Benzoxazole-based Zn(II) and Cu(II) Complexes Overcome Multidrug-resistance in Cancer

GABRIELLA SPENGLER¹, ANNAMÁRIA KINCSES¹, BÁLINT RÁCZ¹, BORISZ VARGA^{1,2}, GENKI WATANABE³, RYOSUKE SAIJO³, HIROSHI SEKIYA⁴, EIJI TAMAI⁴, JUN MAKI⁴, JOSEPH MOLNÁR¹ and MASAMI KAWASE³

¹Department of Medical Microbiology and Immunobiology,
Faculty of Medicine, University of Szeged, Szeged, Hungary;

²Department of Rheumatology and Immunology, University of Szeged, Szeged, Hungary;

³Faculty of Pharmaceutical Sciences, Matsuyama University, Matsuyama, Japan;

⁴Department of Infectious Diseases, College of Pharmaceutical Sciences,
Matsuyama University, Matsuyama, Japan

Abstract. *Background/Aim:* Multidrug resistance (MDR) represents a significant impediment to successful cancer treatment. In this study, novel metal [Zn(II), Cu(II), Mg(II), Ni(II), Pd(II), and Ag(I)] complexes of 2-trifluoroacetylbenzoxazole previously synthesized and characterized by our group were tested for their MDR-reversing activity in comparison with the free ligands in L5178Y mouse T-lymphoma (MDR) cells transfected with human ATP-binding cassette sub-family B member 1 (ABCB1; P-glycoprotein) gene. *Materials and Methods:* Cytotoxic and antiproliferative effects of the complexes were assessed by the thiazolyl blue tetrazolium bromide (MTT) method. Modulation of ABCB1 activity was measured by rhodamine 123 accumulation assay using flow cytometry. The apoptosis-inducing activity of some complexes was also tested on the multidrug resistant L5178Y mouse T-lymphoma cells, using the annexin-V/propidium iodide assay. *Results:* When compared to the free ligand, a remarkable enhancement in MDR reversal and cytotoxic activity was found for the Zn(II) and Cu(II) complexes. The activity of the complexes proved to be up to 29- and 5-fold higher than that of the ligands and the ABCB1 inhibitor verapamil as positive control, respectively. The complexes possessed a remarkable potential to induce apoptosis of MDR cells. *Conclusion:* Our results suggest that the Zn(II) and Cu(II) complexes display significant MDR-reversing activity in a dose-dependent manner and possess

strong cytotoxic activity and a remarkable potential to induce apoptosis in MDR L5178Y mouse T-lymphoma cells.

Multidrug resistance (MDR) of cancer is defined as the insensitivity of cancer cells to the cytotoxic action of various anticancer drugs (1-4). One of the major mechanisms of MDR is the overexpression of efflux pumps, which reduces the accumulation of administered anticancer drugs. Multidrug efflux pumps fall into one of six distinct families of membrane proteins, with the most important MDR transporters of cancer cells belonging to the ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp; ABCB1), multidrug resistance-associated protein-1 (ABCC1) and breast cancer resistance protein (ABCG2) (5).

One promising approach to overcome MDR mediated by P-gp is based on the development of MDR-reversal agents that possess efflux pump inhibitor properties (6-8). A number of MDR-reversal agents of synthetic or natural origin have been reported and some of them have reached the stage of clinical trials. However, none of them has been approved for clinical use because of undesirable side-effects (9, 10). Therefore, it is important to continue research in this area in order to discover new inhibitors of primary efflux pumps.

The discovery and development of new metal-based chemotherapeutics is an ever-growing area of research in medicinal chemistry. Coordination of organic compounds with metal causes drastic changes in the biological properties of both the ligand and the metal moiety. Therefore, many metal complexes had been designed and synthesized in order to explore their pharmacological and antitumor activity (11-14). The platinum-based drug cisplatin is one of the commonly used drugs in the clinic in the treatment of various types of human cancer, but its serious side-effects and resistance by cancer cells confine its clinical application (15). In order to curb increasing resistance, it is a necessity

Correspondence to: Masami Kawase, Faculty of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan. Tel: +81 899267098, Fax: +81 899267162, e-mail: kawase@g.matsuyama-u.ac.jp

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to design potential alternatives. Recently, several ruthenium-based complexes, such as NAMI-A, KP1019, KP1339, and TD1433, have been developed, of which the latter two are currently undergoing clinical trials (16, 17). In this endeavor, transition metals such as Cu, Zn, Ni, Ag, and Au are considered to be promising candidates for designing such therapeutic agents because of their biocompatibility and their endogenous presence in living system as co-factors in several enzymes (18-21).

