2]$, **1**	7.1	2.7	<
$[Co(Mon)_2(H_2O)_2]$, **2**	-	2.9	<
$[Mn(Mon)_2(H_2O)_2]$, **3**	-	2.7	<

"<" – the CC_{50} value was not determined because in all concentrations tested the cell viability was lower than 50%

"-" – the CC_{50} value was not determined because in all concentrations tested the cell viability was higher than 50%

Table 2. Cytotoxicity assay using cell line A431-MRP (CC_{50}, μM)

Compound	24 h	48 h	72 h
Monensic acid	15.4	4.1	<
$[Ca(Mon)_2(H_2O)_2]$, **1**	9.6	2.3	<
$[Co(Mon)_2(H_2O)_2]$, **2**	12.5	2.0	1.8
$[Mn(Mon)_2(H_2O)_2]$, **3**	15.0	2.4	<

"<" – the CC_{50} value was not determined because in all concentrations tested the cell viability was lower than 50%

"-" – the CC_{50} value was not determined because in all concentrations tested the cell viability was higher than 50%

Table 3. Cytotoxicity assay using cell line A431-MDR (CC_{50}, μM)

Compound	24 h	48 h	72 h
Monensic acid	15.4	4.1	0.9
$[Ca(Mon)_2(H_2O)_2]$, **1**	-	5.0	0.6
$[Co(Mon)_2(H_2O)_2]$, **2**	-	2.8	1.4
$[Mn(Mon)_2(H_2O)_2]$, **3**	-	6.4	<

"<" – the CC_{50} value was not determined because in all concentrations tested the cell viability was lower than 50%

"-" – the CC_{50} value was not determined because in all concentrations tested the cell viability was higher than 50%

Table 4. Cytotoxicity assay using cell line A431-ABCG2 (CC_{50}, μM)

Compound	24 h	48 h	72 h
Monensic acid	34.7	3.3	0.8
$[Ca(Mon)_2(H_2O)_2]$, **1**	10.6	0.6	<
$[Co(Mon)_2(H_2O)_2]$, **2**	-	2.5	<
$[Mn(Mon)_2(H_2O)_2]$, **3**	-	<	<

"<" – the CC_{50} value was not determined because in all concentrations tested the cell viability was lower than 50%

"-" – the CC_{50} value was not determined because in all concentrations tested the cell viability was higher than 50%

Table 5. Cytotoxic concentration 90 (CC_{90}, μM) of the compounds in drug sensitive and multidrug resistant tumor cells

Compound	Cell line				
	A431		A431-MDR	A431-MRP	
	48h	72h	72h	48h	72h
Monensic acid	14.2	5.4	25.8	13.3	4.9
Complex **1**	14.3	2.6	11.6	13.0	7.0
Complex **2**	10.2	3.3	6.9	11.6	15.1
Complex **3**	5.3	2.4	3.2	11.7	3.2

Table 6. Cytotoxicity assay using cell line Lep3 (CC_{50}, μM)

Compound	24 h	48 h	72 h
Monensic acid	18.4	4.3	6.4
$[Ca(Mon)_2(H_2O)_2]$, **1**	9.1	3.9	2.0
$[Co(Mon)_2(H_2O)_2]$, **2**	7.2	3.9	3.7
$[Mn(Mon)_2(H_2O)_2]$, **3**	4.3	5.5	5.5

"<" – the CC_{50} value was not determined because in all concentrations tested the cell viability was lower than 50%

"-" – the CC_{50} value was not determined because in all concentrations tested the cell viability was higher than 50%

Table 7. Effect of MonH and metal(II) complexes on colony-forming ability of tumor cells

Compound / Cell line	Concentration (μM) *		
	A431	MRP	MDR
Monensic acid	≥ 5	≥ 20	≥ 5
$[Ca(Mon)_2(H_2O)_2]$, **1**	≥ 2.5	≥ 12.5	≥ 7.5
$[Co(Mon)_2(H_2O)_2]$, **2**	≥ 5	≥ 10	≥ 12.5
$[Mn(Mon)_2(H_2O)_2]$, **3**	≥ 2.5	≥ 5	≥ 5

* Concentration at which the compound inhibits the colony-forming ability of tumor cells

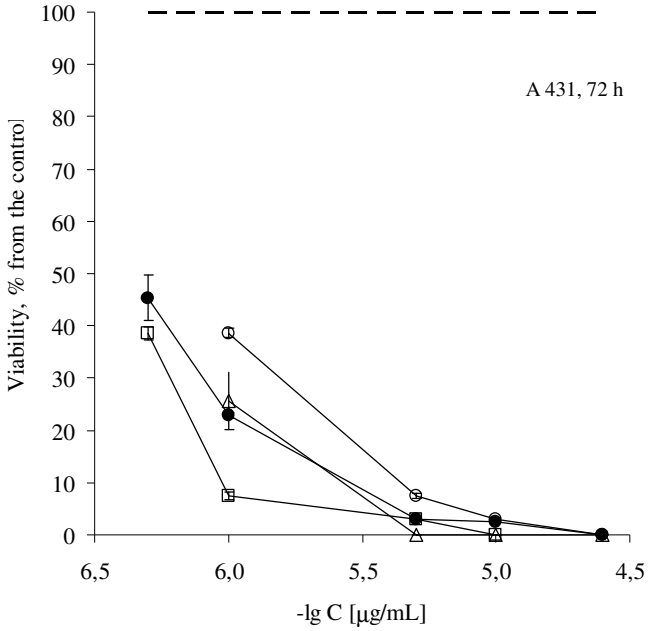


Fig. 1. Effect of Monensin and complexes **1-3** on viability of cell line A431 on 72 h (○ - MonH, ● – **1**, Δ - **2**, □ – **3**, --- control)

