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Dual inhibitors of P-glycoprotein and tumor cell growth: (Re)discovering thioxanthones

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ABSTRACT

For many pathologies, there is a crescent effort to design multiple ligands that interact with a wide variety of targets. 1-Aminated thioxanthone derivatives were synthesized and assayed for their *in vitro* dual activity as antitumor agents and P-glycoprotein (P-gp) inhibitors. The approach was based on molecular hybridization of a thioxanthone scaffold, present in known antitumor drugs, and an amine, described as an important pharmacophoric feature for P-gp inhibition. A rational approach using homology modeling and docking was used, to select the molecules to be synthesized by conventional or microwave-assisted Ullmann C–N cross-coupling reaction. The obtained aminated thioxanthones were highly effective at inhibiting P-gp and/or causing growth inhibition in a chronic myelogenous leukemia cell line, K562. Six of the aminated thioxanthones had GI_{50} values in the K562 cell line below 10 μM and 1-{[2-(diethylamino)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**37**) had a GI_{50} concentration (1.90 μM) 6-fold lower than doxorubicin (11.89 μM) in the K562Dox cell line. The best P-gp inhibitor found was 1-[2-(1*H*-benzimidazol-2-yl)ethanamine]-4-propoxy-9*H*-thioxanthen-9-one (**45**), which caused an accumulation rate of rhodamine-123 similar to that caused by verapamil in the K562Dox resistant cell line, and a decrease in ATP consumption by P-gp. At a concentration of 10 μM, compound **45** caused a decrease of 12.5-fold in the GI_{50} value of doxorubicin in the K562Dox cell line, being 2-fold more potent than verapamil. From the overall results, the aminated thioxanthones represent a new class of P-gp inhibitors with improved efficacy in sensitizing a resistant P-gp overexpressing cell line (K562Dox) to doxorubicin.

1. Introduction

Resistance to chemotherapy represents one of the major obstacles to cancer treatment. Multidrug resistance (MDR) can be broadly defined as a phenomenon by which tumor cells *in vivo*, and cultured cells *in vitro*, show simultaneous resistance to a variety of structurally and functionally dissimilar cytotoxic and xenobiotic compounds [1]. P-glycoprotein (P-gp), an ABC (ATP-binding cassette) super-family member [2,3], is a membrane transporter that actively extrudes a set of structurally unrelated compounds, namely chemotherapeutic agents, out of the cells [4] conferring the MDR phenotype in cancer [5]. Despite promising *in vitro* results obtained for several generations of P-gp inhibitors, disappointing clinical trials demand for new drugs and strategies to reverse the P-gp-mediated MDR phenotype [6,7].

Simultaneous modulation of multiple biological targets can be beneficial for treatment of diseases with complex etiologies such

Abbreviations: ABC, ATP-binding cassette; ATP, adenosine-5′-triphosphate; BLAST, basic local alignment search tool; DNA, deoxyribonucleic acid; GI_{50}, inhibition of cell growth (the concentration needed to reduce the growth of treated cells to half that of untreated cells); MDR, multidrug resistance; MFI, mean fluorescence intensity; MRP, multidrug resistance proteins; MRP-1, multidrug resistance protein 1; MW, molecular weight; NBD, nucleotide binding domain; P-gp, P-glycoprotein; Rh123, rhodamine-123; RLU, relative light unit; RMSD, root-mean-squared error displacement; SE, standard error; SRB, sulphorhodamine-B; TM, transmembrane (one α-helix); TMD, transmembrane domain (six α-helixes).

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as cancer [8,9]. Recently, there has been a paradigm shift in drug design towards the development of multifunctional drugs, i.e., drugs that aim multiple targets [8,9]. The benefits of this approach include improved compliance, efficacy and often reduced side effects in comparison with polypharmacy [8]. In addition, some multifunctional drugs may work better at normalizing the pathology than a single targeted compound. This strategy has already been used for the design of multifunctional agents for HIV [10] and several neurodegenerative diseases [11,12]. Podophyllotoxin derivatives were recently described to fight simultaneously cancer and MDR [13]. Nevertheless, a structure-based design strategy to obtain antitumor agents which are concomitantly P-gp inhibitors has never been attempted before. The aim of this work was the design of P-gp inhibitors which would also behave as cancer cell growth inhibitors.

Thioxanthones are S-heterocycles with a dibenzo-γ-thiopyrone scaffold and they are an important class of molecules showing interesting biological properties, namely antitumor activity [14,15]. Also, dibenzo-γ-pyrones (xanthones) have already been described as potential P-gp inhibitors, binding in the ATP-binding site [16]. Therefore, thioxanthones combine structural features that make them promising as dual ligands.

The first thioxanthones described as potential antitumor agents were hycanthone (**1**) [17] and lucanthone (**2**) [18], used in therapeutics as antischistosomal agents (Fig. 1). However, mutagenicity [19], probably resulting from the methylene moiety directly linked to C-4, was reported as a major drawback, leading to their withdraw [20]. Later, SR233377 (**3**) [15,21] and SSR271425 (**4**) [14,22,23] (Fig. 1) were tested in clinical trials as antitumor agents but showed cardiotoxicity. In contrast, for the photoinitiators 2-isopropyl-9*H*-thioxanthen-9-one (ITX, **5**) [24], and 1-chloro-4-propoxy-9*H*-thioxanthen-9-one (**6**), *in vivo* genotoxicity studies do not indicate a genotoxic potential, and compound **6** was considered not to be a dangerous substance according to GHS and to Directive 67/548/EEC [25]. Hence, to continue the search for new potential anticancer drugs with a thioxanthonic scaffold, we considered compound **6** to be a suitable chemical substrate and we introduced an amine side chain to pursuit a hybridization approach.

In this work, computational studies, synthesis and subsequent investigation of cell growth inhibitory effects and P-gp modulation for a series of new thioxanthonic derivatives are described, as schematized in Fig. 2. A virtual library of molecules was built by merging a thioxanthonic scaffold with P-gp inhibitors' pharmacophoric features, i.e., a planar system of rings (thioxanthonic scaffold) and a ionizable group such as an amine [26] (Fig. 2I). Those thioxanthonic derivatives were docked into P-gp homology models (Fig. 2II); the molecules with the best docking scores were then synthesized (Fig. 2III) and their P-gp inhibition and cell growth inhibitory effects experimentally assessed using several techniques (Fig. 2IV–VIII).

2. Material and methods

2.1. Preparation of a library of virtual thioxanthones and known P-gp inhibitors/ligands

About one thousand thioxanthones, 22 known P-gp inhibitors from the 1st, 2nd, and 3rd generation, thirty-six flavonoids that are known to bind the P-gp ATP-binding site [27] (Supplementary data, Annex B), were drawn and minimized using AM1 semi-empirical method, with a gradient energy minimization method until the energy change between steps was lower than 0.01 kcal mol^{-1} (Hyperchem, Hypercube, USA). The algorithm used was the Polak-Ribiere (conjugate gradient).