The benzoxazole heterocycle system is an important pharmacophore and privileged structure in medicinal chemistry (22, 23). Its derivatives, and particularly, 2-substituted benzoxazoles, exert various biological activities such as anticancer, antimicrobial, antiviral, and antifungal (24-28).

In previous studies, we synthesized new Zn(II), Mg(II), Ni(II), Cu(II), Pd(II), and Ag(I) complexes of 2-trifluoroacetylbenzoxazole ligand, of which the later displayed significant antibacterial activities (29). In the present study, 2-trifluoroacetylbenzoxazoles were used as active ligands to coordinate with transition metals because these compounds are highly desirable ligands for their ability to coordinate with metal center, good planarity, highly hydrophobic DNA interactions and mimicry of the purine nucleus (30, 31).

In order to identify specific and selective MDR reversal agents, we have evaluated the cytotoxic and MDR reversing potential of three ligands (compounds 1-3, Figure 1) and their eight metal complexes (compounds 4-11; Figure 1) in parental L5178Y mouse T-lymphoma cell line and its human *ABCB1* gene-transfected (MDR) subline. In addition, the capacity of potent MDR modulators as apoptosis inducers was also evaluated.

In this article, we report the MDR-modulating activities of metal complexes and the identification of selective MDR modulators of the *ABCB1* pump in cancer cells.

Materials and Methods

Compounds. The syntheses of the ligands and metal complexes were described previously (29) and their structures are shown in Figure 1. The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain).

Cell lines. The L5178Y mouse T-cell lymphoma cells (FDA, Silver Spring, MD, USA) were transfected with pHaMDR1/A retrovirus (7). The *ABCB1*-expressing MDR cell line was selected by culturing the infected cells with colchicine. Parental L5178Y mouse T-cell lymphoma cells and the human *ABCB1*-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, 200 mM L-glutamine, and penicillin-streptomycin mixture at 100 U/l and 10 mg/l, respectively.

Assay for cytotoxic effect. The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in 100 μ l

of McCoy's 5A medium then 1×10^4 cells in 100 μ l of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C for 24 h. At the end of the incubation period, 20 μ l of thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) solution (from a 5 mg/ml stock) were added to each well. After incubation at 37°C for 4 h, 100 μ l of sodium dodecyl sulfate (Sigma-Aldrich) solution (10% in 0.01 M HCl) were added to each well and the plates were further incubated at 37°C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA) (7). Inhibition of cell growth was determined according to the following formula:

$$100 - \left[\frac{OD_{\text{sample}} - OD_{\text{medium control}}}{OD_{\text{cell control}} - OD_{\text{medium control}}} \right] \times 100 \quad \text{Eq 1}$$

Where IC_{50} was defined as the inhibitory dose that reduce the growth of the cells exposed to the compound by 50%.

Fluorescence uptake assay. The density of the L5178Y parental and MDR cell lines were adjusted to 2×10^6 cells/ml, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf tubes. The test compounds were added at a final concentration of 2 and 20 μ M and the samples were incubated for 10 min at room temperature. Verapamil (Sigma-Aldrich) was applied as positive control at 20 μ M. DMSO was added to the negative control tubes in the same volume as had been used for the tested compounds. No activity of DMSO was observed. Next, 10 μ l (5.2 μ M final concentration) of rhodamine 123 (Sigma-Aldrich) were added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and re-suspended in 1 ml phosphate buffered saline for analysis. The fluorescence of the cell population was measured with a PartecCyFlow® flow cytometer (Partec, Münster, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with untreated cells (7). A fluorescence activity ratio (FAR) was calculated on the basis of the measured fluorescence values *via* the following equation:

$$FAR = \frac{MDR_{\text{treated}} / MDR_{\text{control}}}{parental_{\text{treated}} / parental_{\text{control}}} \quad \text{Eq 2}$$

Apoptosis assay. The assay was carried out using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Calbiochem, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The density of the mouse MDR T-lymphoma cell suspension was adjusted to approximately 1×10^6 cells/ml. The cell suspension was distributed into 0.5 ml aliquots (5×10^5 cells) to a 24-well microplate and the compounds were added at a final concentration of 10 μ M. The apoptosis inducer 12H-benzo[a]phenothiazine (M627) was applied as positive control at a final concentration of 20 μ M (32). The cells were incubated in the presence of the compounds for 1 h in a water-bath (37°C). The samples were then centrifuged and the culture medium was removed, the cells were washed with phosphate-buffered saline and fresh medium was added to the cells. The 24-well plates were incubated overnight at 37°C with 5% CO_2 . On the following day, the samples were then centrifuged at $2000 \times g$ for 2 min at room temperature and