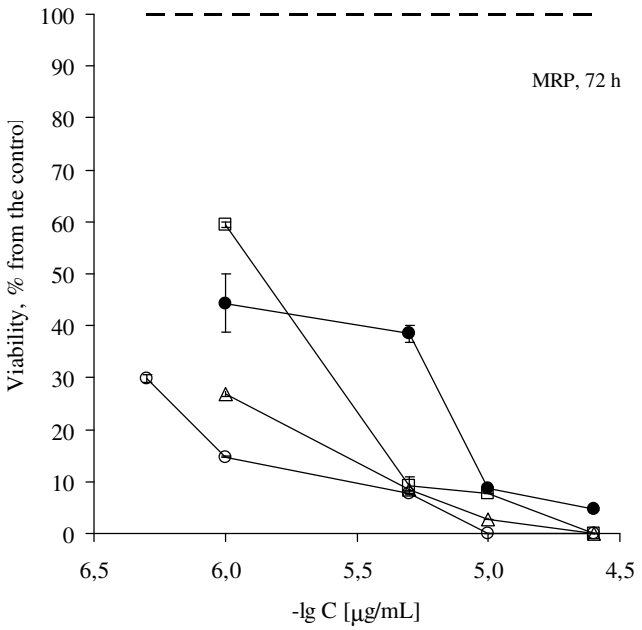


Fig. 2. Effect of Monensin and complexes **1-3** on viability of cell line A431-MRP on 72 h (○ - MonH, ● – **1**, Δ - **2**, □ – **3**, --- control)

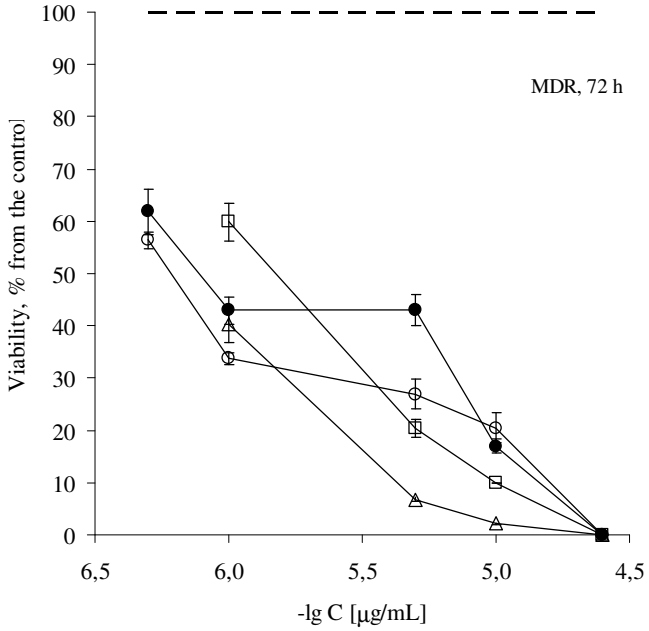


Fig. 3. Effect of Monensin and complexes **1-3** on viability of cell line A431-MDR on 72 h (○ - MonH, ● – **1**, Δ - **2**, □ – **3**, --- control)

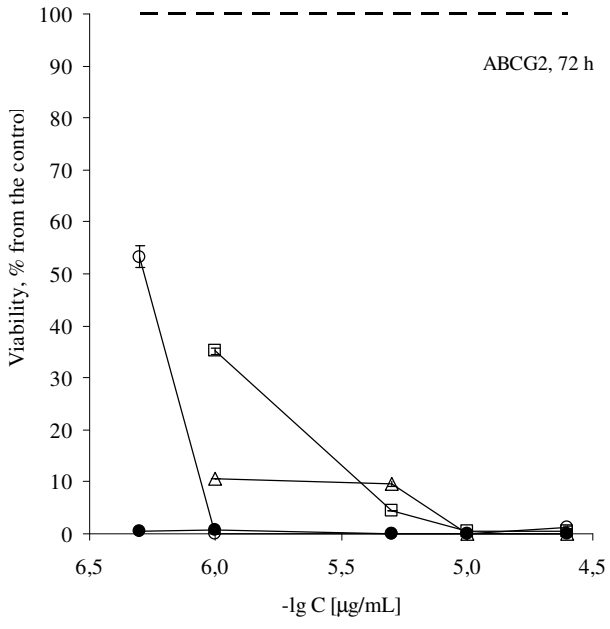


Fig. 4. Effect of Monensin and complexes **1-3** on viability of cell line A431-ABCG2 on 72 h (○ - MonH, ● – **1**, Δ - **2**, □ – **3**, --- control)