2.2. Docking of virtual thioxanthones

Virtual screening was carried out on a commodity PC running Linux Ubuntu 6.06. The software eHiTS from SimBioSys Inc. [28,29] was used for active site detection and docking. Docking was performed on P-gp models built using Sav1866 as template (Supplementary data, Annex A). No special preparation of the 3D structures was carried out because eHiTS automatically evaluates

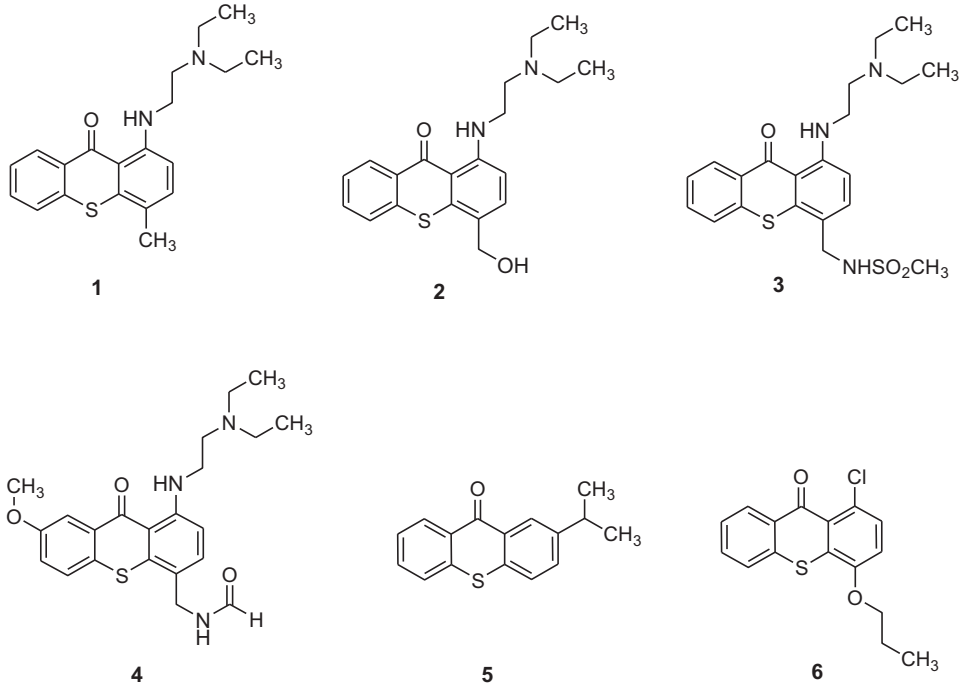


Fig. 1. First thioxanthones described as potential antitumor agents: hycanthone (**1**) and lucanthone (**2**), SR233377 (**3**) and SSR271425 (**4**); thioxanthones used safely as photoinitiators: 2-isopropyl-9*H*-thioxanthen-9-one (**5**) and 1-chloro-4-propoxy-9*H*-thioxanthen-9-one (**6**).

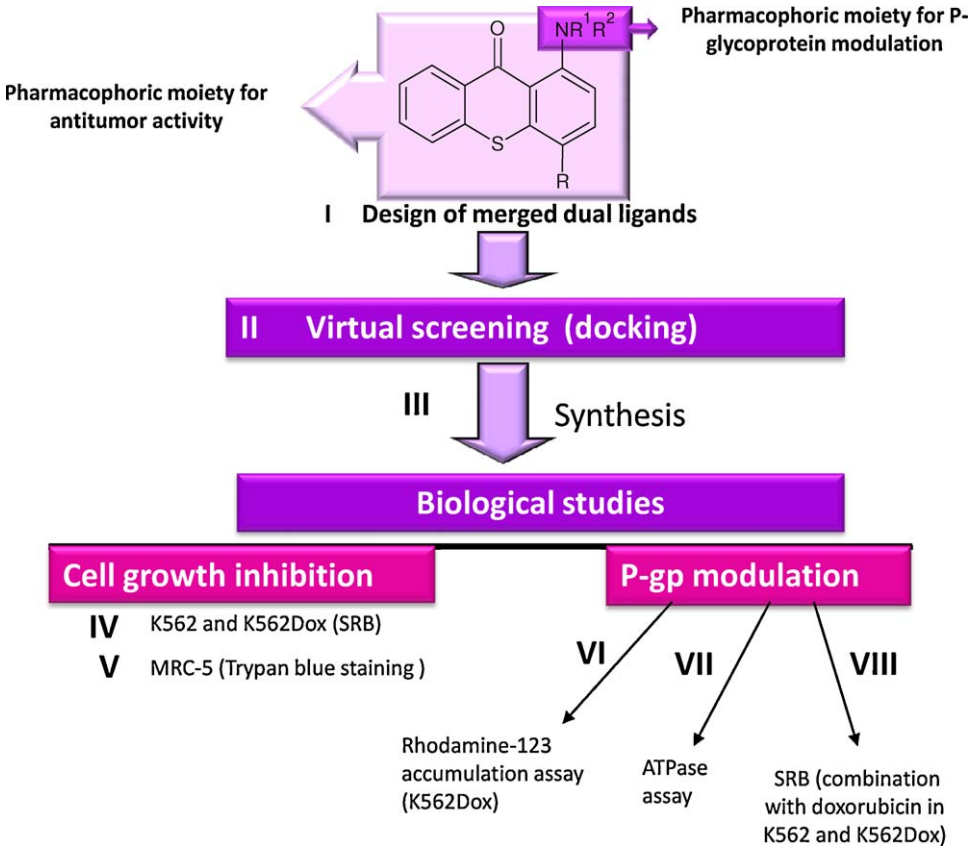


Fig. 2. Schematic representation of cheminformatic and laboratorial procedures. (I) Dual ligands design by merging a thioxanthonic scaffold with an amine. (II) Docking studies. (III) Synthesis. (IV) Sulphorhodamine-B assay (SRB) for investigation of tumor cell growth inhibition in K562 and K562Dox cell lines. (V) Trypan blue exclusion assay for investigation of cellular viability in a fibroblast cell line (MRC-5). (VI) Rhodamine-123 cellular accumulation assay and (VII) ATPase assay for P-gp inhibition screening. (VIII) SRB assay in cells treated with a combination of doxorubicin and the test compound.

all of the possible protonation states for ligands and receptor. eHiTS ran using the residues at: (i) the ATP-binding site on nucleotide-binding domains (NBD) and (ii) the drug binding pocket on the interface of the TMD as clip files. The docking is performed, by default, within a 7 Å margin around those residues. The input files of the molecules (virtually designed thioxanthones) were in *mol* format. The docking accuracy was set to 2, the number of docking poses was set to 5, and *sdf* was chosen as the output file format. Open Babel [30] was used to manipulate the various file formats of ligands. PyMol from DeLano [31] and Chimera from UCSF [32] were used for visual inspection of results and graphical representations.

2.3. Synthesis

1-Chloro-4-propoxy-9*H*-thioxanthen-9-one (**6**) and amines (**7**–**28**) were purchased from Sigma–Aldrich (Spain). The solvents used were products pro analysis or HPLC grade from Sigma–Aldrich and Fluka. Microwave reactions were performed using a MicroSYNTH 1600 from Millestone (ThermoUnicam, Portugal) synthesizer in sealed reaction vessels (10 mL). The synthetic procedures and spectral data are detailed in Supplementary data D. Purifications of compounds were performed by flash chromatography using Merck silica gel 60 (0.040–0.063 mm), liquid–liquid extraction (NMP:water and diethyl ether system) and preparative thin layer chromatography (TLC) using Merck silica gel 60 (GF_{254}) plates (Darmstadt, Germany). Reaction progression were controlled by TLC performed using silica plates HF_{254} (l = 0.2 mm) (Merck, Darmstadt, Germany) (ethyl acetate:acetone:TEA 80:20:1 or 90:10:1). Compounds were visually detected on visible light, by absorbance at 254 and/or 365 nm. Melting points were obtained in a Köfler microscope. IR spectra were measured on an ATI Mattson Genesis series FTIR spectrophotometer (Dekalb, IL, USA), software WinFirst v.2.10 (Madison, WI, USA) in KBr microplates (cm^{-1}). ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker Avance 300 instrument (300.13 MHz for ^1H and 75.47 MHz for ^{13}C) (Blue lion biotech, Snoqualmie, WA, USA). Chemical shifts are expressed in (ppm) values relative to tetramethylsilane (TMS) as an internal reference (Supplementary data, Annex E). Elemental analysis results were obtained in the services of C.A.C.T.I., Vigo, Spain. The purity of each compound was determined by HPLC-DAD analysis. All tested compounds, whether synthesized or purchased, possessed a purity of at least 95%. The compounds were synthesized and purified by the described procedures (in Supplementary data D).