Figure 1. The structures and molecular weights of the ligands (compounds **1-3**) and metal complexes (compounds **4-11**).

the supernatant was removed and the cells were re-suspended in 0.5 ml fresh serum-free medium. After this procedure, the apoptosis assay was carried out according to the rapid protocol of the kit by which cells were stained with FITC-labelled annexin V and propidium iodide (PI) and then analyzed by flow cytometry. This discriminated between necrotic (annexin V⁻/PI⁺), viable (annexin V⁻/PI⁻), early apoptotic (annexin V⁺/PI⁻), and late apoptotic (annexin V⁺/PI⁺) cells. The fluorescence of cell populations was analyzed immediately using a PartecCyFlow® flow cytometer.

Results

We screened our in-house library of metal complexes and related compounds, most of them previously synthesized in our laboratory (29). The metal complexes **4-11** were easily prepared in moderate to good yields by the reaction of 2-trifluoroacetylbenzoxazole ligands (compounds **1-3**) with metal salts (29). The structures of compounds **1-11** screened for their MDR-modulating activities are shown in Figure 1

The cytotoxic activity of ligands and their metal complexes was evaluated using MTT assay in two tumor cell lines, parental L5178Y mouse T-lymphoma cells and its human *ABCB1*-gene transfected MDR subline. These MDR

cells overexpress the ABCB1 protein that is responsible for drug efflux. The IC₅₀ values are presented in Table I, in which the chemotherapeutic drug cisplatin was used as positive control. The results indicated that the cytotoxicity of Zn(II) complexes **4-6** was greater than those of the ligands **1-3**. This observation suggested the advantages of Zn(II) complexes over their parent ligands in practical applications as MDR-modulating agents. The selectivity index (SI) for each compound was calculated based upon the comparison of the sensitivity (IC₅₀) of parental (chemosensitive) to MDR (chemoresistant due to overexpression of ABCB1) cell lines. When the SI value is greater than 1, resistance in the MDR cells is reversed by a compound. Thus, selective cytotoxic activity was observed in the case of compounds **4** (SI=6.9), **5** (SI=2.7), **3** (SI=2.6), **2** (SI=2.3) and **9** (SI=2.1) in the MDR cells relative to the parental cells, suggesting that the compounds were able to modulate or block the ABCB1 transporter. On the other hand, compounds **1**, **6**, **7**, **8**, and **11** were equally active against both parental and MDR because the SI did not show any significant discrimination between parental and MDR cell lines. The Pd(II) complex **10** was not toxic against the MDR cells (SI<0.20). All compounds

Table I. Cytotoxic activity of ligands **1-3** and metal complexes **4-11** on parental and multidrug-resistant (MDR) cancer cells.

Compound	IC ₅₀ (μM) ^a		Selectivity index ^b
	Parental cells	MDR cells	
1	6.05±1.47	6.76±0.75	0.89
2	7.68±0.56	3.41±0.54	2.25
3	8.87±0.44	3.37±0.41	2.63
4	3.44±0.21	0.50±0.39	6.88
5	6.43±0.25	2.35±0.58	2.74
6	2.22±0.38	2.05±0.06	1.08
7	1.25±0.11	1.35±0.09	0.93
8	2.88±0.46	3.31±0.31	0.87
9	23.38±1.78	10.99±0.49	2.13
10	19.71±3.98	>100	<0.20
11	1.21±0.09	1.58±0.14	0.77
Cisplatin	8.67±0.23	11.20±0.95	0.77

IC₅₀: 50% Inhibitory concentration. ^aData are the average±SD of three independent experiments. ^bSelectivity index=IC₅₀ (parental cells)/IC₅₀ (MDR cells).

except Mg(II) complex **9** and Pd(II) complex **10** were more cytotoxic against both parental and MDR cells than cisplatin used as a positive control. In particular, the cytotoxicity of the Cu(II) complex **7** and Ag(I) complex **11** against the parental and the MDR cells was up to 7-to 9-fold higher than that of cisplatin.