Table 8. Hierarchic orders of cell lines according to their drug sensitivity

Compound	Hierarchic order
Monensic acid	A431= MRP=Lep3 >ABCG2 ≥ MDR
Complex **1**	A431=MRP=ABCG2>MDR
Complex **2**	A431= Lep3>MDR≥ MRP> ABCG2
Complex **3**	A431≥ MRP = MRD > ABCG2

*All hierarchic orders start with the most sensitive cell line (i.e. with the lowest CC_{50} and CC_{90} value); MRP = A431-MRP, MDR = A431-MDR, ABCG2=A431-ABCG2

Table 9. Relative resistance values (48 h) * of MonH and **1-3**

Compound	MRP	MDR	ABCG2
Monensic acid	0.85	0.85	0.69
$[Ca(Mon)_2(H_2O)_2]$, **1**	0.85	1.85	0.22
$[Co(Mon)_2(H_2O)_2]$, **2**	0.69	0.97	0.86
$[Mn(Mon)_2(H_2O)_2]$, **3**	0.89	2.37	n.c.

* Relative resistance value was calculated as resistant cell CC_{50}/sensitive cell CC_{50}; n.c. – not calculated

IV. DISCUSSION

Monensin, an antibiotic produced by fermentation of a strain of *Streptomyces cinnamonensis*, is a carboxylic Na^+/H^+ ionophore used worldwide in veterinary medicine as a growth promoter in beef cattle and feed additive against coccidiosis in chickens and pigs [31]-[33]. The increasing interest to biological activity of Monensin in recent years has been stimulated by the data concerning antitumor properties of this compound. It has long been considered that activation of the Na^+/H^+ antiporter and the resulting pH changes participate in many cellular functions such as proliferation, differentiation and apoptosis [34]-[38]. In particular, Monensin is known to transport Na^+ ions into cell while protons are transported out of cells, resulting in intracellular alkalization [39], [40]. In 1995 Zhu and Loh [34] reported that Monensin caused apoptosis as well as intracellular alkalization in HL-60 human leukemia cells. It has also been noticed that the cytotoxic action of drugs such as Monensin may be due to a decrease in the cellular ATP level [41].

The antitumor activity of Monensin has been extensively studied by Park et al. [11]-[15]. The authors have reported that the antibiotic significantly inhibits proliferation of cultured cell lines established from renal cell carcinoma (inhibitory concentration 50, IC_{50}, of about 2.5 μM), colon cancer ($IC_{50} \approx$ 2.5 μM), myeloma ($IC_{50} \approx 1$ μM), lymphoma ($IC_{50} \approx 0.5$ μM) and myelogenous leukaemia ($IC_{50} \approx 0.5$ μM). In addition, Monensin suppresses *in vivo* the growth of murine leukemia WEHI-3BD cells in BALB/c mice [11]. It has been found in our investigations that Monensic acid and its metal(II) complexes decrease significantly viability and proliferation of cultured cell lines established from some of the most common and aggressive human malignancies such as glioblastoma multifirme (8 MGBA) and cancers of the breast (MCF-7), lung (A549), liver (HepG2) and uterine cervix (HeLa). The compounds are shown to be also highly effective against virus-transformed tumor cells expressing *v-myc* (LSCC-SF-Mc29, derived from a transplantable chicken hepatoma induced by the myelocytomatosis virus Mc29) and *v-src* (LSR-SF-SR, established from a transplantable rat sarcoma induced by Rous sarcoma virus strain Schmidt-Ruppin) gene. The members of both gene families – *Myc* and *Src*, are well known to be involved in pathogenesis of a wide variety of malignancies in humans and animals [42]-[43].

In the present study we have demonstrated for the first time that MonH and its metal(II) complexes significantly decrease viability and proliferation not only of drug sensitive human skin carcinoma cell line A431 but also of its multidrug resistant clones A431-MDR, A431-MRP and A431-ABCG2 that express MDR1, MRP1 or ABCG2 gene.

Therapy resistance is the major limitation for the successful treatment of human cancers especially at the disseminated stage. Cells in culture have served as an useful tool not only for the study of the mechanism(s) of drug resistance but also for searching new strategies and agents that can help us to overcome this problem [44]-[49]. Multidrug resistance (MDR) includes numerous strategies by which tumor cells can elude not only the cytotoxic effect of the drug(s) employed in chemotherapy but also can loose their sensitivity to a broad spectrum of drugs with neither obvious common targets nor structural homology. Decreased accumulation of drugs, increased efflux, reduced apoptosis and greater activity of DNA-repairing systems are some of the most studied mechanisms involved in MDR. ATP-binding cassette (ABC) proteins are present in all known living species, with a relatively conserved structure. To date, 49 different ABC genes have been identified in the human genome, divided into seven classes (A-G) based on sequence similarities. The multidrug ABC transporters are plasma membrane glycoproteins which cause chemotherapy resistance in cancer by actively extruding a large variety of therapeutic compounds from malignant cells. However, the same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues, especially in secretory organs, at the sites of absorption, and blood-brain barriers. Our decision to use A431-MDR, A431-MRP and A431-ABCG2 cell lines as experimental models is not occasional. The three major multidrug resistance ABC proteins are namely MDR1 (ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1) and ABCG2 (placenta-specific ABC transporter, ABCP/breast cancer resistance protein, BSRP/mitoxantrone resistance protein, MXR). MDR1 and MRP1 can transport a large variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates. The transport properties of ABCG2 are overlapping both with that of MDR1 and of MRP type proteins, thus these three proteins form a specific network in chemo-defense mechanism(s) [44]-[46].

