Verapamil and Its Derivative Trigger Apoptosis through Glutathione Extrusion by Multidrug Resistance Protein MRP1

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ABSTRACT

This study demonstrates that verapamil and a newly synthesized verapamil derivative, NMeOHI2, behave as apoptosis-inducing proteins in multidrug resistance protein 1 (MRP1)-expressing cells. When treated with either verapamil or NMeOHI2, surprisingly, baby hamster kidney-21 (BHK) cells transfected with human MRP1 were killed. Because parental BHK cells were not, as well as cells expressing an inactive (K1333L) MRP1 mutant, this indicated that cell death involved functional MRP1 transporter. Cell death was identified as apoptosis by using annexin V-fluorescein labeling and was no longer observed in the presence of the caspase inhibitor Z-VAD-FMK. In vitro, both verapamil and its derivative inhibited leukotriene C4 transport by MRP1-enriched membrane vesicles in a competitive manner, with a K i of 48.6 μM for verapamil and 5.5 μM for NMeOHI2 and stimulated reduced glutathione (GSH) transport 3-fold and 9-fold, respectively. Treatment of MRP1-expressing cells with either verapamil or the derivative quickly depleted intracellular GSH content with a strong decrease occurring in the first hour of treatment, which preceded cell death beginning at 8–16 h. Furthermore, addition of GSH to the media efficiently prevented cell death. Therefore, verapamil and its derivative trigger apoptosis through stimulation of GSH extrusion mediated by MRP1. This new information on the mechanism of induced apoptosis of MDR cells may represent a novel approach in the selective treatment of MRP1-positive tumors.

INTRODUCTION

Failure to achieve complete and durable responses from cancer chemotherapy is a common clinical problem that limits the curative potential of anticancer drugs in clinical oncology. Multidrug resistance (MDR) is believed to be a major cause of treatment failure. The “classical” MDR phenotype, characterized by a typical cross-resistance pattern against structurally and functionally unrelated anticancer agents, results from the overexpression of one or several membrane proteins belonging to the ATP-binding cassette transporter superfamily, which can actively extrude anticancer drugs from the cell at the expense of ATP hydrolysis. Two of the best characterized examples of these transporters are the P-glycoprotein (P-gp) and the multidrug resistance protein 1 (MRP1), encoded by the human ABCB1 and ABCG2 genes, respectively (1, 2). A number of clinical studies indicate that the presence of these drug-resistance proteins can be of prognostic significance (3).

Although MRP1 and P-gp share the ability to confer resistance to a broad spectrum of anticancer agents, only MRP1 transports organic anions, many of which are conjugated to reduced glutathione (GSH), sulfate, or glucuronate (4). Drugs such as vincristine and daunorubicin are cotransported with GSH (5, 6), and physiological substrates of MRP1 include GSH (4) and leukotriene C4 (LTC4; Ref. 7).

Many efforts have been undertaken to identify agents able to reverse the MDR mediated by MRP1 and P-gp, taking into account their clinical relevance. Although many potential modulators have been reported for P-gp, very few have been described for MRP1 (3); these include flavonoids that were demonstrated to modulate transport and ATPase activities of MRP1 (8, 9). The well-known calcium channel blocker, verapamil, is a reference for P-gp inhibition (10–13). However, verapamil has been reported in most instances to be only weakly, or not at all, effective to restore drug sensitivity in MRP1-overexpressing cells (14–16). The basis for this apparently variable effect of verapamil on MRP1-associated resistance is unknown. However, if verapamil alone poorly inhibited LTC4 transport by MRP1, its inhibition in the presence of GSH was enhanced >20-fold (5, 16–18). One of the major obstacles that has emerged in the testing of various chemosensitizers in clinical trials has been their intrinsic toxicity. This was the case for verapamil, of which the optimal testing of efficiency to reverse MDR was hampered by the dose-limiting cardiovascular toxicity associated with its administration (19). One potential solution to this problem is the development of more effective and less toxic derivatives.

In this study, we wished to clarify the effects of verapamil on MRP1-stably transfected cells, because the mechanism of inhibition was not fully understood. We found that verapamil and the newly synthesized NMeOHI2 derivative (Fig. 1) acted as apoptogens on MRP1-stably transfected baby hamster kidney-21 (BHK-21) cells through GSH extrusion by MRP1. This finding may provide a novel approach in the selective treatment of MRP1-positive cancers.