2.4. HPLC chromatographic conditions

The HPLC analysis was performed in a Finnigan Surveyor–Autosampler Plus and LC Pump Plus (Thermo Electron Corporation, Waltham, MA, USA), equipped with a diode array detector TSP UV6000LP, and using a C-18 column (5 μm, 250 mm × 4.6 mm I.D.), from Macherey-Nagel (Deuren, Germany). Xcalibur2.0 SUR 1 software (Thermo Electron Corporation, Waltham, MA, USA) managed chromatographic data. Acetonitrile was of HPLC grade from Merck. HPLC ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA). The mobile phases were degassed for 15 min in an ultrasonic bath before use. It is used as an isocratic elution of MeOH:H_2O basified with TEA (1%) or acidified with CH_3COOH (1%) at a constant flow rate of 1.0 mL min^{-1}.

2.5. Biological activity

2.5.1. Cell lines

K562 (human chronic myelogenous leukemia, erythroblastic; ECACC, Europe Collection of Cell Cultures, UK), K562Dox (derived from K562 by doxorubicin stimulated overexpression of P-gp; kind gift from Prof. J.P. Marie, Paris, France), and MRC-5 (human lung fibroblasts which undergo between 60 and 70 doublings before senescence) cell lines were routinely maintained in RPMI-1640 (with Hepes and Glutamax, Gibco®, Invitrogen, Germany), with 10% or 5% fetal bovine serum (FBS, Gibco®, Invitrogen, Germany) and incubated in a humidified incubator at 37 °C with 5% CO_2 in air. All experiments were performed with cells in exponential growth, with viabilities over 90% and repeated at least three times. K562Dox cells were maintained by treating them with 1.0 μM doxorubicin every two weeks in order to maintain the P-gp overexpression, and all experiments were performed at least four days after this administration and in doxorubicin-free medium.

2.5.2. Compounds

SRB and rh123 were obtained from Sigma Aldrich. Verapamil, quinidine and mibefradil (known P-gp inhibitors) were used as controls (Sigma Aldrich, Spain).

2.5.3. Flow cytometry determination of rhodamine-123 accumulation

K562 andK562Dox (5×10^6 cells mL^{-1}) were incubated for 1 h at 37 °C in the presence of 10 or 20 μM of the test compounds, and with 1 μM rh123. K562Dox and K562 cells alone as well as K562Dox cells in the presence of the known P-gp inhibitors verapamil, mibefradil, quinidine and hycanthone (**2**) [33] (10 and 20 μM) were used as controls. After the incubation time, cells were washed twice, re-suspended in ice cold PBS and kept at 4 °C in the dark until analysis in the flow cytometer. At least 20,000 cells per sample were counted and analysed by flow cytometry (Epics XL-MCL, Beckman Coulter, USA). Cells shown in forward scatter and side scatter were electronically gated and acquired through the FL1 channel. The amount of fluorescence was plotted as a histogram of FL1 within the gate. Data acquisition was performed using WINMDI 2.9 (TSRI, USA) to determine median fluorescence intensity values (MFI). For simple interpretation, the ratio of rh123 accumulation was calculated as $(MFI_{K562Dox+Drug} - MFI_{K562Dox})/MFI_{K562Dox}$. Results represent the average of at least three independent experiments.

2.5.4. Determination of ATPase activity

The ATPase activity of P-gp was determined using the luminescent ATP detection kit (P-gp-Glo Assay Kit, Promega, Germany) according to the manufacturers' recommendation [34]. Test compounds at 200 μM or sodium vanadate at 20 μM (positive control, noncompetitive inhibitor) or verapamil, mibefradil, and quinidine at 200 μM (positive controls, competitive inhibitors) in buffer solution were incubated with 0.5 mg mL^{-1} P-gp and 5 mM MgATP at 37 °C for exactly 40 min, and the remaining ATP was detected after 20 min resting at room temperature, as a luciferase-generated luminescent signal. Results are presented as the average of three independent experiments. % Values of RLU (relative light unit) was calculated in relation to non-treated control (NT). It was calculated using the following formula that fits the NT control to zero for easier interpretation: $\% \text{ RLU} = (RLU_{test} - RLU_{NT})/RLU_{NT} \times 100$.

2.5.5. Sulphorhodamine-B assay

K562 and K562Dox cells were plated into 96-well tissue culture plates at 5×10^4 cells mL^{-1}. After 24 h, cells were treated with serial dilutions of the test compound. Following 48 h treatment, cell growth was assayed using the SRB assay. The GI_{50} values for the thioxanthonic derivatives (concentration resulting in 50% inhibition of cell growth) were calculated from the plotted results.

The ability of the tested compounds to decrease doxorubicin GI_{50} was evaluated in K562 and K562Dox cells by SRB assay, as previously described [33]. K562 and K562Dox cells were plated into 96-well tissue culture plates at 5×10^4 cells mL^{-1}. After 24 h, cells were treated with serial dilutions of doxorubicin in combination with 1 or 10 μM of the test compound. Following 48 h treatment, cell growth was assayed using the SRB assay. The GI_{50} values for doxorubicin (concentration resulting in 50% inhibition of cell growth) were calculated from the plotted results. The ratio of doxorubicin GI_{50} decrease was obtained by calculating the GI_{50} of doxorubicin alone/GI_{50} of doxorubicin in the presence of the tested compound.

2.5.6. Viable cell number by trypan blue exclusion assay

Adherent MRC-5 cells were plated (2×10^5 cells mL^{-1} in 96-well plates). 24 h after plating, cells were treated with complete medium (blank), with the solvent of the compounds (DMSO, control), or with the following compounds: **30**, **33**, **37**, **38**, **41**, **43**, **45**, and **48** in a concentration corresponding to their GI_{50} on K562 cell line. Viable cell number was assessed following 48 and 72 h of incubation with the compounds using the trypan blue exclusion assay (results not shown).

2.5.7. Statistical analysis

Data was expressed as the mean ± SE and analysed by the Student's *t*-test. *P*-values below 0.05 were considered statistically significant.

