1. Introduction

Drug resistance is a crucial determinant of the failure of chemotherapy especially in metastatic solid tumors (Duan et al., 2009). There is a general consensus that the establishment of multidrug resistance (MDR) phenotype depends on several modifications likely to occur into cells as a result of tumorigenic mechanisms and exposure to different chemotherapeutic agents. However, the commonest determinant of this multidrug resistance is over-expression of ABC transporters, in particular of ABCB1, ABCG2 and ABCC1.

In tumors, the main involved and characterized transporter was the ABCB1 (Duan et al., 2009) the first human ABC transporter gene discovered and characterized (Chen et al., 1986). It is responsible for transport of several different drugs, including anticancer drugs, including anthracyclines, vinca alkaloids, taxanes, etoposide, imatinib, camptothecins, etc. (Takara et al., 2006). The correlation between ABCB1 expression and response to chemotherapy has been demonstrated in acute myelocytic leukemia as well as in breast, liver, adrenocortical, renal and colon cancer (Han et al., 2000; Trock et al., 1997; Chung et al., 1997; Chevillard et al., 1996; Filipits et al., 1996). ABCG2 confers resistance to anthracyclines, and is mainly expressed in breast, gastrointestinal, testicular and oral cancers (Allen and Schinkel, 2002; Diestra et al., 2002; Scheffer et al., 2000). ABCC1 has been shown to be mainly involved in glutathione-linked organic compounds transport and expressed in many resistant tumor cells. Its expression has been demonstrated in acute myelocytic leukemia, in breast and lung cancer (Cole and Deedey, 1998; Leith et al., 1999; Filipits et al., 1996; Nooter et al., 1996; Consoli et al., 2002; Young et al., 1999).

The large occurrence of these ABC transporters and their correlation with MDR phenotype has suggested to attack MDR cells either at chemotherapy start or at recurrence with the ambitious aim to stabilize chemotherapy efficacy and prolong its effects over time. Nevertheless, over the last few years three generations of MDR regulators have been tested. The failure of clinical trials with the first two generations of these compounds was ascribable to several severe side effects (Ferry et al., 1996). Finally, the third generation of MDR drugs was shown to exhibit a high affinity to MDR transporters and activity at nanomolar concentrations.
(Seeig and Gatlik-Landwojtowicz, 2005; Colabufo et al., 2008a, 2008b; Azzariti et al., 2006). Clinical trials with some of these third-generation compounds showed unencouraging results thus suggesting to explore other approaches, including multiple inhibition of other MDR transporters and/or cell targets, liposome encapsulation of anticancer agents, etc. (Nobili et al., 2006).

Starting from sigma2 receptor agonists design, we recently synthesized PB28 which showed more than its expected activity on this “not yet known” receptor affecting also ABCB1 (Azzariti et al., 2006). The demonstration of the involvement of this transporter, together with ABCG2 and ABCC1, in establishing multidrug resistance in tumors led us to conduct further structural studies focused into the synthesis of new MDR inhibitors with a higher selective activity (Colabufo et al., 2008a, 2008b).

In the present study, we investigated the in vitro characteristics of the most promising compound of a previous selection, MC70 (Colabufo et al., 2008a, 2008b), confirming that this compound: i) acts as an inhibitor of ABCB1, ii) has antitumor efficacy, and iii) is likely to play a role as an enhancer of chemotherapy activity.

2. Materials and methods

2.1. Cell culture and reagents

MCF7/ADR (resistant to Adriamycin or doxorubicin) and colon cancer cell line Caco-2 were kindly provided by Prof. G. Zupi (IRE, Rome, Italy) and Dr. A. Cavallini (National Institute for Digestive Diseases, I.R. C.C.S. Saverio de Bellis, Castellana Grotte, Bari, Italy), respectively. MCF7/ADR and Caco-2 cells were respectively cultured in vitro in RPMI and DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 50,000 U/ml penicillin and 80 μM streptomycin in a humidified incubator at 37 °C with an atmosphere containing 5% CO2.

Cell culture reagents were purchased from Celbio s.r.l. (Milano, Italy). Culture Plate 96-wells plates were purchased from PerkinElmer Life Science and Calcein-AM from Molecular Probes (Eugene, OR). Verapamil was purchased from Tocris Bioscience (Bristol, United Kingdom).

2.2. Synthesis of MC70

MC70 was previously synthesized although chemical procedure allowed the compound to be produced in low yield, furthermore intermediates exhibited chemical instability (Colabufo et al., 2008a). For these reasons, another synthetic procedure to prepare MC70 has recently been planned (Fig. 1). 4’-Hydroxy-4-biphenylylcarboxylic acid (1) reacted with iodomethane and cesium carbonate in DMF obtaining the corresponding methylester (2). The crude extract condensed with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline in the presence of Na2CO3 in CH3CN, afforded the amide 3, which was reduced by LiAlH4 in THF dry obtaining MC70 in a high yield (92%).