MATERIALS AND METHODS

Materials. [Glycine-2-3H]-GSH (52 Ci/nmol) and [14,15,19,20-3H]-LTC4 (158 Ci/nmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Reduced glutathione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LTC4, nucleotides, creatine phosphate, creatine phosphokinase, MgCl2, dithiotreitol, acivicin, (±) verapamil, penicillin-streptomycin, and fetal bovine serum were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Triton X-100 was from Merck (Darmstadt, Germany), caspase inhibitor I (Z-VAD-FMK) from Calbiochem (Darmstadt, Germany), and methotrexate from Rhône-Poulenc (Moutrouge, France). The NMeOHI2 derivative of verapamil was synthesized at the Laboratoire de Chimie Biomimétique (Grenoble, France) by Barattin et al.4

Cell Lines. BHK-21 cells stably transfected with either wild-type or (K1333L) mutant MRP1 have been described previously (20, 21). A similar expression level of MRP1 and mutant MRP1 were found, whereas no MRP1 expression was detected on control BHK-21 cells by Western blot analysis (20, 21). Cells were grown at 37°C in 5% CO2 in culture medium containing 1% FBS.

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penicillin-streptomycin and 5% fetal bovine serum, in the presence of 200 μM methotrexate for transfected cells.

Membrane Vesicle Transport Studies. Inside-out plasma membrane vesicles were prepared from the MRPI-transfected or the untransfected BHK-21 cell lines using a nitrogen cavitation procedure as described previously (20).

ATP-dependent uptake of [3H]LTC4 was measured by incubating MRPI-enriched membrane vesicles (3 μg protein/30 μl reaction volume) for 6 min at 37°C in 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM MgCl2, 200 mM [3H]LTC4 (17.54 nCi), 4 mM ATP, with (2 mM) or without GSH, and increasing concentrations of verapamil or its derivative NMeOHI2 (0–100 μM) dissolved in DMSO (2.5% final concentration). The reaction was stopped by high dilution and rapid filtration through a nitrocellulose membrane, which was then counted by liquid scintillation, as described (20). GSH alone induced a 30% inhibition, which was taken into account. Within each experiment, determinations were carried out in triplicate. To characterize the type of the inhibition by either verapamil or NMeOHI2, LTC4 was included at increasing concentrations ranging from 15 to 160 μM, and GSH was added at a 5 μM final concentration.

[3H]GSH and NMeOHI2 transport assays were carried out using the rapid filtration method as described (22). Uptake was measured for 30 min at 37°C on MRPI-enriched vesicles (25 μg protein in 50-μl reaction volume), 4 mM ATP, 10 mM MgCl2, 10 mM creatine phosphate, 100 μg/ml creatine phosphokinase, 10 mM dithiothreitol, and 200 μM [3H]GSH (100 μM; 120 nCi). Verapamil and NMeOHI2 were dissolved in DMSO and added to the indicated concentrations. The final DMSO concentration never exceeded 2.5%. To minimize GSH catabolism during transport, vesicles were preincubated with 500 μM acivicin for 30 min at room temperature. Uptake was stopped by addition of excess ice-cold Tris sucrose buffer. Data were corrected for the control amount of [3H]GSH recovered in the presence of 4 mM AMP.

Cytotoxicity Assay and Cell Survival Determined by MTT Assay. Plasma membrane integrity was monitored by measuring the activity of cytosolic lactate dehydrogenase (LDH) in the culture supernatant, using the Cytotoxicity Detection kit LDH from Roche Molecular Biochemicals (Meylan, France). Cells were incubated at 37°C for 17 h before addition of verapamil or NMeOHI2. LDH activity was assessed in the absence (○) or presence (△) of 2 mM reduced glutathione (GSH). Data points represent means of triplicate determinations; bars, ±SD.