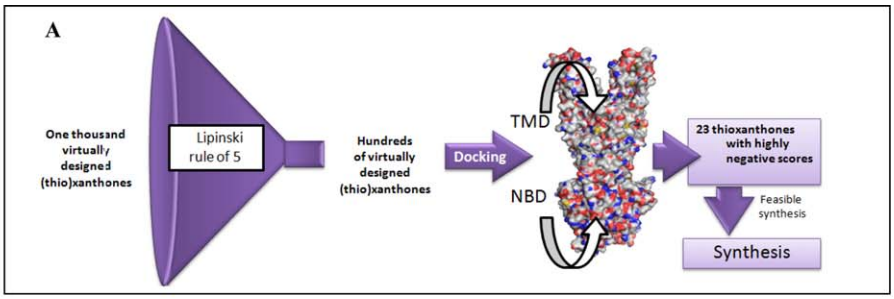
3. Results

3.1. Design and docking of thioxanthones

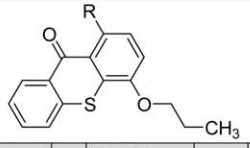
Docking was performed using a dataset of approximately 1000 virtual new aminated thioxanthones (Table 1A) and two P-gp models constructed using the homologous Sav1866 from *Staphylococcus aureus* (Supplementary data, Annex A) as template. The designed molecules resulted from a merging strategy for dual ligands [8,9] (represented in Fig. 2I) between a thioxanthonic scaffold, associated to antitumor activity [15,22,23], with an 4-alkoxyl or 4-hydroxyl group; and an amine group at C-1, associated to P-gp inhibitory activity [26,35]. The introduction of an amine side chain, namely an aminoalkylamino group, on the thioxanthonic nucleus seems to be also an important feature for the expression of cytotoxicity [36–38]. Thus, amine structures [39,40] such as the *N*,*N*-diethylethane-1,2-diamine, present in thioxanthones SR233377 (**3**) and SR271425 (**4**), and in several DNA intercalating agents, were also used in the design of 1-aminated thioxanthonic derivative. Additionally, the presence of an amine in these derivatives allows the formation of salts, which improves compound water solubility [41].

Computational filters were applied to the initial library of aminated thioxanthones, in order to select the molecules that complied with the Lipinski rules of five [42], and other features described as important for P-gp inhibition, such as log *P* value of at least 2.92 or higher [26,35] (Table 1A). The molecules that respected these cutoffs were docked into the drug binding pocket formed by the interface between TMD1 and TMD2 and in the ATP-binding site located on the NBD of P-gp. Twenty-three aminated thioxanthones with the best docking scores (on NBD or TMD) (Table 1B and C) and with a rapid synthetic protocol, were selected to be synthesized, starting from thioxanthone **6** and amines **7**–**28**. These molecules were selected based on the following cut-offs: molecules with NBD docking scores lower than −3.0 kJ mol^{-1}, and/

Table 1
(A) Scheme of the virtual screening protocol; best scoring virtually designed thioxanthones with (B) a 4-propoxyl or (C) a 4-hydroxyl substituent at C-4.



B



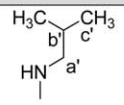
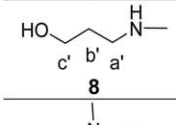
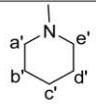
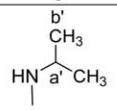
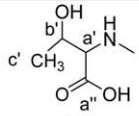
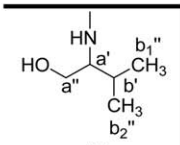
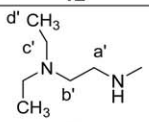
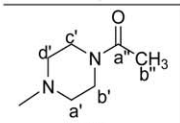
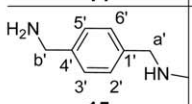
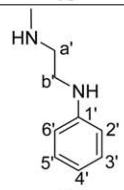
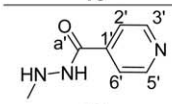
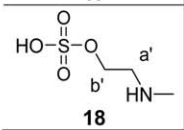
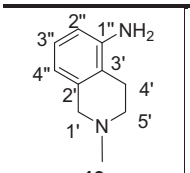
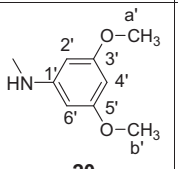
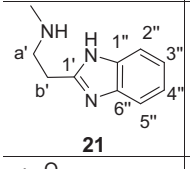
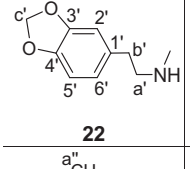
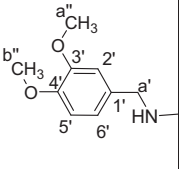
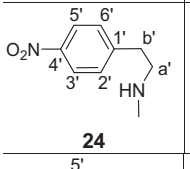
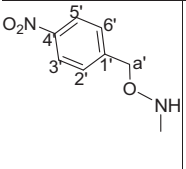
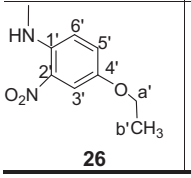
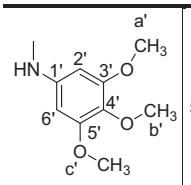
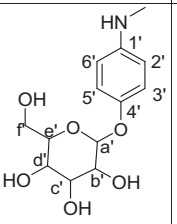
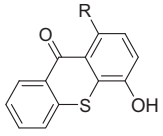
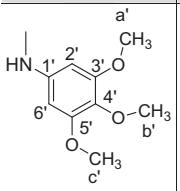
Amine (R)	Compound	Docking scores on a) NBD b)TMD (kJ mol^{-1})	MW	LogP
 7	31	a) -2.666 b) -2.012	327	5.64
 8	32	a) -4.625 b) 0.44	343	3.71
 9	33	a) -3.246 b) 0.005	353	5.45
 10	34	a) -2.998 b) -2.212	357	5.19
 11	35	a) -3.789 b) -1.135	371	3.77
 12	36	a) -4.177 b) -3.933	371	4.87
 13	37	a) -3.383 b) -1.482	384	5.02
 14	38	a) -3.328 b) -1.431	396	3.76
 15	39	a) -5.236 b) -0.536	404	4.88
 16	40	a) -4.629 b) 0.94	404	5.64
 17	41	-3.252 -1.106	405	3.87
 18	42	a) -4.335 b) -1.568	409	3.07

Table 1 (*Continued*)

 19	**43**	a) -3.755 b) -3.52	416	5.45
 20	**44**	a) -4.267 b) -2.638	421	5.90
 21	**45**	a) -4.981 b) 0.317	429	4.15
 22	**46**	a) -5.084 b) -0.324	433	5.86
 23	**47**	a) -3.217 b) -1.214	435	5.74
 24	**48**	a) -5.184 b) -1.017	436	5.11
 25	**49**	a) -5.953 b) -1.505	438	5.03
 26	**50**	a) -3.428 b) -2.19	450	4.85
 27	**51**	a) -3.644 b) 1.169	451	5.88
 28	**52**	a) -5.441 b) -1.972	539	3.76

C 

Amine (R)	Compound	Docking scores on a)NBD b)TMD (kJ mol^{-1})	MW	LogP
 27	**55**	a) -2.493 b) -2.554	409	3.86

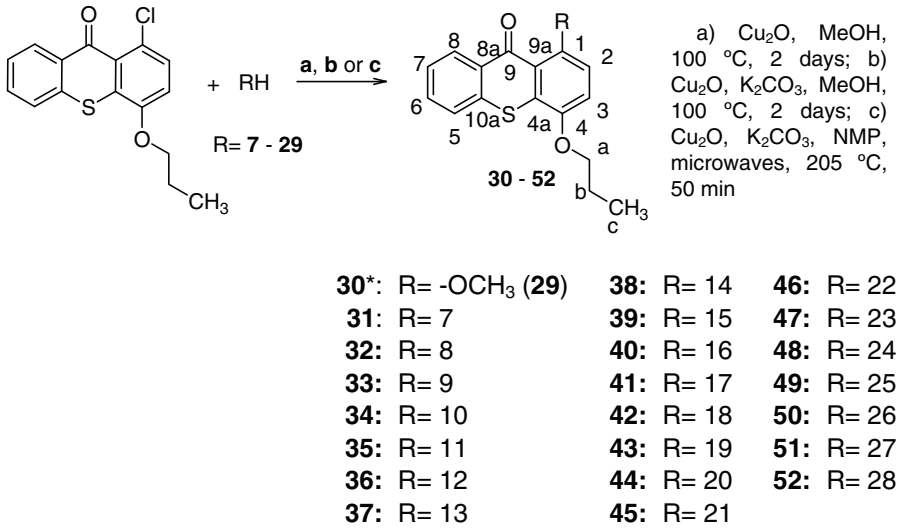
LogP= logarithm of the partition coefficient; MW= molecular weight

or with TMD docking score lower than -2.0 kJ mol^{-1} (see examples of discarded molecules in Supplementary data, Annex C).