2.3. Efflux of rhodamine-123 experiment

MCF7/ADR cells were seeded onto glass coverslips at a density of 15,000/cm2, and used for fluorescence measurements after 4 days. Cells were incubated for 45 min into a medium containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1.6 mM MgCl2, 11 mM glucose, 25 mM HEPES (pH 7.4), and 10 μM Rhodamine-123 (Rho-123), either in the absence or in the presence of an increasing concentration of the drug under investigation. The coverslip was then mounted on a specially constructed open chamber (0.5 ml), fixed on the stage of an inverted Nikon TE2000-S microscope. Excitation wavelength (488 nm) was generated by Poly-chrome IV (Till Photonics; Gräfelfing, Germany). Emission wavelength (525 nm, XF3017 filter by Omega Optical, Brattleboro, VT, USA) was detected by a PC-interfaced Micromax camera (RS Princeton Instruments; Trenton, NJ, USA). Workbench 2.2 software by Axon Instruments (FHC Inc., Bowdoin, ME, USA) was used either to drive the light emission and the camera or to quantify fluorescence values from a number of regions of interest. Cells were perfused (2 ml/min) with the incubation medium, without Rh-123, containing the tested compound. Experiments were run at 22 °C and repeated with at least 3 different cell preparations.

2.4. Calcein-AM experiments

These experiments were carried out as reported by Feng et al. with minor modifications (Feng et al., 2008). Each cell line (50,000 cells per well) was seeded into 96-wells black culture plate with 100 μl medium and allowed to become confluent overnight. Test compounds were solubilized in 100 μl of culture medium and added to monolayers. The 96/well plate was incubated at 37 °C for 30 min. Calcein-AM was added in 100 μl of Phosphate Buffered Saline (PBS) to yield a final concentration of 2.5 μM and the plate was incubated for 30 min. Each well was washed 3 times with ice-cold PBS. Saline buffer was added to each well and the plate was read to Victor3 fluorometer (PerkinElmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Under these experimental conditions Calcein cell accumulation in the absence and in the presence of the compounds tested was evaluated and the fluorescence basal level was estimated by untreated cells. In treated wells the increase in fluorescence compared to baseline was measured. The IC50 values were determined by fitting the fluorescence increase percentage versus log [dose]. Verapamil was used as a reference compound.

2.5. Genome analysis

Samples were prepared as described by Bevilacqua et al. (2008). Data were deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE26250 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26250). Microarray data analysis was performed using customized R language-based script (Gentleman et al., 2004), using Bioconductor packages for quality control analysis, data normalization, and identification of differentially expressed genes (Tusher et al., 2001). In more detail, the package “arrayQuality” performed array quality on spotted arrays, while the limma (Linear Models for Microarray Analysis) and marray packages were used together for chip normalization and background correction. Significant analysis of Microarrays (SAM), with a two class unpaired option, was used to identify the genes distributed between two conditions (treated vs non-treated). SAM analysis was performed using R package “samr” (Tusher et al., 2001).

2.6. Cell proliferation assay

Determination of cell growth inhibition was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay and by cell counting. The MTT assay for each concentration responsible for 50% inhibition of cell growth (IC50) determination and drug combination effectiveness was performed as described by Azzariti et al. (2008). For IC50 determination in Caco-2 cells, doxorubicin was given at concentrations of 1, 5, 10, 25, 50 and 100 μM for 1 day. IC50 was defined as the drug concentration yielding a fraction of affected (non surviving) cells = 0.5, compared with untreated controls and was calculated utilizing CalcuSyn ver.1.1.4 software (Biosoft, UK). In the combination studies, MC70 was given at 2 and/or 20 μM and the chemotherapeutic agents at the concentration reported in each experiment.
2.7. Cell cycle analysis

Cells were harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 ml of 70% ethanol at −20 °C, and washed once in ice-cold PBS. The pellet was re-suspended in PBS containing 1 mg/ml RNase, 0.01% NP40 and the cellular DNA was stained with 50 μg/ml propidium iodide (PI) (Sigma). Cells were stored in ice for at least 1 h prior to analysis. Cell cycle determinations were performed using a FACScan flow cytometer (Becton Dickinson), collecting several data (dot plot FSC vs SSC; dot plot FL2A vs FL2W and histogram FL2A). Histograms (FL2A) were interpreted using CellQuest software, provided by the manufacturer.

2.8. Cell apoptosis assay

Apoptosis detection was further investigated by the Cell Death ELISA<sup>plus</sup> kit (Roche Molecular Biochemicals, Milan, Italy). The test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by biotinylated antihistone-coupled antibodies, and their enrichment in the cytoplasm is calculated as the absorbance of sample cells/absorbance of control cells. The enrichment factor was used as a parameter of apoptosis and shown on the Y-axis as mean ± SE. Experiments were performed according to manufacturer's instructions.