The possibility that ionophores could modulate multidrug resistance has already been reported. However, considerable confusion exists regarding the mechanism(s) by which ionophores affect resistant (and sensitive parental) cells - ATP depletion, intracellular pH modification, membrane potential

variation, modulation of P-glycoprotein activity are under consideration. The interaction of various ionophore antibiotics with P-glycoprotein (the product of MDR1 gene) has been investigated [50]. Dysregulation of ion exchange has been supposed to play a central role in the evolution of drug resistance in tumor cells [51]-[53].

Multidrug resistant tumor cells exhibit an altered pH gradient across different cell compartments, which favors a reduced intracellular accumulation of antineoplastic drugs and a decreased therapeutic effect, respectively. It has been reported that the activity and expression of Na^+/H^+ exchanger (Monensin) are increased in doxorubicin-resistant (HT29-dox) human colon carcinoma cells in comparison with doxorubicin-sensitive HT29 cells [54]. Studies using carboxylic ionophores such as Monensin have indicated that these agents can modulate anthracycline toxicity apparently by causing an enhancement of its accumulation [55], [56].

The effects of Monensin liposomes on drug resistance reversal, induction of apoptosis and expression of multidrug resistance (MDR) genes in drug resistant cancer cells have been also studied. Thus, the combination of doxorubicin (2.5 μg/mL) with Monensin liposomes (20×10^{-8} M) induced apoptosis in approximately 40% of doxorubicin-resistant human breast tumour (MCF-7/dox) cells, whereas doxorubicin (2.5 μg/mL) or Monensin liposomes (20×10^{-8} M) by themselves produced minimal apoptosis (< 10%) in MCF-7/dox cells [57]. In another study, small unilamellar stealth Monensin liposomes (SMLs) have shown considerable effect as a potentiator in combination with adriamycin in overcoming drug resistance of HL-60 human leukemia cells [58]. These data suggest that the delivery of Monensin via liposomes can provided an opportunity to overcome drug resistance.

Undoubtedly, the most intriguing question is whether Monensin and its metal (II) complexes could be considered as potential antitumor and/or MDR reversing agents. The answer is not easy and requires a complex and multistep approach. What can we say at the moment?

On one hand, as it was discussed above, the anticancer activity of Na^+/H^+ ionophores and especially of Monensin has been established. In this study we present data that MonH and its metal(II) complexes decrease viability and proliferation of cultured human drug sensitive and multidrug resistant skin cancer cells. The effective concentrations at which these compounds reduce the viable cells by 50% (CC_{50}) and 90% (CC_{90}) and suppress the ability of tumor cells to grow in semi-solid medium are found to be very low (Tables 1-5, 7). These results are not surprising and could be explained at least partially by the ability of Monensin to induce significant alterations in the treated cells such as changes in pH (intracellular alkalization) and ATP content; early mitochondrial damage and energy deficit; cell cycle arrest (in G1 or G1-M phase) and apoptosis and/or necrosis efflux [11]-[15], [59]. It has to be mentioned here that in our experiments the antitumor activity of Monensin and its complexes has been proved both in short-term monolayer (24-72 h, performed by MTT test) and long-term 3D (16 days, colony-forming method) cultures. The compounds examined generally are toxic to resistant cells at concentrations similar or even lower to those that are required to inhibit viability and growth of parental A431 cells: in many cases the relative resistance values were found to be in the range of 0.85 - 0.97 (Table 9). As can be seen, there are only few exceptions such as $[Ca(Mon)_2(H_2O)_2]$ (**1**) that is toxic to resistant A431-ABCG2 cells at concentration approximately 5 times lower as compared to sensitive A431 cells. The A431-MDR cells are approximately twice more resistant than A431 cells.

On the other hand, the well known toxicity of Monensin as well as the fact that Monensin and its complexes are not water soluble raised the question whether such compounds could be utilized therapeutically in cancer treatment and control. The mechanisms of Monensin toxicity have been ascribed to cellular ion imbalance, calcium overload and lipid peroxidation and disintegration of cell membranes [31], [60]. The primary target organs of the inophores are heart, diaphragm, kidney and skeletal muscle [61]. One of the tasks of our study was to evaluate comparatively the cytotoxic effects of the compounds investigated on tumor and non-tumor cells. Although the Lep3 cells were found to be more resistant to the toxic activity of MonH and its metal(II) complexes (Table 6) as compared to the skin cancer cells especially after 72 h of treatment, these cells still showed relatively high sensitivity – CC_{50} are in the range of 2.0-18.4 μM. This phenomenon could be explained by the nature of Lep3 cells - it is widely accepted that embryonic cells resemble to some extent tumor cells due to their low differentiation and high proliferative potential.