3.2. Synthesis

Thioxanthones **31**–**52** (Scheme 1) were synthesized by reaction between 1-chloro-4-propoxy-9H-thioxanthen-9-one (**6**) and a primary or a secondary amine (**7**–**28**, Table 1) by C(1)-N Ullmann cross coupling.

The synthesis of aminated thioxanthones was initially investigated by a classical copper mediated cross-coupling reaction, using amine in excess (two equivalents of amine, **7**–**28**) and thioxanthone **6** as limitating reagent, Cu_2O [43] in catalytic amounts (5% equiv.) at 100 °C in a closed vessel for 1–2 days (Scheme 1, procedure a). However, the reaction was only successful for simple aliphatic amines (such as **8**, with a yield of 30%). Traditional copper-mediated Ullmann couplings generally have low functional group tolerance and irreproducible results [44,45], and require harsh reaction conditions, such as high temperatures and the use of strong bases [46]. The addition of K_2CO_3 (Scheme 1, procedure b) provided a alkaline media and allowed the synthesis of **36** with a 25% yield and of **47** with a 75% yield. As microwave-promoted



Scheme 1. Copper-catalyzed nucleophilic aromatic substitution of thioxanthones. *Obtained as a secondary product by reaction of **6** with methanol (procedure a).

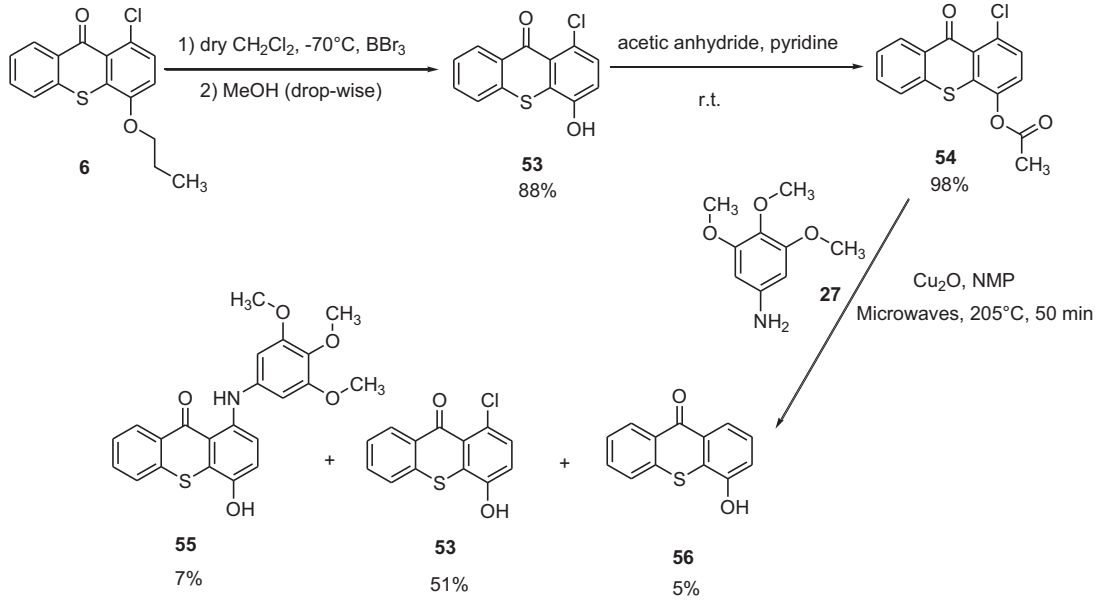
synthesis is an area of crescent interest in N–C cross-coupling reaction [47], the heating source was also modified (Fig. 2, procedure c) [48,49]. Additionally, the solvent was changed to *N*-methyl-pyrrolidone (NMP), an aprotic, alkaline solvent, more appropriate for microwave heating. In some cases, a mixture of NMP:water [50,51] was used in order to improve the solubility of the alkaline catalyst and the amine, allowing the reaction to proceed (amines **11**, **21**, and **28**). Under these conditions, the amination reaction worked not only for alkylamines (e.g. **12** and **13**) but also for cyclic amines (e.g. **14** and **19**) and aromatic amines (e.g. **20**, **26**, and **27**) and allowed to obtain the desirable aminated thioxanthones (**36**–**38**, **43**, **44**, **50**, and **51**, respectively).

To obtain compound **55**, a dealkylation of **6** with boron bromide, BBr_3 [52] followed by an acetylation was performed (Scheme 2). Reaction of compound **54** with 3,4,5-trimethoxyaniline (**27**) furnished directly the desirable 4-hydroxy-1-aminated derivative **55** along with compounds **53** and **56** (Scheme 2).

In summary, twenty-three new aminated thioxanthones predicted from the docking study to be potential P-gp modulating agents were synthesized in reasonable yields to perform biological assays. Other thioxanthonic derivatives obtained were also investigated in the biological assays (**30**, **53**, **54**, and **56**). The structure elucidation (Supplementary data, Annexes D and E) of the synthesized compounds was based on spectral data (IR, ^{1}H NMR, ^{13}C NMR, HMBC, and HSQC) and on elemental analysis.

3.3. Flow cytometry rhodamine-123 assay

An indirect way to analyse P-gp activity is by the evaluation of the mean fluorescence intensity (MFI) of cells exposed to rhodamine-123 (rh123), a known P-gp fluorescent substrate, together with the potential P-gp inhibitor [53–55]. Quantification of the relative fluorescence accumulation can be used as an indirect way to study the individual potency of inhibitors [56]. This flow cytometry method for P-gp functional evaluation has been used for decades [57,58] and was chosen to initiate the present study. However, rh123 is a substrate not only for P-gp but also for MRP [59] and expression of both P-gp and MRP has been reported to



Scheme 2. Synthesis of compound **55**.