2.9. Wound healing assay

Confluent monolayer of Caco-2 or MCF7/ADR cells was wounded and treated with MC70 at 2 μM and 20 μM concentrations or left untreated (control). The plates were photographed 0, 6, 24, 30, 48 and 54 h post-wounding. Cell migration was quantified by counting the wound width after the plates were treated, utilizing ImageJ® analysis software. Results are given as migration length and are accounted for by the average per field ± S.D. of three independent experiments.

2.10. Western blot analysis

Protein extracts were obtained by homogenization in RIPA buffer (0.5 M NaCl, 1% Triton X100, 0.5% NP40, 1% deoxycholic acid, 3.5 mM SDS, 8.3 mM Tris HCl pH 7.4, 1.6 mM Tris base) and treated with 1 mM phenylmethylsulfonyl fluoride (PMSF). Total proteins were measured and analyzed as described in Azzariti et al. (2008). Expression levels were evaluated by densitometric analysis using Quantity One software (Biorad, Hercules, CA). β-actin expression levels were used to normalize the sample values.

2.11. Antibodies

All monoclonal antibodies utilized were provided by Cell Signalling-USA and Sigma-Aldrich, St. Louis, MO-USA. A mouse-HRP and a rabbit-HRP (Amersham Pharmacia Biotech, Upsala Sweden) were used as secondary antibodies.

2.12. Lactate dehydrogenase activity

Cells undergoing necrosis typically exhibit rapid swelling, loss of membrane integrity, and release of lactate dehydrogenase (LDH). This enzyme activity into the culture medium was measure as described by Bernt and Bergmeyer (1963) and expressed as percent of maximum LDH released 24 h after the induction of glutamate neurotoxicity (Atlante et al., 2005). An aliquot (100 μl) of culture medium (2 × 10<sup>6</sup> cells/2 ml culture medium) was added to 2 ml of 50 mM Tris–HCl buffer pH 7.4 in the presence of 0.2 mM NADH. The assay reaction was started by adding 0.6 mM pyruvate. 2.13. Statistical analysis

All in vitro experiments were performed in triplicate, and results were expressed as mean ± standard deviation (S.D.) unless otherwise indicated. Statistical differences in in vitro and in vivo data were assessed by ANOVA, followed by the Student-Newman–Keuls test, and by Tukey's test to find which means are significantly different from one another. P-values lower than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism software package version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

To obtain a large amount of MC70, the drug was prepared as described in the “Materials and methods” section and summarized in Fig. 1.

In a previous screening effort, we had been able to select some promising molecules, MC18 and MC70, with EC50s in Caco-2 cells monolayer assay of 1.64 μM and 0.05 μM, respectively and in respect to standard compound, PB28 (EC50 = 0.55 μM) (Colabufo et al., 2008a, 2008b) and in the present study we explored in more detail MC70, the most promising of these molecules.

The characterization of MC70 activity was carried out utilizing PB28 and MC18 as standard compounds, together with Verapamil, the “classic” ABCB1 inhibitor. PB28 is a sigma-2 ligand which affects ABCB1 activity and its derivative MC18 exhibits a stronger ABCB1 inhibitory action (Colabufo et al., 2008a; Azzariti et al., 2006). The cells utilized in this study were MC7F/ADR, a breast cancer cell line where ABCB1 expression was induced by doxorubicin exposure, HT-29, Lovo and Caco-2, colon cancer cell lines, the last of which used as a conventional model to investigate transport across membranes involving ABC transporters.

Our study envisaged: basic characterization of MC70 efficacy as an inhibitor of MDR transporters, investigation of its anticancer behavior and the exploration of the molecular and cellular mechanisms underlying this latter.

3.1. Basic characterization

3.1.1. MDR-1 modulation

The MCF7/ADR cell line was treated with Rhodamine-123, a fluorescent ABCB1 substrate that permeates the cells at high concentrations or left untreated. Expression of ABCB1 activity, we can exit the cells by passive efflux or by ABCB1-mediated extrusion. In the presence of an ABCB1 inhibitor, at each concentration being tested, Rhodamine-123 efflux decreased in a time-dependent manner (Altenberg et al., 1994).

In order to challenge MC70, MC18, and PB28 as potential inhibitors of ABCB1 activity, we first preloaded MCF7/ADR cells with Rhodamine 123 (Rho-123) and then measured the efflux of Rho-123 in the presence of the inhibitor tested.