The rhodamine 123 (a substrate of ABCB1) exclusion assay was used to assess the potential ABCB1-mediated MDR-reversing activity of compounds **1-11**. In this assay, the FAR was evaluated for the accumulation ratio of rhodamine 123 in MDR and parental cells (Table II). The reversal of MDR means that the accumulation of rhodamine 123 by the MDR cells should be similar to the accumulation by the chemosensitive parental cells, and the FAR should therefore be greater than 1. Verapamil, a well-known modulator (33), was used as positive control. The data in Table II reveal that complexes of Zn(II) (compounds **4**, **5** and **6**), Cu(II) (compound **7**) and Ag(I) (compound **11**) were potent MDR-reversing agents, while the ligands (compounds **1**, **2** and **3**) and complexes of Ni(II), Mg(II) and Pd(II) (compounds **8**, **9** and **10**) were inactive. It is noted that compounds **4** (FAR=28.0), **6** (FAR=16.9) and **7** (FAR=19.3) displayed a remarkable inhibition of MDR at a concentration of 20 μM. To study whether MDR reversal activity had a dose–effect dependence, four potent compounds **4-7** were tested at several concentrations (2, 10, 15 and 20 μM) and their FARs are presented in Table III. At 2 μM, these compounds were weak modulators. However, at 10-20 μM, the FAR values of these compounds were higher than 10 (11.0-29.4), leading us to classify them as strong MDR modulators (34). These results are of particular interest, since

 Table II. Multidrug resistance (MDR)-reversal activities of ligands **1-3** and metal complexes **4-11** on MDR cancer cells. The inhibition of ABCB1 transporter is evident when the fluorescence activity ratio (FAR) exceeds 1.

Compound	Conc. μM	FSC	SSC	FL-1	FAR
Verapamil	20	1937	1005	4.91	5.64
1	20	2022	1027	0.90	0.96
2	20	2020	1017	1.04	1.11
3	20	2005	1055	0.89	0.95
4	20	1617	1286	26.1	28.0
5	20	1582	1320	4.49	4.81
6	20	1585	1392	15.8	16.9
7	20	1717	1228	18.0	19.3
8	20	1937	1080	0.87	0.93
9	20	1794	1032	0.67	0.79
10	20	1811	1081	0.97	0.82
11	20	1330	1573	1.91	2.25
DMSO	2.0%	1835	1024	0.73	0.83

FSC: Forward scatter count of cells in the sample (cell size ratio); SSC: side scatter count of cells in the samples; FL-1: mean fluorescence intensity of the cells.

the MDR-reversing activity of verapamil at 20 μM is approximately 5. In addition, for compounds **5**, **6** and **7**, the effect was somewhat lower at higher concentration, meaning that the applied concentrations exceeded an optimum substrate concentration. The maximum effect was found at the lowest dose applied.

Next, the effect of some metal complexes (compounds **4-7** and **9-11**) on apoptosis induction in the MDR cells was studied. An apoptosis inducer, M627, was used as the positive control (32). The compounds were tested at 10 μM and the results are summarized in Table IV. Complexes **5**, **6** and **7** had the capacity to induce early apoptosis. Several complexes induced early apoptosis with potency decreasing in the order **11**>M627>**6**>**7**>**5**. Late apoptosis was induced by compounds **4**, **5**, **6**, **7**, and **11**: the most potent compounds were **5** and **6** as they induced late apoptosis in 69.6% and 59.1% of the cell population, respectively. Complexes **4**, **5**, **6**, **7**, and **11** led to cell death of 5.58%, 4.12%, 4.53%, 2.81%, and 3.81%, respectively. The Ag(I) complex **11** was able to induce significant apoptosis, similar to the potent MDR modifier M627. Apoptosis induction by Mg(II) complex **9** and Pd(II) complex **10** was less pronounced compared to the effective apoptosis inducers **4**, **5**, **6**, and **7**. These data show that the potent MDR modulators possessed a remarkable potential to induce apoptosis in MDR cells.

Discussion

The problem in cancer treatment is the frequent occurrence of MDR. A recent approach in therapy is to develop tailored treatment protocols targeting specific pathways of cellular

Table III. Fluorescence activity ratio (FAR) values for metal complexes **4-7** on multidrug-resistant (MDR) cancer cells at different concentrations. The inhibition of ABCB1 transporter is evident when FAR exceeds 1.

Compound	Concentration (μ M)			
	2	10	15	20
Verapamil	-	-	-	5.11
4	1.35	22.23	14.60	29.37
5	1.57	23.49	14.98	5.67
6	1.48	11.00	24.08	17.33
7	0.99	16.30	16.59	12.89
DMSO*	0.73	-	-	-

*The concentration of DMSO was 2.0%.

proliferation or MDR. Therefore, an urgent task is to find more effective, modulators of low toxicity to inhibit MDR. A number of metal complexes, for examples, ruthenium complexes (35) and Cu(II), Zn(II), and Mn(II) *N*-(2-hydroxyacetophenone)glycinates (11) have also been shown to be strong MDR-reversing agents. In spite of advances in development of a number of good candidates for modulating MDR, we are still far away from being able to conclude that these agents would be applied clinically.