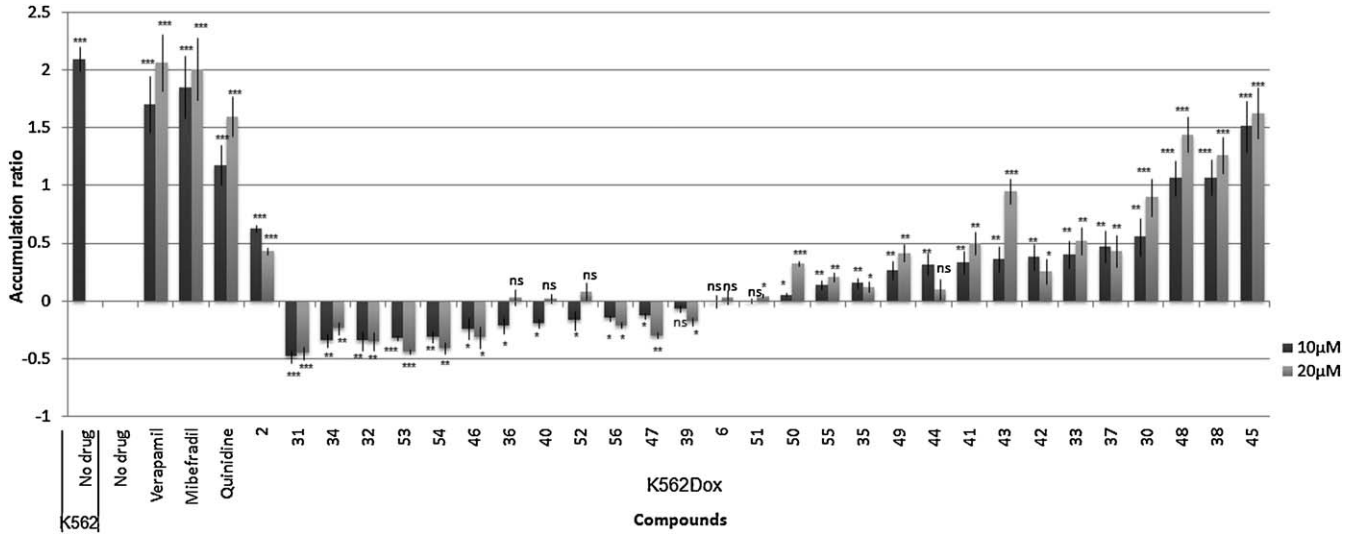


Fig. 3. Relative accumulation ratios of rh123 after 1 h of incubation in K562 or K562Dox cell lines. K562Dox alone (no drug) is represented as zero for easier interpretation. Accumulation ratio superior to zero correspond to rh123 accumulation superior to K562Dox, i.e., potential P-gp inhibitors. Results are the mean ± SE of three independent experiments. Results are the average of three independent experiments ± SE. Statistical significance was tested by paired *t*-test using the untreated K562Dox (second entry) as control. *** Indicates $P < 0.001$; ** indicates $0.001 < P \leq 0.01$; * indicates $0.01 < P \leq 0.05$; ns indicates not significant, i.e., $P > 0.05$ ($n = 3$ independent experiments).

occur in leukemic cells [60]. We have previously failed to detect MRP-1 by Western Blot in either K562 or K562Dox cell lines, whereas we confirmed that P-gp is expressed in K562Dox cell line but not in K562 cell line [33]. The results from the effect of the test compounds **2**, **6**, **30**–**56** in the cellular accumulation of rh123 are shown in Fig. 3.

After K562 cells were incubated with rh123, accumulation ratio increased when compared with K562Dox, as it was expected for a cell line which does not express P-gp (Fig. 3). Untreated K562Dox cells rh123 accumulation was considered residual (Fig. 3). Verapamil, quinidine and mibefradil, known first-generation P-gp inhibitors [61], and hycanthone (**2**), were used in this study as controls. As expected, they caused a rh123 accumulation on K562Dox cells similar to the one observed on the sensitive cell line, K562 (Fig. 3). After K562Dox cells were incubated in the presence of the test compounds (**2**, **6**, **30**–**56**), the accumulation ratios varied. In the case of fifteen of the investigated compounds (compounds **30**, **33**, **35**, **37**, **38**, **41**–**45**, **48**–**51**, and **55**, Fig. 3), together with the controls (verapamil, quinidine, mibefradil and compound **2**), an increase in the accumulation ratio of rh123 was observed, an effect compatible with P-gp inhibition. Particularly, 1-[2-(1*H*-benzimidazol-2-yl)ethanamine]-4-propoxy-9*H*-thioxanthen-9-one (**45**), 1-(4-acetylpiperazin-1-yl)-4-propoxy-9*H*-thioxanthen-9-one (**38**) and 1-{[2-(4-nitrophenyl)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**48**) showed an effect similar to that elicited by the known P-gp inhibitor, quinidine, and higher than the known thioxanthone hycanthone (**2**). A dose-dependent response was clearly observed for compound **48**, whereas there was no clear evidence of a dose-dependent response for compounds **38** and **45**. In contrast, twelve derivatives (compounds **31**, **32**, **34**, **36**, **39**, **40**, **46**, **47**, **52**–**54**, and **56**, Fig. 3) showed a significant decrease in the accumulation ratio, an effect compatible with P-gp activation. Although the building block **6** had no effect on P-gp function (Fig. 3), the side product **30** revealed an interesting rh123 accumulation rate.

3.4. P-gp ATPase assay

The rate of P-gp ATP hydrolysis allows to discriminate between compounds that increase the ATP consumption by P-gp (i.e., competitive inhibitors or substrates) and compounds that reduce the use of ATP either by acting directly on ATP-binding site or indirectly by blocking an allosteric site relevant for P-gp activity (i.e., noncompetitive inhibitors) [62,63]. Therefore, to elucidate if the thioxanthonic derivatives that caused accumulation of rh123 were either competitive inhibitors (substrates for transport by P-gp), or P-gp noncompetitive inhibitors, effects on the ATPase activity of P-gp were measured using human P-gp membranes [33,64]. The results for the fifteen potential P-gp inhibitors (compounds **30**, **33**, **35**, **37**, **38**, **41**–**45**, **48**–**51**, and **55**) along with the building block **6** and four controls (sodium orthovanadate, verapamil, mibefradil and quinidine) are shown in Fig. 4.

Sodium orthovanadate is an inhibitor of P-gp ATPase activity [65] and as expected caused significant increase in % relative light unit (% RLU), whereas verapamil, mibefradil and quinidine are P-gp substrates and competitive inhibitors, showing decreased % RLU (Fig. 4).

Compounds **30**, **33**, **35**, **38**, **41**, **42**, **45**,and **48**–**51** caused a significant increase in the % RLU in relation to the untreated (NT), i.e., they were found to be P-gp ATPase inhibitors (noncompetitive P-gp inhibitors). They may prevent ATP hydrolysis by targeting the P-gp ATP binding site directly [16,66] or by binding to an allosteric residue [67,68] which causes inhibition of P-gp function with resulting inhibition of ATPase activity. Compounds such as **37**, **43**, **44**, and **55** behave similarly to verapamil, mibefradil, and quinidine, i.e., they increased the hydrolysis of ATP through P-gp, indicating that they are competitive inhibitors of P-gp, possibly being themselves transported by the pump. In summary, from the sixteen compounds that had caused accumulation of rh123 in the cells, four are acting as competitive and eleven as noncompetitive inhibitors of P-gp (docking poses of two of the best P-gp inhibitors, **43** and **45**, are described on Supplementary data, Annex F). Furthermore, there was a correlation between docking scores on NBD and experimental results for noncompetitive ATPase inhibitors, as well as between docking scores on TMD and experimental results for competitive ATPase inhibitors (Supplementary data, Annex G).