The efflux of Rho-123 from intracellular volume takes place through a passive pathway and/or via an ABCB1-mediated extrusion. This efflux is quantitatively described by the following equation

\[ F_{Rho-123} = F_0 e^{-kt} + \text{background} \]  

or, in a logarithmic form:

\[ \ln(F_{Rho-123} - \text{background}) = \ln(F_0) - kt \]  

where \( F_{Rho-123} \) is the Rho-123 fluorescence measured, \( F_0 \) is \( F_{Rho-123} \) at \( t = 0 \), and \( k \) is the rate constant (Eidelman and Cabantchik, 1989). In Eq. 2, when \( F_{Rho-123} \) is reduced to 1/2 of its initial value (\( F_0 \)), \( t = \ln(2)/k \); this value is referred to as \( t_{1/2} \), and is conventionally used to compare different rates.
As is shown in Fig. 2A inset, we measured the fluorescence of Rho-123 in a region of the coverslip devoid of cells, referred to as (a) area, as well as in several cells like the 3 border cells referred to as (b) areas. In the main panel of Fig. 2A the following values were plotted versus t: i) the values indicated as “background”, which were measured in (a) area plus 5 fluorescence arbitrary units (the contribution by unloaded cells as it was measured in control experiments); ii) the values indicated as “$F_{\text{Rho-123}}$” and calculated by averaging the fluorescence values from 18 cells; iii) the values indicated as “$\ln(F_{\text{Rho-123}} - \text{background})$” and calculated by using the experimental data. On the upper trace, only the points lining on a straight line fit the exponential decay function (Eq. 2) and make it possible to estimate $F_0$ and $k$, and finally to calculate $\tau_{1/2}$, with a least squares regression program (GraphPad Prism Software, version for Windows (1998); GraphPad Software, Inc.: San Diego, CA).

The efflux of Rho-123 was measured in the absence of inhibitors and in the presence of 0.2 mM Verapamil or increasing PB28 or MC18 or MC70 concentrations (from 0.1 nM to 100 μM).

The results of our experiments are summarized in Fig. 2B, where the $\tau_{1/2}$ values calculated are plotted versus log[drug]. MC18 was ineffective on the Rho-123 efflux. The inhibition by PB28 was constant up to the highest concentration used. Finally, the inhibition by MC70 was dose-dependent and similar to that by 0.2 mM Verapamil. These findings reflected the results observed in $[\text{H}]$ ABCB1-mediated vinblastine inhibition transport in Caco-2 monolayer (Colabufo et al., 2008a, 2008b; Azzariti et al., 2006).

These results are consistent with our previous findings (Colabufo et al., 2008a), which highlighted an increase in intracellular concentration of doxorubicin of 6.1 ± 0.1 folds after MC70 exposure, thus confirming the marked ability of MC70 to act as a pure inhibitor of ABCB1.

3.1.2. ABCG2 and ABCC1 modulation

Another way to study MDR proteins activity is to monitor calcine accumulation in transfected MDCK cells, canine cells specifically transfected with cDNA of each human ABC transporters. Intact cells are incubated with AcetoxyMethyl (AM) esters of Calcein (Calcein-AM), a lipophilic ABCB1 substrate that diffuses across the plasma membrane into the cell, where it is hydrolyzed by endogenous cytoplasmic esterases into highly fluorescent Calcein. The hydrolyzed compound is not

![Fig. 1. Strategy to synthesize MC70.](image_url1)

![Fig. 2. Efflux of Rhodamine-123 and effect of MC18, MC70, PB28 and Verapamil on the $\tau_{1/2}$ of the Rhodamine-123 efflux from MCF 7/Adr cells.](image_url2)
a MDR proteins substrate, and since it is hydrophilic, it cannot cross the cell membrane via passive diffusion. Thus, a rapid increase in the fluorescence of cytoplasmic Calcein can be monitored. The MDR pump, present in the plasma membrane, rapidly effluxes the Calcein-AM before its entry into the cytosol thus determining a reduction in the fluorescent signal due to the accumulation of Calcein. The mechanism of this assay is schematically illustrated in Fig. 3A. The evaluation of ABCB1 activity in the presence of pump inhibitors can be carried out in a competitive manner. The compounds that block MDR pumps inhibit Calcein-AM efflux increasing fluorescent Calcein accumulation.

MC18 and MC70 were found to inhibit both ABCG2 and ABCC1 with different potencies.

In MDCK-BCRP, MC70 displayed a marked activity (EC$_{50}$ = 1.50 μM), MC18 showed an effect overlapping that of MC70 (EC$_{50}$ = 2.97 μM) and PB28 was found to be slightly less active than the former two (EC$_{50}$ = 5.37 μM). The corresponding curves are reported in Fig. 3B.