Previously, we reported that the ligand **1** had showed no obvious cytotoxicity [50% cytotoxicity concentration (CC_{50})=630 μ M] against human gingival fibroblasts and was more cytotoxic to two human oral tumor cell lines (HSC-2: CC_{50} =43 μ M vs. 4.1 μ M for doxorubicin; and HSG: CC_{50} =79 μ M) (36). In the present investigation, we developed new metal coordination compounds of the ligand **1** with the aim of obtaining suitable MDR-reversing agents. As a result of screening to obtain possible lead structures bearing metal, the strongest ability as MDR reversers and a many-fold activity when compared to verapamil was found for Zn(II) and Cu(II) complexes. Thus, the three parent ligands **1-3** showed no obvious MDR-modulating activity, while complexes **4-6** displayed a remarkable inhibition of MDR, indicating that ligand coordination plays an important role in the active complexes. Such increased activity of the metal complexes can be explained on the basis of the Overtone concept and Tweedy chelation theory (37-41). It is important to note that ABC transporters are primary efflux pumps deriving their energy from the hydrolysis of ATP. MDR modulators are believed to bind to the transmembrane domains of ABCB1 which leads to inhibition of ABC transporters (42-44). A number of studies revealed that lipophilicity is an important factor which controls MDR-modulating activity (6, 45). On chelation, the polarity of the metal ion is

Table IV. Effect of compounds **4-7** and **9-11** (at 10 μ M) on apoptosis induction in multidrug-resistant (MDR) cancer cells^a.

Compound	Early apoptosis (%)	Late apoptosis (%)	Cell death (%)
A ⁻ PI ⁻	0.33	0.12	1.89
A ⁻ PI ⁺	0.10	0.16	3.21
A ⁺ PI ⁻	9.53	0.018	0.02
A ⁺ PI ⁺	6.70	2.53	0.53
M627	26.1	9.05	0.79
4	8.56	18.7	5.58
5	19.8	69.6	4.12
6	23.0	59.1	4.53
7	22.0	11.2	2.81
9	8.69	1.95	0.59
10	10.1	3.01	0.76
11	30.3	20.4	3.81
DMSO*	8.67	2.28	0.65

^aControl: A⁺: annexin V-FITC staining; A⁻: without annexin V-FITC; PI⁺: propidium iodide staining; PI⁻: without propidium iodide; M627: 12*H*-benzo[a]phenothiazine (20 μ M) as positive control. *Concentration 1.0%.

reduced to a greater extent due to the overlapping of ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Moreover, delocalization of the π electrons over the whole chelate ring is increased and lipophilicity enhances penetration of the complexes into the transmembrane domains of ABCB1. Therefore, a significant increase in MDR-modulating activity with Zn(II) and Cu(II) complexes can be explained on the basis of the above described Overtone concept and Tweedy chelation theory.

Conclusion

This study has identified a novel class of ABCB1 inhibitors, Zn(II) and Cu(II) complexes. These compounds demonstrate high potency against ABCB1 which far exceed that of the reference drug verapamil. We can conclude that the Zn(II) and Cu(II) complexes can not only be considered as effective anti-MDR agents, but also as apoptosis inducers and may be attractive lead compounds for further development as MDR-reversing agents. Hence, these potent metal complexes should be pursued in a variety of directions. Firstly, molecular mechanisms by which metal complexes bind to ABCB1 should be investigated, which will give insight for further designing of potent ABCB1 inhibitors. Among various metal ions, copper and zinc play a prominent role in the active sites of many metalloproteins. Studies have indicated that many zinc and copper complexes exhibit favorable antiproliferative activity against tumor cells, which have been attributed to their interaction with DNA (although

their ability to bind to DNA largely depends on the coordinated ligand) (19-21). Secondly, evaluation of the activity of metal complexes against other efflux transporters such as MRP and BCRP should be carried out. Thirdly, the substituents of the benzoxazole ring in the ligand should be evaluated for MDR-modulating properties and selective toxicity for neoplasms.

The ease of synthesis and the small size of these compounds make them potential candidates for structure–activity relationships as efflux pump inhibitors in multidrug efflux systems.

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