3.5. Growth inhibition assay

The cell growth inhibitory effect of twenty seven thioxanthones (**30**–**56**) was examined in the K562 and K562Dox cell lines by the sulphorhodamine-B assay (SRB) and results are presented in Table 2A and B, respectively. On K562 cell line, the GI_{50} values for

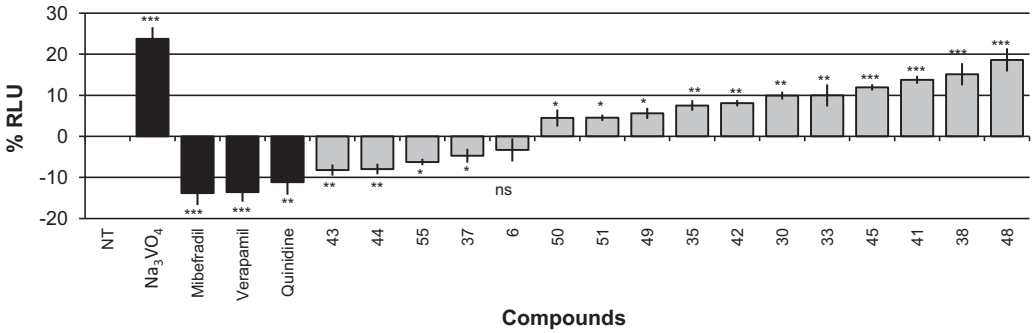


Fig. 4. P-gp ATPase assay. Results are % of RLU (relative light unit) in relation to non-treated (NT) control. NT control luminescence is represented as zero for easier interpretation. % Values of RLU superior to zero correspond to inhibitors of P-gp ATPase, i.e., noncompetitive P-gp inhibitors. % Values of RLU inferior to zero correspond to substrates of P-gp, i.e., competitive P-gp inhibitors. Results are the mean ± SE of three independent experiments. Na_3VO_4, verapamil, mibefradil, and quinidine were used as controls. Statistical significance was tested by paired *t*-test using the NT-control as a negative control. *** Indicates $P < 0.001$; ** indicates $0.001 < P \leq 0.01$; * indicates $0.01 < P \leq 0.05$; ns indicates not significant, i.e., $P > 0.05$ ($n = 3$).

Table 2
Cytotoxic effects of thioxanthones **30**–**56** on sensitive K562 (A) and resistant K562Dox cells (B).

(A)

Compound	GI_{50} (K562) (μM)
37	1.90 ± 0.15
43	3.00 ± 0.48
33	3.72 ± 1.47
55	4.38 ± 0.44
30	4.47 ± 1.93
41	4.81 ± 4.21
50	12.98 ± 0.36
54	13.57 ± 2.96
51	15.57 ± 3.15
35	16.22 ± 0.48
39	16.50 ± 3.06
45	16.99 ± 2.33
49	18.13 ± 4.35
44	19.23 ± 0.98
48	20.96 ± 2.08
36	21.47 ± 2.61
53	22.73 ± 0.64
38	29.79 ± 3.02
34	52.95 ± 1.47
52	59.45 ± 2.77
42	60.58 ± 2.01
56	74.32 ± 7.16
46	92.92 ± 3.33
47	104.71 ± 7.29
31	>150
32	>150
40	>150
Verapamil	>150
Doxorubicin	0.06 ± 0.01

(B)

Compound	GI_{50} (K562Dox) (μM)
37	1.95^{ns} ± 0.34
43	11.89^{*} ± 1.17
33	12.59^{*} ± 0.69
41	15.38^{*} ± 0.53
45	19.43^{ns} ± 0.76
30	19.60^{*} ± 2.73
38	37.31^{*} ± 2.08
48	41.56^{*} ± 5.48
Verapamil	>150
Doxorubicin	11.62^{*} ± 1.14

Results are mean ± SE of at least three independent experiments, except for compounds with $GI_{50} > 150$ μM (two independent experiments).

* Values of K562Dox GI_{50} are statistically different ($P < 0.05$) from the K562 GI_{50}.

ns: values of K562Dox GI_{50} are not statistically different ($P > 0.05$) from the K562 GI_{50}.

twenty-four of the compounds (**30**, **33**–**39**, **41**–**56**) were in the range 1.9–104.7 μM (Table 2A). Six compounds showed a $GI_{50} < 10$ μM (**30**, **33**, **37**, **41**, **43**, **55**). The most potent cell growth inhibitor thioxanthone was 1-{[2-(diethylamino)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**37**), with a GI_{50} of 1.9 μM. Eight of the thioxanthones that presented the best P-gp and cell growth inhibitory activity (**30**, **33**, **37**, **38**, **41**, **43**, **45**, and **48**) were further investigated on the K562Dox cell line (Table 2B). As expected, the GI_{50} values in K562Dox cell line were generally higher than in the K562 cells. Six of the compounds investigated on K562Dox cell line showed a GI_{50} value < 20 μM (**30**, **33**, **37**, **41**, **43**, and **45**), indicating that they might have both P-gp and cell growth inhibitory activity. Noteworthy, compound **37** was 9-fold more potent than doxorubicin in this cell line.

The growth inhibitory effect of the thioxanthonic derivatives was also assayed on a nontumor cell line model, MRC-5. There was no significant decrease in viable cell number of cells treated with compounds **30**, **33**, **37**, **38**, **41**, **43**, **45**, and **48**, when compared to DMSO solvent control, 48 and 72 h after treatment (results not shown).

To investigate the ability of these newly designed dual inhibitors to reverse the MDR phenotype, a doxorubicin sensitization assay in the P-gp overexpressing cell line (K562Dox) was performed (Table 3A). To understand if the growth inhibitory effect on this cell line was due to their dual actions, a sensitization assay was also performed on K562 cell line (Table 3B). We hypothesized that if a compound was able to decrease doxorubicin GI_{50} in K562Dox, without decreasing doxorubicin GI_{50} in K562 cell line, then its effect should be mainly due to P-gp inhibition. On the other hand, if a compound decreases doxorubicin GI_{50} on both K562 and K562Dox cell lines, then its effect should be due to both cell growth inhibitory activity and P-gp inhibition. Results of the SRB assay on cells treated with a combination of the thioxanthonic derivative (**30**, **33**, **37**, **38**, **41**, **43**, **45**, or **48**) and doxorubicin are presented in Table 3. Two concentrations of the thioxanthonic derivatives were used, 1 and 10 μM, which are below the GI_{50} of the compounds tested alone.

At 10 μM, verapamil caused a 5.9-fold decrease in doxorubicin GI_{50} on K562Dox cell line. At the same concentration, compound **45** decreased the doxorubicin GI_{50} by 12.6-fold, followed by compound **38** with a 8.9-fold decrease, and by compound **48** with a 7.5-fold decrease (Table 3A). Compound **45** at 10 μM was approximately 2-fold more potent than verapamil, and compounds **38** and **48** were approximately 1.5-fold more potent than verapamil, probably due to a dual activity as P-gp and cell growth inhibitors, since these compounds also reduced doxorubicin GI_{50} in the sensitive K562 cell line (Table 3B).

Table 3
Cytotoxic effects of doxorubicin in combination with newly synthesized thioxanthones (at 1 or 10 μM) on resistant K562Dox (A) and sensitive K562 cells (B).