In MDCK-MRP-1, MC18 displayed an inhibition effect higher than that of MC70 (EC$_{50}$ = 2.80 μM and EC$_{50}$ = 9.30 μM, respectively) as shown in Fig. 3C. By contrast, PB28 was found to be an ABC11 inducer for all the concentrations tested. The plot of Fig. 3D highlights a dose–dependent decrease in Calcein cell accumulation compared to the baseline level. In both cell lines, Verapamil was tested as a reference compound showing submicromolar inhibition activity on the ABCG2 pump (EC$_{50}$ = 0.40 μM) and a marked effect on the ABCC1 pump (EC$_{50}$ = 4.40 μM).

The curves of tested compounds for inhibiting ABCG2 activity are representative of three independent experiments (one-way ANOVA: P < 0.0001). Results of the Tukey test were Verapamil vs PB28: P < 0.001; Verapamil vs MC18: P = 0.001; Verapamil vs MC70: P > 0.05; PB28 vs MC18: P = 0.001; PB28 vs MC70: P > 0.05; MC18 vs MC70: P > 0.05.

The curves of compounds for inhibiting ABCC1 activity are representative of three independent experiments (one-way ANOVA: P < 0.0001). Results of the Tukey test were Verapamil vs PB28: P < 0.001; Verapamil vs MC18: P < 0.05; Verapamil vs MC70: P > 0.05; PB28 vs MC18: P < 0.001; PB28 vs MC70: P > 0.05; MC18 vs MC70: P > 0.05.

These findings highlight that the chemical modification progressively introduced in the lead compound (PB28), increased the ability to affect ABCB1 and in a different manner ABCG2 and ABCC1.

3.2. Anticancer activity

After a preliminary, confirmatory characterization of the activity of the compound under study as a modulator of ABC transporters, especially of ABCB1, we focused on its ability to affect, both directly and indirectly, cancer progression and/or conventional chemotherapy effectiveness.

3.2.1. Kinetic data: cell growth inhibition

3.2.1.1. MC70 plus doxorubicin, a substrate of ABCB1. Analyses were carried out in breast and colon cancer cells utilizing doxorubicin as a conventional chemotherapeutic drug. Doxorubicin was utilized at the concentration inducing only a slight inhibition of cell growth (about 10%) to better highlight its activity when combined to MC70. MC70 was utilized at 2 and 20 μM, the high concentration has already
been shown to inhibit ABCB1 and induce doxorubicin accumulation into cells (data not shown). In MCF7/ADR cells, a 1 day-doxorubicin dose of 50 μM was used, which could be assumed to induce only a slight inhibition in 1 day since it was the IC50 value after 3 days drug exposure, as reported in our previous (Azzariti et al., 2006). In Caco-2 cells, the choice of the concentration relied on the data reported in the literature studies suggesting that this agent is able to induce cell death in different ways as a function of its different concentrations (Rebbaa et al., 2003). We determined the IC50 value for doxorubicin, which was 50 μM (data not shown) and the concentration selected for the study were 100 nM and 50 μM in a view to highlighting combination effectiveness as well as the different mechanisms of death induced by doxorubicin as a function of the concentration utilized.

In MCF7/ADR, 2 days MC70 exposure inhibited cell growth, passing from 85% to 80% at 2 and 20 μM, respectively; in Caco-2 cells, 20 μM MC70 showed a dramatically increased efficacy reaching a 50% inhibition level.

The different behavior, shown in the two cell lines, was even inverse and more evident when the drug was given together with doxorubicin. In breast cancer cells, combined exposure to MC70 and doxorubicin resulted in a dramatic increase in cytotoxicity, from 8% to 42 and 90% of cell growth inhibition when chemotherapy was preceded by the compound under investigation, at 2 and 20 μM. Conversely, in colon cancer cells, the two drugs did not exhibit any increased effectiveness when administered together compared to when given alone as demonstrated by the fact that, in samples incubated with various concentrations of combined drugs, cell growth was similar to the value obtained by the most effective drug as a single agent, as shown in Fig. 4A.

3.2.1.2. MC70 plus topotecan and CPT-11, substrates of ABCG2. Our previous results had highlighted a poor ability on the part of MC70 to enhance doxorubicin toxicity in Caco-2 cells. However, doxorubicin is not the first line drug for colon cancer patient’s treatment and our aim is to propose a novel promising combination therapeutic approach.

To this end, we investigated MC70 plus “classic” chemotherapy with topoisomerase I inhibitors, such as CPT-11 and topotecan which are substrates of ABCG2 that interact with the compound in question. In three colon cancer cell lines, pre-exposure to MC70, at slight inhibition-inducing concentrations (10–40%), resulted in increased cytotoxicity of topotecan and CPT-11 (Fig. 4B). Under this experimental condition the reduction of cell growth was significant in both Lovo and Caco-2 cells as reported in Fig. 4B.