Regarding DMSO used as a solvent, it should be noted that it is not a natural ingredient of human's body and several systemic side effects derived from the application of this compound have been reported. At the same time, DMSO was proved to be a cell-differentiation agent, a hydroxyl radical scavenger, an antidote to the extravasation of vesicant anticancer drugs, a topical analgetic, etc. DMSO is one of the most common solvents for the *in vivo* administration of water-insoluble substances [62]. Is it possible to solve the problem with toxicity and solubility of MonH and its metal(II) complexes? In relation to our study, at least two facts have to be mentioned here: i) in many cases the effective concentrations (CC_{50}, CC_{90}, CIC) of MonH and its complexes are found to be less than 5 μM, a value which corresponds to ≈ 3.5 μg/mL (in the case of MonH) and ≈ 7 μg/mL (in the case of metal(II) complexes). The simple calculations reveal that the amount of DMSO in these solution is only 0.35% and 0.70%, respectively; ii) water-insoluble compounds could be suitable for liposome drug delivery systems that are one of the most promising new technologies for targeted cancer therapy.

Another question that requires interest and has to be discussed here, is whether (and how) the presence of the biometal(II) ion does influence the cytotoxic and cytostatic properties of Monensic acid. As it was found in our previous

investigations, the isostructural metal(II) compounds possess generally enhanced activity against Gram-positive aerobic and anaerobic bacteria in comparison to Monensic acid [20], [23]-[25] and were also demonstrated to possess more pronounced antitumor activity against a wide variety of cultured animal and human cancer cell lines [26]-[28]. The results shown in the present study also indicate that Ca(II), Co(II) and Mn(II) complexes of MonH express relatively better cytotoxic and antiproliferative effects against human skin carcinoma drug sensitive (parental) and multidrug resistant cell lines as compared to MonH. Something more, among the compounds investigated, the complex of Mn(II) with MonH, $[Mn(Mon)_2(H_2O)_2]$ (**3**), exhibits the best cytotoxic and cytostatic properties especially on the growth of 3D colonies in semi-solid medium (Table 7).

Another compound that undoubtedly has to be mentioned here, is $[Co(Mon)_2(H_2O)_2]$ (**2**) - the relative resistance values of this complex were calculated to be less than 1 (Table 9), which suggests that the compound is probably more active in resistant as compared to sensitive skin carcinoma cells.

These data are not surprising because both compounds (**2** and **3**) were also shown to decrease significantly the viability and growth of cultured human glioblastoma (8MGBA), hepatoma (HepG2), breast cancer (MVF-7) and cervical carcinoma (HeLa) cells and in some cases their activities were found to be comparable (MCF-7 cells, 48 h) and even higher (8MGBA cells, 24-72 h) than those of some of the most widely used in clinical oncology antitumor drugs (cisplatin, 5-fluorouracil, daunorubicin) [26]-[28]. The existence of some variations in antineoplastic properties of the compounds tested that share very similar chemical structure could be explained by at least two reasons: i) the influence of the metal ions – Ca(II), Co(II) and Mn(II) are known to play different roles in biological systems, and ii) the cell specific response.

V. CONCLUSION

In summary, Monensic acid and its metal(II) complexes could be considered as promising cytotoxic and cytostatic agents effective in drug sensitive as well as in drug resistant human skin cancer tumor cells. Additional experiments are required to clarify in details the potential antineoplastic activity of these compounds and their mechanism(s) of action.

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REFERENCES

[1] A. Agtarap, J. W. Chamberlin, M. Pinkerton, and L. K. Steinrauf, "The structure of monensic acid, a new biologically active compound," *J. Am. Chem. Soc.*, vol. 89, no. 22, pp. 5737-5739, Oct. 1967.

[2] B. C. Pressman, "Biological application of ionophores," *Ann. Rev. Biochem.,* vol. 45, pp. 501-529, 1976.

[3] P. H. Stern, "Ionophores: chemistry, physiology and potential applications to bone biology," *Clin. Orthop. Rel. Res.*, vol. 122, pp. 273-298, Jan.-Feb. 1977.

[4] P. C. Augustine, C. K. Smith, H. D. Danforth, and M. D. Ruff, "Effect of ionophorous anticoccidials on invasion and development of Emeria: comparison of sensitive and resistant isolates and correlation with drug uptake," *Poultry Sci.*, vol. 66, no. 6, pp. 960-965, Jun 1987.

[5] S. D. Folz, B. L. Lee, L. H. Nowakowski, and G. A. Conder, "Anticoccidial evaluation of halofuginone, lasalocid, maduramicin, monensin and salinomycin," *Vet. Parasit.*, vol. 28, no. 1-2, pp. 1-9, 1988.

[6] D. A. Kevin II, D. A. F. Meujo, and M. T. Hamann, "Polyether ionophores: broad-spectrum and promising biologically active molecules for the control of drug resistant bacteria and parasites," *Exp. Opi. Drug Discov.*, vol. 4, no. 2, pp. 109-146, Feb. 2009.

[7] B. G. Cox, N. van Truong, J. Rzeszotarska, and H. Schneider, "Stability constants of complexes of monensin and lasalocid with alkali-metal and alkaline-earth-metal ions in protic and polar aprotic solvents," *J. Chem. Soc., Faraday Trans. I,* vol. 80, no. 12, pp. 3275-3284, 1984.

[8] M. Mimouni, S. Perrier, I. Pointud, and J. Juillard, "Selectivity of bacterial ionophore monensin for monovalent metal cations. Solvent effects in methanol and biphasic water-organic systems," *J. Sol. Chem.*, vol. 22, no. 9, pp. 769-785, 1993.