RESULTS

Effects of Verapamil and Its Derivative NMeOHI2 on LTC4 Transport by MRPI-Enriched Membrane Vesicles. Verapamil and the newly synthesized NMeOHI2 derivative, of which the structures are shown in Fig. 1, were tested for their ability to inhibit ATP-dependent [3H]LTC4 uptake. Iodine atoms were introduced with the aim to potentially increase the affinity for MRPI as it was described for iodinated derivatives of chalcones in the case of P-gp (24). The modification concerned the B phenyl ring of verapamil (Fig. 1A) and the newly synthesized NMeOHI2 derivative, of which the structures are shown in Fig. 1. The MTT colorimetric assay, as described previously (23), was used to assess the sensitivity of control cells and MRPI-transfected BHK-21 cells to verapamil or NMeOHI2 in the absence or presence of GSH (from 5 to 25 μM). Exogenous GSH addition had no significant effect on survival of BHK-21 control cells; for clarity, these data have been omitted in Fig. 10.

Analysis of Propidium Iodide Incorporation and Phosphatidylserine Exposure by Flow Cytometry. Control cells or MRPI-transfected BHK-21 cells (1.5 × 106) were seeded into 6-mm plates and cultured for 17 h before the addition of verapamil or NMeOHI2. The DMSO final concentration was 0.02%. At various incubation times (0, 3, 8, 16, and 24 h), attached and floating cells were harvested and resuspended with cold PBS for immediate treatment and analysis of propidium iodide incorporation and Annexin-V-fluorescein labeling, according to the manufacturer’s instructions (Annexin-V-FLUOS Staining kit; Roche Molecular Biochemicals, Meylan, France). Triplicate samples for each experimental condition were analyzed using FL2 and FL1 channels respectively of a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

Total Cellular Glutathione Content. The total cellular glutathione content (GSH + oxidized glutathione) was measured using the colorimetric method of Bioxytech GSH/oxidized glutathione–412 assay (OxisResearch, Portland, OR). Cells (1.8 × 106) were seeded into 100-mm plates and cultured for 17 h before addition of verapamil (10 μM) or NMeOHI2 (1 μM). DMSO concentrations did not exceed 0.02%. At various incubation times (up to 9 h), attached and floating cells were harvested, washed by 1 ml of cold PBS, harvested again, and resuspended in 200 μl of cold PBS. For experiments in the presence of exogenous GSH, cells were extensively washed by cold PBS. One hundred fifty μl of the suspension were treated according to the manufacturer’s instructions, and absorbance at 412 nm was monitored for 3 min. The content of total glutathione was quantified by comparison with known glutathione standards. Protein titration was performed on the remaining 50 μl by the Lowry method assay (Pierce, Brehières, France) after protein precipitation with trichloroacetic acid. The measured total glutathione concentrations were expressed per mg protein.

Fig. 1. Structure of verapamil (A) and its derivative, NMeOHI2 (B).
MRP1-enriched membrane vesicles (IC$_{50}$ ~50 μM). The inhibitory potency was strongly enhanced by coincubation with 2 mM GSH (IC$_{50}$ ~8 μM). In contrast, the inhibition of [3H]LTC$_4$ transport by verapamil could only be observed in the presence of GSH with an IC$_{50}$ >100 μM (data not shown). When the concentrations of both [3H]LTC$_4$ and inhibitor were varied, Lineweaver-Burk analysis of the data indicated that verapamil (Fig. 3A) and NMeOHI$_2$ (Fig. 3B), in the presence of GSH, behaved as competitive inhibitors of LTC$_4$ transport, with respective apparent $K_i$ values of 48.6 ± 4.9 and 5.45 ± 1.4 μM. The newly synthesized derivative is therefore a 10-fold stronger inhibitor of MRP1-mediated LTC$_4$ transport than verapamil and might constitute a potential modulator of MRP1-mediated MDR.

**Hypersensitivity of MRP1-Transfected BHK-21 Cells to Verapamil and Its Derivative.** Before studying the ability of both compounds to modulate MRP1-mediated MDR, their toxic effects were investigated on both control and MRP1-transfected BHK-21 cells (Fig. 4). Surprisingly, the latter cells were killed by verapamil (IC$_{50}$ ~1 μM) or NMeOHI$_2$ (IC$_{50}$ ~0.1 μM), whereas only little, if any, cytotoxicity was observed on control, untransfected BHK-21 cells. To check whether the MRP1 transporter was directly involved in hypersensitivity, BHK-21 cells transfected with an inactive MRP1 (K1333L) mutant (20) were analyzed for their sensitivity toward verapamil and its derivative. The results in Fig. 4 show that no cytotoxicity was observed on the cells expressing mutant MRP1, directly demonstrating the necessity of a functional MRP1 transporter to observe hypersensitivity.