3.2.2. Migration analysis

MC70 is a derivative of lead compound PB28 with high sigma-2 receptor affinity and only moderate selectivity toward sigma-1 receptors (Colabufo et al., 2008a). Megalizzi et al. demonstrated that the inhibition of sigma-1 receptors is responsible for a reduced ability of cells to migrate (Megalizzi et al., 2007). Therefore, we evaluated the effect of MC70 on cellular motility by wound healing assay in our in vitro model. Monolayer of untreated cells (control) showed a quite complete wound healing within 48 h conversely, monolayers of cells treated with MC70, at 2 and 20 μM, showed a clear wound width after 48 h and until 54 h, in all of the three experiments conducted (pictures

Fig. 4. Drug(s)-dependent cell growth inhibition. Cells were incubated with MC70 for 2 days and doxorubicin was administered during the second day. Cell survival was determined by MTT assay as described in Materials and methods section.
not shown). **Fig. 5** shows both pictures and data related to Caco-2 cells with a special focus on the modification that takes place 6 h after drug exposure which reflects the ability of the compound under study to reduce cell migration. The results reported are similar to those obtained in the breast cancer cells, thus demonstrating the ability of the compound to inhibit cell migration in a time-dependent fashion.

### 3.3. Mechanisms investigation

Our quest for an explanation for MC70 activity as anticancer agents, other than direct inhibition of ABCB1, led us to explore the intracellular effect of the compound in more detail. We assessed whether MC70 acts through cell transcription modulation or directly, by modifying specific signaling pathways. We then proceeded and checked whether i) MC70 inhibits cell proliferation through a cytostatic or cytotoxic behavior, when given alone or in combination, and whether ii) cell cycle perturbation, apoptosis and/or necrosis induction were involved. Finally, the MC70-dependent modulation of proteins specifically involved in survival, proliferation and migration pathways was investigated.

#### 3.3.1. Genomic analysis

The in vitro kinetic results demonstrated a clear activity for MC70 as an enhancer of doxorubicin activity in MCF7/ADR and thus, genome-wide microarray (Affymetrix GeneChip Human Gene 1.0 ST) analysis of treated cells was performed in this cell line in order to establish a possible direct impact of MC70 on the cell transcription machinery.

MCF7/ADR cells were treated with MC70, and separately with standard compounds MC18 and PB28, for 3 days and RNAs were prepared, hybridized on chips and analyzed. This agent did not modulate any mRNA expression likely to be related to antitumoral activity compared to both untreated cells and MC18- or PB28-treated cells. The genes most highly ranked by SAM were mainly related to inflammation and not directly connected with any cancer pathway (Table 1).

#### 3.3.2. Cell cycle modulation

A strong perturbation of cell cycle progression is likely to be responsible for the observed kinetic data, a hypothesis that has been investigated in our model. MC70 induced a marked concentration-dependent G2/M phase cell accumulation. This phenomenon was reversed after drug exposures of 24 h and 48 h in Caco-2 and MCF7/ADR, respectively (**Fig. 6A**). The evaluation of cell cycle perturbation was also carried out when our compound was given together with doxorubicin and the anthracycline induced a marked G2/M phase cell accumulation which was found to be partially overcome by a 2-day pre-exposure to MC70 in MCF7/ADR cells (**Fig. 6B**). As shown, when administered alone doxorubicin seemed to induce polyploidy, a phenomenon related to the establishment of senescence already reported by Kikuchi et al. (2010), when the drug was utilized at sub-toxic concentration. In our experimental model, 24 h doxorubicin at 50 μM induced less than 10% of cell growth inhibition.

**Table 1**: MC70 modulated genes most highly ranked by SAM analysis.

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<th>Symbol</th>
<th>Cytoband</th>
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<th>Score (d)</th>
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<th>q-value (%)</th>
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**Fig. 5**: MC70 ability to modulate cellular motility. Wound healing assay was performed at 6–54 h with 2–20 μM MC70. Caco-2 cell migration was quantified with ImageJ® analysis software and results are expressed as percentage of migration area vs time exposure.
thus allowing to consider this concentration as sub-toxic. In Caco-2 cells, MC70 plus 100 nM doxorubicin had a similar behavior (Fig. 6C). Determining this behavior was instead difficult at 50 μM due to the large amount of necrotic cells (data not shown).

3.3.3. Apoptosis induction

Most of the anticancer agents utilized in cancer treatment kill cells by inducing apoptosis, this led most scientists to focus their attention on the study of the apoptotic program rather than on other mechanisms of cell death. However, anthracyclines, including doxorubicin, have been shown to act by killing cells in three different ways: senescence, apoptosis and necrosis (Rebbaa et al., 2003).

In this study, we explored the possibility that the observed reduction of cell growth by MC70 and doxorubicin, administered alone or in combination, was due to apoptosis induction. Apoptosis was evaluated as DNA laddering by Elisa determination. Normalized data are reported in Fig. 7A. 20 μM MC70 induced a slight but statistically validated induction of apoptosis in both cell lines which was completely blocked when the drug was given in association with doxorubicin.