[9] M. Mimouni, M. Hebrant, G. Dauphin, and J. Juillard, "Monovalent cation salts of the bacterial ionophore monensin in methanol. Structural aspects from NMR experiments," *J. Chem. Res*., vol. S6, pp. 278-279, 1996.

[10] F. G. Riddell, "Structure, conformation and mechanism in the membrane transport of alkali metal ions by ionophoric antibiotics," *Chirality*, vol. 14, no. 2-3, pp. 121–125, Feb. 2002.

[11] W. H. Park, M. S. Lee, K. Park, E. S. Kim, B. K. Kim, and Y. Y. Lee, "Monensin-mediated growth inhibition in acute myelogenous leukemia cells via cell cycle arrest and apoptosis," *Intern. J. Cancer*, vol. 101, no. 3, pp. 235-242, Sept. 2002.

[12] W. H. Park, J. G. Seol, E. S. Kim, W. K. Kang, Y. H. Im, C. W. Jung, B. K. Kim, and Y. Y. Lee, "Monensin-mediated growth inhibition in human lymphoma cells through cell cycle arrest and apoptosis," *Brit. J. Haem.*, vol. 119, no. 2, pp. 400-407, Nov. 2002.

[13] W. H. Park, E. S. Kim, C. W. Jung, B. K. Kim, and Y. Y. Lee, "Monensin-mediated growth inhibition of SNU-C1 colon cancer cells via cell cycle arrest and apoptosis," *Intern. J. Oncology*, vol. 22, no. 2, pp. 377-382, Feb. 2003.

[14] W. H. Park, C. W. Jung, J. O. Park, K. Kim, W. S. Kim, Y. H. Im, M. H. Lee, W. K. Kang, and K. Park, "Monensin inhibits the growth of renal cell carcinoma cells via cell cycle arrest or apoptosis," *Intern. J. Oncology*, vol 22, no. 4, pp. 855-860, April 2003.

[15] W. H. Park, E. S. Kim, B. K. Kim, and Y. Y. Lee, "Monensin-mediated growth inhibition in NCI-H929 myeloma cells via cell arrest and apoptosis," *Intern. J. Oncology*, vol. 23, no. 1, pp. 197-204, July 2003.

[16] N. K. Chirase, L. W. Greene, G. T. Schelling, and F. M. Byers, "Effect of magnesium and potassium on microbial fermentation in a continuous culture fermentation system with different levels of monensin or lasalocid," *J. Anim. Sci*., vol. 65, no. 6, pp.1633–1638, Dec. 1987.

[17] K. A. Johnson, "An assessment of monensin's effect upon the ruminant small intestine: Na^+/K^+–ATPase activity in mucosal biopsies and net nutrient absorption," *Diss. Abstr., Intern. B Sci. Eng*., vol. 48, no. 7, p. 1855, 1988.

[18] S. A. Hamidinia, O. I. Shimelis, B. Tan, W. L. Erdahl, C. J. Chapman, G. D. Renkes, R. W. Taylor, and D. R. Pfeiffer, "Monensin mediates a rapid and selective transport of Pb^{2+} - possible application of monensin for the treatment of Pb^{2+} intoxication," *J. Biol. Chem.*, vol. 277, no. 41, pp. 38111-38120, Oct. 2002.

[19] P. Dorkov, I. N. Pantcheva, W. S. Sheldrick, H. Mayer-Figge, R. Petrova, and M. Mitewa, "Synthesis, structure and antimicrobial activity of manganese(II) and cobalt(II) complexes of the polyether ionophore

antibiotic sodium monensin A," *J. Inorg. Biochem*., vol. 102, no. 1, pp. 26-32, Jan. 2008.

[20] I. N. Pantcheva, M. Io. Mitewa, W. S. Sheldrick, I. M. Oppel, R. Zhorova, and P. Dorkov, "First divalent metal complexes of the polyether ionophore monensin A: X-ray structures of $[Co(Mon)_2(H_2O)_2]$ and $[Mn(Mon)_2(H_2O)_2]$ and their properties," *Curr. Drug Discov. Techn*., vol. 5, no. 2, pp. 154-161, June 2008.

[21] I. N. Pantcheva, P. Dorkov, V. N. Atanasov, M. Mitewa, B. L. Shivachev, R. Nikolova, H. Mayer-Figge, and W. S. Sheldrick, "Crystal structure and properties of the copper(II) complex of sodium monensin A," *J. Inorg. Biochem*., vol. 103, no. 10, pp. 1419-1424, Oct. 2009.

[22] J. Ivanova, I. N. Pantcheva, M. Mitewa, S. Simova, H. Mayer-Figge, and W. S. Sheldrick, "Crystal structures and spectral properties of new Cd(II) and Hg(II) complexes of monensic acid with different coordination modes of the ligand," *Cent. Eur. J. Chem*., vol. 8, no. 4, pp. 852-860, Aug. 2010.

[23] I. N. Pantcheva, R. Zhorova, M. Mitewa, S. Simova, H. Mayer-Figge, amd W. S. Sheldrick, "First solid state alkaline-earth complexes of monensic A acid: X-ray crystal structure of $[M(Mon)_2(H_2O)_2]$ (M = Mg, Ca), spectral properties and cytotoxicity against Gram-positive bacteria," *BioMetals*, vol. 23, no. 1, pp. 59-70, Feb. 2010.