**Verapamil and NMeOHI$_2$-Induced Apoptosis in MRP1-Transfected BHK-21 Cells.** To additionally characterize the type of cell death occurring under these conditions, we studied a typical event of apoptosis, namely the presence of phosphatidylserine at the outer face of the plasma membrane in cells cultured in the presence of either 1 μM NMeOHI$_2$ or 10 μM verapamil. As shown in Fig. 5, phosphatidylserine exposure was indeed revealed, after 24-h incubation with either verapamil or its derivative, by the binding of annexin V-fluorescein. Apoptosis was confirmed after staining of picnotic nuclei with Hoechst 33358 (data not shown). Kinetic monitoring of induced apoptosis showed that the binding of annexin V-fluorescein became apparent between 8 and 16 h after the addition of 10 μM verapamil or 1 μM NMeOHI$_2$ (data not shown).

Activation of the caspase family of proteases is also a central biochemical event occurring in apoptotic cells. Therefore, we looked for caspase activation in treated MRP1-transfected BHK-21 cells. As expected, the wide range inhibitor Z-VAD-FMK, when added 90 min before either verapamil (Fig. 6A) or NMeOHI$_2$ (Fig. 6B), prevented cell death when used at 50–100 μM, which demonstrated that caspases were indeed required for cell death.

**Strong Glutathione Depletion in Treated MRP1-Transfected Cells.** Verapamil has been reported previously to stimulate MRP1-mediated GSH uptake by membrane vesicles prepared from MRP1-transfected cells (17) and in MRP1-overexpressing human leukemia cells (18). To investigate whether a similar event occurred in our BHK cell lines, the total cellular glutathione level was comparatively meas-
A cellular depletion of GSH in treated control and MRPI-transfected BHK-21 cells, as a function of time after the treatment. Both verapamil (Fig. 7A) and NMeOHI2 (Fig. 7B) induced a strong and fast (in <1 h) decrease in total cellular glutathione content, whereas no significant decrease was observed with untransfected control cells (Fig. 7) or BHK-21 cells expressing the inactive MRPI (K1333L) mutant (data not shown).

The uptake of [3H]GSH by MRPI-enriched membrane vesicles was measured in the presence of increasing concentrations of either verapamil or NMeOHI2 (Fig. 8). The low basal [3H]GSH uptake observed (2.51 ± 1.54 pmol/mg/min) was markedly stimulated 3- or 9-fold, respectively, in the presence of verapamil or NMeOHI2 (Fig. 8), with apparent $K_a$ of ~10 μM and 1 μM, respectively. The demonstration that GSH transport by MRPI was highly stimulated by NMeOHI2 indicated that the MRPI transporter might be responsible for the intracellular depletion of GSH in treated MRPI-transfected BHK-21 cells.

**Prevention of Apoptosis by Exogenous GSH Addition.** Decrease of total glutathione was observed as a very early event in response of MRPI-transfected BHK-21 cells to modulators. To check if this lowered intracellular GSH indeed played a role in the apoptotic pathway leading to cell death, we tested the effect of adding exogenous GSH on the MRPI-transfected BHK-21 cell viability, in concomitance with verapamil and its derivative. We first checked the prevention of intracellular total GSH loss by exogenous GSH addition. The Fig. 9 shows on the one hand the ability of exogenous GSH to enter both MRPI-transfected BHK-21 cells and untransfected cells (increasing up to 3- or 4-fold the control GSH level) and on the other hand the complete compensation of total GSH loss induced by verapamil and its derivative. For the effect on cell viability, we used the MTT assay, because the LDH assay was incompatible with the presence of GSH. Fig. 10, which shows the survival of MRPI-transfected cells in the presence of modulators (Fig. 10, A and B) and various concentrations of exogenous GSH, indicates that a nearly total prevention against apoptotic death was obtained with 25 mM exogenous GSH.