	(A) K562Dox		(B)K562	
	GI_{50} (μM)	Ratio of doxorubicin GI_{50} decrease	GI_{50} (nM)	Ratio of doxorubicin GI_{50} decrease
Doxorubicin (Dox)	11.62 ± 1.14	–	61.67 ± 1.27	–
Dox + 10 μM Verapamil	$1.97 \pm 0.03^{***}$	5.90	$55.76 \pm 1.12^{*}$	1.11
Dox + 10 μM **45**	$0.92 \pm 0.16^{***}$	12.6	$15.89 \pm 0.6^{***}$	3.88
Dox + 10 μM **38**	$1.30 \pm 0.25^{***}$	8.94	$6.34 \pm 0.02^{***}$	9.73
Dox + 10 μM **48**	$1.56 \pm 0.16^{***}$	7.45	$9.65 \pm 1.07^{***}$	6.39
Dox + 1 μM Verapamil	$4.46 \pm 0.21^{**}$	2.61	58.45 ± 1.17^{ns}	1.05
Dox + 1 μM **37**	$3.15 \pm 0.32^{**}$	3.69	$4.71 \pm 0.40^{***}$	13.09
Dox + 1 μM **45**	$7.17 \pm 0.20^{*}$	1.62	58.31 ± 0.9^{ns}	1.06
Dox + 1 μM **48**	$7.36 \pm 0.16^{*}$	1.58	57.16 ± 1.80^{ns}	1.08
Dox + 1 μM **38**	$8.50 \pm 0.33^{*}$	1.37	$51.84 \pm 0.52^{*}$	1.19
Dox + 1 μM **41**	$9.60 \pm 0.27^{*}$	1.21	$49.11 \pm 0.62^{**}$	1.26
Dox + 1 μM **43**	$9.64 \pm 0.21^{*}$	1.21	$13.00 \pm 0.88^{***}$	4.74
Dox + 1 μM **33**	11.57 ± 0.37^{ns}	1.00	$16.37 \pm 1.00^{***}$	3.77
Dox + 1 μM **30**	11.70 ± 0.34^{ns}	0.99	$42.20 \pm 1.45^{**}$	1.46

The selected concentrations of test compounds are below the GI_{50} of the tested compounds on each cell line when tested alone. Statistical significance was tested by paired *t*-test using the doxorubicin alone GI_{50} of each cell line as the control. *** Indicates $P < 0.001$; ** indicates $0.001 < P \leq 0.01$; * indicates $0.01 < P \leq 0.05$. ns indicate not significant, i.e., $P > 0.05$ ($n = 3$ independent experiments). The ratio of doxorubicin GI_{50} decrease was calculated as follows: GI_{50} of doxorubicin alone/GI_{50} of doxorubicin in the presence of the test compound.

When compounds were tested at 1 μM, compounds **37**, **45**, **48**, and **38** were the ones which had a greater effect in reducing the GI_{50} of doxorubicin in the K562Dox cell line. In contrast, in K562 cell line, compounds **45** and **48** at 1 μM did not cause any alteration in the GI_{50} of doxorubicin, suggesting that the sensitization effect of those two compounds seen in the K562Dox cell line is due to their P-gp inhibitory activity. On the other hand, compound **37** at 1 μM caused a 13-fold decrease on doxorubicin GI_{50} on the K562 cell line ($GI_{50} = 4.71 \pm 0.40$ nM), suggesting that the effects of compound **37** are not only due to P-gp inhibition but also and mainly due to cell growth inhibition. Additionally, compound **38** at 1 μM caused a 1.2 fold-reversal on this cell line, which implies that, besides the P-gp inhibitory effect of this compound, which seems predominant, a significant antileukemic effect is also observed. Compounds **30**, **33**, and **43**, sensitized K562 cells more than K562Dox cells to the effect of doxorubicin, suggesting that their effect is mostly on cell growth.

4. Discussion

The synthesis of 1-aminated thioxanthones was performed by Ullman cross-coupling, using conventional or microwave heating. Both methods yielded the desirable compounds. The presence of a base was favorable and increased the yields of reaction. After purification and structural elucidation, a rh123 accumulation assay was performed in order to select the thioxanthones which were interfering with the efflux of rh123 by P-gp (Fig. 3). Noteworthy, the most potent compounds (potential P-gp inhibitors) present two nitrogen atoms (amine or nitro groups) separated by an alkyl (**37**, **38**) or alkyl-aryl chain (**45** and **48**).

From the sixteen compounds that had caused accumulation of rh123 in the cells, four are acting as competitive inhibitors (increased P-gp ATPase activity compared to non-treated control) and eleven as noncompetitive inhibitors of P-gp (decreased P-gp ATPase activity comparing to non-treated control). When comparing these results (Fig. 4) with the ones obtained on the rh123 accumulation assay (Fig. 3), general agreement between the outcomes of the two assays could be observed. Indeed, most of the compounds with the highest accumulation ratio (>1.0) in the rh123 accumulation assay, such as **30**, **38**, **45**, and **48** also showed a high activity in the ATPase assay. Nevertheless, compound **41**, which presented a high % value of RLU in the ATPase assay, showed an accumulation ratio of rh123 <0.5. This discrepancy in the activity of some compounds measured by two assays is probably due to the fact that the rh123 accumulation assay uses cells and the ATPase assay uses membrane fractions, and therefore, the latter does not suffer interferences by factors related to drug permeability [69,70].

The study of the cell growth inhibitory effect of twenty seven thioxanthones (**30**–**56**) revealed that six compounds present GI_{50} values < 10 μM (**30**, **33**, **37**, **41**, **43**, **55**) in the K562 cell line. In most cases, the GI_{50} values in the K562Dox cell line were higher than in the K562 cells. Nevertheless, the most potent cell growth inhibitor thioxanthone, 1-{[2-(diethylamino)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**37**), showed similar GI_{50} values (1.9 μM) in both tumor cell lines, being 9-fold more potent than doxorubicin in K562Dox cells. Indeed, compound **37** which is structurally similar to the known antitumor thioxanthones **1**–**4**, was shown in the present work to be a potent inhibitor of tumor cell growth. Further studies are needed in order to investigate the effect of compound **37** on cellular proliferation, cell cycle and apoptosis. Our future work will focus on these aspects as well as on the molecular mechanism of action of some of the most promising compounds, such as compound **37**. The possible mechanisms that will be investigated will include intercalation with DNA, as has been previously reported for the known lucanthone and hycanthone [71].

Concerning the capacity of MDR reversal of P-gp overexpressing cell line K562Dox, compound **45** at 10 μM was approximately 2-fold more potent than verapamil, and compounds **38** and **48** were approximately 1.5-fold more potent than verapamil (Table 3A), probably due to a dual activity as P-gp and cell growth inhibitors, since these compounds also reduced doxorubicin GI_{50} in the sensitive K562 cell line (Table 3B). In spite of the lower rh123 accumulation on K562Dox in comparison to verapamil (Fig. 3), compounds **38** and **48** at 10 μM succeed to sensitize resistant K562Dox cells due to its dual activity as P-gp and cell growth inhibitors.

These results suggest that the dual effect of some 1-aminated thioxanthones have a sensitization effect to a traditional chemotherapeutical agent such as doxorubicin, which might be a valuable strategy to explore further in the design of efficient dual activity molecules.

5. Conclusion

The rational merged scaffolds approach rendered a new class of dual activity agents (cell growth and P-gp inhibitors),

aminoalkylthioxanthones. We identified 1-{[2-(diethylamino) ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**37**) as a potent cell growth inhibitor. We also recognized 1-[2-(1*H*-benzimidazol-2-yl)ethanamine]-4-propoxy-9*H*-thioxanthen-9-one (**45**) and 1-{[2-(4-nitrophenyl)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one (**48**) as dual noncompetitive inhibitors of P-gp and inhibitors of cell growth, being more active than verapamil in sensitizing a P-gp overexpressing cell line to doxorubicin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.10.004.

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