[24] I. N. Pantcheva, J. Ivanova, R. Zhorova, M. Mitewa, S. Simova, H. Mayer-Figge, and W. S. Sheldrick, "Nickel(II) and zinc(II) dimonensinates: crystal structure, spectral properties and bactericidal activity," *Inorg. Chim. Acta*, vol. 363, no. 8, pp. 1879-1886, May 2010.

[25] R. Zhorova, M. Marina, I. N. Pantcheva, and M. Mitewa, "Effect of monensic acid and its metal(II) complexes on growth of the Gram-positive anaerobic *Clostridium perfringens*," unpublished.

[26] M. Mitewa, I. Pantcheva, and R. Alexandrova, "Antitumor activity of the polyether ionophorous antibiotic monensin and its metal(II) complexes," in *Recent Researches in Modern Medicine*, O. Braissant, H. Wakamatsu, I. Kuo-Kang, K. Allegaert, Y. Lenbury, A. Wachholtz, Eds.,WSEAS Press, 2011, pp. 439-444.

[27] R. Alexandrova, I. N. Pantcheva, and M. Mitewa, "Cytotoxicity of divalent metal complexes of monensin against human tumor / non-timor cell lines," paper in preparation, 2011.

[28] R. Alexandrova, I. N. Pantcheva, and M. Mitewa, "Activity of monensin complexes on virus-transformed animal tumor cell lines," paper in preparation, 2011.

[29] N.B. Elkind , Z. Szentpétery, A. Apáti, C. Ozvegy-Laczka, G. Várady, O. Ujhelly, K. Szabó, L. Homolya, A. Váradi, L. Buday, G. Kéri, K. Német, and B. Sarkadi, "Multidrug transporter ABCG2 prevents tumor cell death induced by the epidermal growth factor receptor inhibitor Iressa (ZD1839, Gefitinib)," *Cancer Res*., vol. 65, no. 5, pp. 1770-1777, March 2005.

[30] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *J. Immunol. Meth.*, vol. 65, no. 1-2, pp. 55-63, Dec. 1983.

[31] W. G. Bergen and D. G. Bates, "Ionophores: their effects on production, efficiency and mode of action," *J. Anim. Sci.*, vol. 58, no. 6, pp. 1465–1483, June 1984.

[32] L. Dowling, "Ionophore toxicity in chickens: A review of pathology and diagnosis," *Avian Pathol.,* vol. 21, no. 3, pp. 355-368, 1992.

[33] S. A. Sassman and L. S. Lee, "Sorption and degradation in soils of veterinary ionophore antibiotics: monensin and lasalocid," *Environ. Toxicol. Chem.,* vol. 26, no. 8, pp.1614-1621, Aug. 2007.

[34] W. H. Zhu and T. T. Loh, "Effects of Na^+/H^+ antiport and intracellular pH in the regulation of HL-60 cell apoptosis," *Biochim. Biophys. Acta – Mol. Cell Res.,* vol. 1269, no. 2, pp. 122-128, Nov. 1995.

[35] S. Grinstein, D. Rotin, and M. J. Mason, "Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation," *Biochim. Biophys. Acta,* vol. 988, no. 1, pp. 73-97, Jan. 1989.

[36] M. Bental, and C. Deutsch, "^{19}F-NMR study of primary human T lymphocyte activation: effects of mitogen on intracellular pH," *Am. J. Physiol.*, vol. 266, no. 2, pp. 541-551C, Feb. 1994.

[37] G. N. Rao, N. de Roux, C. Sardet, J. Pouyssegur, and B. C. Berk, "Na^+/H^+ antiporter gene expression during monocytic differentiation of HL60 cells," *J. Biol. Chem*., vol. 266, no. 21, pp. 13485-13488, July 1991.

[38] J. M. Cobo, R. Garcia-Cañero, J. G. Valdez, A. M. Barrasso, B. L. Sailer, and H. A. Crissman, "Attenuation of apoptic DNA fragmentation by amiloride," *J. Cell. Physiol.*, vol. 175, no. 1, pp. 59-67, Jan. 1998.

[39] B. C. Pressman and M. Fashin, "Pharmacology and toxicology of the monovalent carboxylic ionophores," *Ann. Rev. Pharm. Toxicol*., vol. 22, pp. 465–490, Apr. 1982.

[40] K. Nakazato and Y. Hatano, "Monensin-mediated antiport of Na^+ and H^+ across liposome membrane," *Biochim. Biophys. Acta*, vol. 1064, no. 1, pp. 103–110, April 1991.

[41] M. F. Mariani, L. Thomas, B. de Feo, and G. D. van Rossum, "Effects of monensin on ATP levels and cell functions in rat liver and lung in vitro," *J. Membr. Biol*., vol. 108, no. 3, pp. 235-246, June 1989.

[42] N. C. Popescu and D. B. Zimonjic, "Chromosome-mediated alterations of the MYC gene in human cancer," *J. Cell Mol. Med.*, vol. 6, no. 2, pp. 151-159, April-June 2002.

[43] G. S. Martin, "The road to Src," *Oncogene*, vol. 23, no. 48, pp. 7910-7917, Oct. 2004.