In the absence of GSH, the IC$_{50}$ values were 6.25 ± 0.1 μM and 1.19 ± 0.04 μM, respectively, for verapamil and NMeOHI2 derivative. By comparison, estimated values of 1 μM and 0.1 μM were, respectively, obtained under different conditions from LDH release measurements (fetal bovine serum was 1%, instead of 5% in the MTT assay, and the culture time was 24 h, instead of 96 h).

**DISCUSSION**

This work demonstrates for the first time that verapamil and its iodinated derivative, NMeOHI2, behave as apoptogens in MRPI-transfected BHK-21 cells, whereas verapamil was generally considered as a conventional modulator of the MDR phenotype.

Verapamil was shown to modulate [3H]LTC$_4$ transport, in a GSH-dependent manner, when using MRPI-enriched membrane vesicles prepared from MRPI-transfected BHK-21 cells, in agreement with the results of Loe et al. (17). The verapamil derivative, NMeOHI2, synthesized with the aim to potentially increase the affinity for MRPI and to lower cardiocytotoxicity, inhibited the [3H]LTC$_4$ transport 10-fold more strongly than verapamil. The inhibition was competitive (Fig. 3) and observed even in the absence of GSH, whereas verapamil inhibition was only apparent in the presence of GSH (17). Both the presence of iodine atoms and phenolic group on the B ring of NMeOHI2 greatly enhanced the affinity for MRPI. Because the higher lipophilicity of dithiane compounds was found to increase MRPI inhibition (25), the effects observed here with the iodinated verapamil derivative might, at least in part, be due to increased lipophilicity. At this stage, the newly synthesized NMeOHI2 derivative could constitute a good candidate for modulating the MRPI-mediated MDR phenotype.

The following data, however, surprisingly revealed a strong cytotoxicity induced by both compounds on MRPI-transfected cells, with an efficiency 10-fold higher for NMeOHI2 than verapamil, in contrast to the absence of any cytoxicity on the parental BHK control cells. This hypersensitivity clearly required an active MRPI transporter, because no or little cytotoxicity was observed...
on cells transfected with an inactive mutant of MRP1 (Fig. 4). Parental BHK control cells showed a slight sensitivity to the NMeOHI₂ derivative (Fig. 4B), whereas no cytotoxicity was observed in the cells transfected with the mutant of MRP1. This was probably due to binding of the verapamil derivative to the inactive MRP1 overproduced in the cell membranes, lowering its entry in the cell. The precise mechanism leading to hypersensitivity of MRP1-transfected BHK-21 toward verapamil and its derivative has then been identified. Firstly, the analysis of phosphatidylserine accessibility to annexin V showed the involvement of apoptosis and caspase activation, because no cell death was observed in the presence of the caspase inhibitor Z-VAD-FMK (Fig. 6). Secondly, kinetic assays revealed that the intracellular glutathione content dramatically decreased, only in MRP1-transfected cells, and reached almost completion in 1 h (Fig. 7), whereas cell death only began between 8 and 16 h after the treatment. Thirdly, the addition of exogenous GSH was itself sufficient to abort the apoptotic development (Fig. 10) probably by entering the cell (by an unknown mechanism) and compensating GSH loss (Fig. 9). However, at this stage, we cannot exclude that exogenous GSH might as well inhibit the efflux of an other relevant compound (such as a membrane lipid involved in apoptosis signaling). Nevertheless, all of these results demonstrate that apoptosis of MRP1-transfected cells induced by either verapamil or its iodinated derivative is correlated with the intracellular GSH content downfall.

The hypothesis of oxidative stress as a universal trigger for apoptosis is well recognized, even for inducing stimuli apparently not related to redox modulation. Indeed, apoptosis has been reported to be associated with glutathione depletion when induced by agents that do not produce any direct oxidative stress (26). This depletion might be the cause of oxidative stress by altering the cell reducing power with extrusion of GSH out of the cell. Oxidized glutathione was neither accumulated in the apoptotic cells nor found in the extracellular medium.