At a dose of 50 μM, this anthracycline was unable to induce apoptosis in both in vitro models, this evidence seems to be in contrast with most literature data on the activation of apoptosis after doxorubicin administration. However, this lack of apoptosis has been already shown in other cell models and largely explained by the identification of at least three different doxorubicin-dependent death mechanisms, such as senescence, apoptosis and necrosis (Rebbaa et al., 2003). Moreover, the resistance to doxorubicin of MCF7/ADR cells depends on the chronic exposure to low doses of this drug (40 nM) which induces the overexpression of ABCB1 a well known ABC transporter involved in determining resistance to anthracyclines. In this model, doxorubicin did not kill cells because its intracellular concentration was too low to act. But, when an ABCB1 inhibitor like MC70 is given together with the chemotherapy drug, its effectiveness is completely

![Fig. 6. MC70 dependent cell cycle modification. Cells were incubated with 20 μM MC70 and/or doxorubicin (at 50 μM and 100 nM in MCF7/ADR and Caco-2, respectively) and cell cycle was analysed by CFM as described in Materials and methods section. In A, percentage of cell accumulation in Go/G1(____) and G2/M (.....) phase were added.](image-url)
recovered (Azzariti et al., 2006). Our results demonstrated that the recovered activity of doxorubicin was not traced to apoptosis induction, but presumably to a different mechanism of action, as clearly shown by the enrichment factor of about 1.

As to the results in Caco-2 cells, we evaluated the possibility that death by doxorubicin might be traced to apoptosis as a function of the drug concentrations utilized, 50 μM and 100 nM. Our data, summarized in Fig. 7, clearly confirmed that the anthracycline induced programmed cell death at a low dose, but at a higher dose, cells died for a different mechanism. However, when MC70 was given together with doxorubicin, apoptosis induction was strongly reduced (Fig. 7A), even if cells died as shown in Fig. 4.

3.3.4. Necrosis induction

To confirm that the cell growth reduction observed at a higher concentration of doxorubicin (Fig. 3) was due to necrosis, the determination of LDH activity was measured (Atlante et al., 2005). In the presence of 50 μM doxorubicin, cell death did not occur via apoptosis, since no DNA laddering (Elisa determination) occurred either in the absence or presence of MC70 (see Fig. 7A), but via necrosis as demonstrated by the increase in lactate dehydrogenase release in the presence of MC70 (Fig. 7B). This increased LDH activity depended on doxorubicin concentration. Actually at a concentration of 100 nM the values obtained after doxorubicin and after MC70 plus doxorubicin were not statistically different (t Student test); contrary to what happened at a 50 μM concentration.

3.3.5. Pathways modulation

To understand the cellular pathways involved in determining the phenotypic effects obtained after MC70 exposure, we analyzed the modulation of AKT and of the three members of the MAPK (mitogen-activated protein kinase) superfamily including ERK, JNK/SAPK and p38 MAPKinesases.

In both MCF7/ADR and Caco-2 cells, MC70 exposure did not modulate the expression level of Akt, Erk1/2, JNK and p38 (data not shown), but it modified the phosphorylation levels of all of them.

In MCF7/ADR cells, a 48 hour-MC70 exposure increased the expression levels of p-Akt, p-p38, p-Erk1/2 and p-JNK with different intensities as reported in Fig. 8. When MC70-treated cells were then exposed to doxorubicin, phosphorylated Akt (Ser473) strongly decreased compared to both drugs in monotherapy. On the contrary,
the three members of the MAPK superfamily were more phosphorylated (Fig. 8A).

In Caco-2 cells, each modulation was less evident. In more detail p-Akt exhibited a behavior similar to that in MCF7/APDR, conversely p-p38 and p-JNK were activated by MC70 alone, but when given in combination their expression levels decreased and overlapped those of doxorubicin-treated cells (Fig. 8). As for p-Erk1/2, MC70 strongly decreased its expression level when given in combination with the anthracycline (Fig. 8).

4. Discussion

The aim of the present study was to check the possibility to overcome MDR utilizing new molecules which specifically inhibit drug efflux thus inducing an increase in chemotherapy effectiveness, through direct and indirect mechanisms. As a matter of fact, MDR inhibitors have been consistently shown to be very promising in clinical studies, but their ultimate clinical therapeutic usefulness remains to be established for solid tumors (Nobili et al., 2006; Kaye, 1998).

In this paper, we report the in vitro characterization of a new compound, MC70, a derivative of a sigma-2 agonist PB28 with expected increased efficacy in ABCB1 inhibition. In a previous study, we had characterized the ability of MC70 to inhibit ABCB1 by three combined assays in Caco-2 cells (Colabufo et al., 2008a). Here, we further characterize this product in terms of its interaction with the ABC transporters involved in cancer multidrug resistance, ABCB1, ABCG2 and ABCC1, and deeply analyze its ability to influence other cell processes, including proliferation, apoptosis and invasion.