[44] S.V. Ambudkar, C. Kimchi-Sarfaty, Z. E. Sauna, and M. M. Gottesman, "P-glycoprotein: from genomics to mechanism", *Oncogene*, vol. 22, no. 47, pp. 7468-7485, Oct. 2003.

[45] B. Saekadi, C. Ozvegy-Laczka, K. Nemet, and A. Viradi, "ABCDG2 - a transporter for all seasons," *FEBS Lett*., vol. 567, no. 1, pp.116-120, June 2004.

[46] E. M. Leslie, R. G. Deeley, and S. P. Cole, "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defence," *Toxicol. Appl. Pharmacol*., vol. 204, no. 3, pp. 216-237, May 2005.

[47] J. Aldrich-Wright, "Platinum drugs, still essential in our fight against cancer," in *Advances in Biomedical Research*, P. Anninos, M. Rossi, T. D. Pham, C. Falugi, A. Bussing, M. Koukkou, Eds.,WSEAS Press, 2010, p. 59.

[48] J. Smieja and A. Swierniak, "Optimal multidrug chemotherapy taking into account drug resistance stemming from gene amplification," in *Proc. WSEAS Intern. Conf. MCBC, MCBE, ICAMSL, ICAI*, Tenerife, Spain, 2002, pp. 255-259.

[49] A. Iliadis and D. Barbolosi, "Optimizing Drug Regimens in Cancer Chemotherapy," in *Proc. WSEAS Intern. Conf. Math. Biol. Ecol*., Corfu, Greece, 2004, pp. 176-181.

[50] M. Naito, T. Hoshino, Y. Matsushita, R. Hirai, and T. Tsurio, "Two types of interaction between P-glycoprotein and ionophore antibiotics," *J. Clin. Pharmacol*., vol. 2, pp. 263-267, 1991.

[51] S. M. Simon and M. Schindler, "Cell biological mechanisms of multidrug resistance in tumors," *Proc. Natl. Acad. Sci. USA*, vol. 91, no. 9, pp. 3497-3504, April 1994.

[52] S. M. Simon, "Role of organelle pH in tumor cell biology and drug resistance," *Drug Discov. Today,* vol. 4, no. 1, pp. 32-38, Jan. 1999.

[53] A. K. Larsen, A. E. Escargueil, and A. Skladanowski, "Resistance mechanisms associated with altered intracellular distribution of anticancer agents," *Pharmacol. Ther.*, vol. 85, no. 3, pp. 217-229, March 2000.

[54] E. Miraglia, D. Viarisio, C. Riganti, C. Costamagna, D. Ghigo, and A. Bosia, "Na^+/H^+ exchanger activity is increased in doxorubicin-resistant human colon cancer cells and its modulation modifies the sensitivity of the cells to doxorubicin," *Intern. J. Cancer*, vol. 115, no. 6, pp. 924-929, July 2005.

[55] M. Sehested, T. Skovsgaard, and H. Roed, "The carboxylic ionophore monensin inhibits active drug efflux and modulates in vitro resistant Ehrlich ascites tumor cells," *Biochem. Pharmacol*., vol. 37, no. 17, pp. 3305-3310, Sept. 1988.

[56] W. D. Klohz and R. W. Steinkampf, "The effect of lysosomotropic agents and secretory inhibitors on anthracycline retention and activity in multiple drug-resistant cells," *Mol. Pharmacol*., vol. 34, no. 2, pp. 180-185, Aug. 1988.

[57] M. S. Shaik, A. Chatterjee, and M. Singh, "Effects of monensin liposomes on the cytotoxicity, apoptosis and expression of multidrug resistance genes in doxorubicin-resistant human breast tumour (MCF-7/dox) cell-line," *J. Pharm. Pharmacol.*, vol. 56, no. 7, pp. 899-907, July 2004.

[58] M. Singh, A. J. Ferdous, and T. L. Jackson, "Stealth monensin liposomes as a potentiator of adriamycin in cancer treatment," *J. Control Release*, vol. 59, no. 1, pp. 43-53, May 1999.

[59] A. C. Souza, F. S. Machado, M. R. Celes, G. Faria, L. B. Rocha, J. S. Silva, and M. A. Rossi, "Mitochondrial damage as an early event of monensin-induced cell injury in cultured fibroblasts L929," *J. Vet. Med. A - Physiol. Pathol. Clin. Med*., vol. 52, no. 5, pp. 230-237, June 2005.

[60] H. H. Mollenhauer, D. J. Morré, and L. D. Rowe, "Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity," *Biochim. Biophys. Acta*, vol. 1031, no. 2, pp. 225–246, 1990.

[61] M. Dacasto, L. Leppa, E. Cornaglia, F. Valenza, M. Carletti, A. Bosio, S. Bosia, G. Ugazio, and C. Nebbia, "Effects of ionophore antibiotic monensin on hepatic biotransformation and target organ morphology in rats," *Pharmacol. Res*., vol. 39, no. 1, pp. 5–10, Jan. 1999.

[62] N. C. Santos, J. Figueira-Coelho, J. Martins-Silva, and C. Saldanha, "Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular and molecular aspects," *Biochem. Pharm*., vol. 65, no. 7, pp. 1035-1041, April 2003.