Our results are consistent with various findings from different sources and allowed us to bring them together. Verapamil and its derivative are described here as apoptogens leading to very fast (≤1 h) GSH decrease despite noninducing oxidative stress, and this kind of process might be extrapolated to other apoptogens, because puromycin (26), anti-Fas/APO-1 antibody (27), and di-phenyleneiodonium (28) have been described to behave similarly. Ghibelli et al. (29) observed a rapid GSH decrease preceding irreversible commitment to cell death, because removal of the apoptogen or forced maintenance of GSH inside the cells led to abortion of apoptotic signaling (29). The apoptosis induced by verapamil or its derivative on MRP1-transfected BHK-21 cells was efficiently prevented by the addition of exogenous GSH, in agreement with the results of van den Dobbelsteen et al. (27) who delayed apoptosis when GSH-diethylesters were used to increase intracellular GSH. Therefore, the apoptotic process can be divided in two distinct phases, induction and execution, the GSH extrusion occurring during the first one. GSH depletion, through disruption of redox equilibrium, caused cytochrome c release and triggered
apoptosis (30). The decrease rate of intracellular GSH content must, thus, be rapid, because no apoptosis was observed after treatment with butionine sulfoximine, an inhibitor of GSH synthesis, leading to GSH depletion over a long period (24 h), but without significant effect during the first hours (29). In the presence of butionine sulfoximine, cells might slowly “adapt” to glutathione deprivation by setting up other ways of maintaining a correct redox equilibrium, which is not possible when deprivation occurs too quickly (several minutes in apoptosis versus 24 h for butionine sulfoximine; Ref. 29).

Taking into account that no cytotoxicity and no GSH decrease were observed on either control cells or cells expressing an inactive (K1333L) MRP1 mutant, the involvement of active MRP1 clearly emerged as being responsible for direct efflux of GSH leading to intracellular depletion. Indeed, the transport of [3H]GSH catalyzed by MRP1-enriched membrane vesicles was highly stimulated by verapamil and its derivative. Our results agree with those of Loe et al. (17) who found a stimulation by verapamil of MRP1-mediated GSH transport and others (18, 31) who reported a fast loss of intracellular glutathione related to MRP1. However, none of these works described the killing effect we have observed and characterized here, which might be due to differences in cell lines and glutathione contents. Some cell types indeed contain higher intracellular GSH levels than others, depending on the tissue role in defense against toxicants and on the balance between GSH use and synthesis.
GSH efflux was hypothesized to occur via specific GSH carriers (27–30) identified, for example, with the inhibitor bromosulfophthalein (28). In contrast, the GSH efflux in our study was supported by MRP1. To our knowledge, only one study has suggested involvement of MRPI-mediated glutathione efflux in the apoptosis of immortalized human keratinocytes induced by UVA (32). If the role of glutathione in apoptosis is controversial and depends on cell type and proapoptotic stimuli, MRPI represents a good candidate for such a strong and rapid depletion, even more if its transport activity is stimulated. Why some cell types escape to death, i.e., are not hypersensitive to verapamil, is not understood at this time and will be the purpose of future investigations.

Apoptotic pathways contribute to the cytotoxic effects of most chemotherapeutic drugs (33). In this work, verapamil and its derivative were shown to kill MRPI-overexpressing cells by activating a common apoptotic pathway, but indirectly through MRPI modulation. A similar specific induction of apoptosis in multidrug-resistant cells was described for P-gp inhibitors, both in leukemic cells (34) where the mechanism remained unknown, although a cytokinesis failure was suggested, and for low verapamil concentrations through activation of P-gp ATPase activity leading to production of reactive oxygen species (35).

Tumor cells susceptible to become resistant to chemotherapy by overexpression of MRPI would constitute a privileged target for verapamil and its derivative NMeOHI2 by allowing specific cell killing, thus preventing the emergence of the MDR phenotype. As nontransported substrates for MRPI (17), verapamil and its derivative could remain active with time and potent even at low concentrations, and, therefore, may constitute potential candidates for clinical application. This kind of molecule could be useful as an additive to chemotherapy mixtures to circumvent MRPI-mediated MDR phenotype by specifically killing MRPI-overexpressing tumor cells without inflammatory damage because apoptotic cells are cleanly eliminated by macrophages. This new explanatory hypersensitivity in MDR cells could lead to a novel approach in the treatment of MRPI-positive tumors. At this time, the verapamil derivative NMeOHI2 appears promising for the design and development of even more potent verapamil derivatives useful for the treatment of MDR in cancer patients.

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