MC70 confirmed to be a better ABCB1 inhibitor compared to its precursors MC18 and PB28. As to MC70 selectivity toward other ABC transporters, we found that all the compounds tested displayed an inhibition activity toward ABCG2, where MC70 displayed a submicromolar potency. Conversely, MC70 and MC18 were found to be inhibitors of ABCB1 while PB28 was observed to stimulate the transporter activity.

The efficacy of MC70 as an anticancer agent in MCF7/APDR and Caco-2 cells and the mechanisms involved in this efficacy were further investigated.

Our compound showed to have an opposite effect in breast and colon cancer cells. In fact, while in breast cells, MC70 exhibited a slight ability to inhibit cell proliferation when given alone, but a strong ability to enhance doxorubicin effectiveness, in colon cells MC70 inhibited cell growth without affecting doxorubicin efficacy. However the molecular pathway underlying MC70 activity in CaCo-2 cells remains to be elucidated. Failure in enhancing doxorubicin cytotoxicity could depend on the poor sensitivity to anthracyclines of the colon cancer model. Our study of the possibility to combine MC70 with topoisomerase I inhibitors in the colon cancer model strongly suggests that this could be a promising therapeutic approach and represent the next step of our investigation.

In addition, our drug induced apoptosis, demonstrated by DNA laddering formation, but when given in combination with high doses of the anthracycline apoptosis was reduced in favor of other types of chemotherapy-dependent cell death. When doxorubicin was given at low doses (at 100 nM), apoptosis was not activated and only a slight induction of necrosis was evident thus suggesting that other mechanisms of cell death might occur under these experimental conditions; this topic will be the next step of our study.

Doxorubicin has already been shown to be able to kill cells in a concentration-dependent manner and via with three different processes: senescence, apoptosis and necrosis (Rebbaa et al., 2003). Moreover, as far as MCF7/APDR is concerned, Yokochi and Robertson have demonstrated a direct correlation between the presence of DNA methyltransferase-1 (DNMT1) and doxorubicin-induced apoptosis. In fact cells lacking DNMT1 and exposed to the drug were not found to exhibit apoptosis (Yokochi and Robertson, 2004; Lin and Nelson, 2003). In Caco-2 cells, Stemppak et al. demonstrated that DNMT1 was expressed. However, the absence of doxorubicin-induced apoptosis could be accounted for by the use of a low drug concentration (Stempak et al., 2005). Our data are consistent with the hypothesis that high doses of doxorubicin kill cells not via apoptosis induction, but rather via necrosis induction.

MC70 inhibited cell invasion as highlighted by the wound healing experiments. We think this characteristic could be ascribed to its residual activity as sigma-1 ligand, which is known to reduce cell migration (Megalizzi et al., 2007).

In our search for possible molecular and cellular mechanisms responsible for all these different phenotypic phenomena, we performed microarray experiments excluding modulation at transcription level. The possibility of using a genome-wide microarray analysis to determine the ability to modulate transcription by the MC70 came from evidence that experimental PB28 decreased ABCB1 expression level (Azzariti et al., 2006). This analysis showed that MC70, like the other two precursors, MC18 and PB28, did not vary the levels of mRNA expression. So only a modulation at an intracellular post-transcriptional level remains to be assumed.

Apoptosis induction, cell growth and invasion migration could be accounted for by modulation of cell cycle, of pAkt and of three MAPKs phosphorylation. Our data demonstrated MC70 ability to induce apoptosis which could be justified by the partial and transient cell accumulation in the G2/M phase probably as a function of JNK increased phosphorylation.

These correlations have already been demonstrated in other cell models (Wang et al., 2010; Yang et al., 2010), but the stimulation of
programmed cell death seemed, in our experiments, reinforced by the combined administration of MC70 plus doxorubicin at low doses in Caco-2 cells; conversely, high doses of the chemotherapeutic drug also together with MC70 provoked cell death via the necrosis pathway as confirmed by an increased release of LDH by cell swelling. The different kind of death mechanism induced by doxorubicin administration reported by Rebbaa et al. (2003) seem to substantiate our results with necrosis which might also be stimulated by MC70 through an increase in the pAkt expression level.

5. Conclusions

The evaluation of the pharmacological activity of MC70 has suggested that it is a novel anticancer agent with both cytostatic and cytotoxic characteristics that, when given in combination, could potentiate doxorubicin effectiveness through the modulation of a large number of cell targets. In our opinion, MC70 could account for a promising therapeutic approach in combination with anthracyclines in the treatment of chemotherapy resistant tumors.

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References


