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Cytotoxicity of cinchona alkaloid organocatalysts against MES-SA and MES-SA/Dx5 multidrug-resistant uterine sarcoma cell lines

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ABSTRACT

Since the first application of natural quinine as an anti-malarial drug, cinchona alkaloids and their derivatives have been exhaustively studied for their biological activity. In our work, we tested 13 cinchona alkaloid organocatalysts, synthesised from quinine. These derivatives were screened against MES-SA and Dx5 uterine sarcoma cell lines for in vitro anticancer activity and to investigate their potential to overcome P-glycoprotein (P-gp) mediated multidrug resistance (MDR). Decorating quinine with hydrogen-bond donor units, such as thiourea and (thio)squaramide, resulted in decreased half-maximal growth inhibition values on both cell lines (1.3–21 μM) compared to quinine and other cinchona alcohols (47–111 μM). Further cytotoxicity studies conducted in the presence of the P-gp inhibitor tariquidar indicated that several analogues, especially cinchona amines and squaramides, but not thiosquaramide, were expelled from MDR cells by P-gp. Similarly to the established P-gp inhibitor quinine, 6 cinchona analogues were shown to inhibit calcein-AM efflux. Interestingly, quinine and didehydroquinine exhibited a marginally increased toxicity against the multidrug resistant Dx5 cells. Collateral sensitivity of the MDR cell line was more pronounced when the cinchona thiosquaramide was complexed with Cu (II) acetate. Based on the results, cinchona derivatives are good anticancer candidates for further drug development.

1. Introduction

Other than being a well-known organocatalyst family,[1] cinchona alkaloids have been widely used in the pharmaceutical industry. Quinine was the first effective drug for the treatment of malaria,[2–3] moreover, quinine and its derivatives have also been investigated as antiarrhythmic,[4] antiviral,[5–6] and antimicrobial agents,[7] and their cytotoxic activities were also confirmed in several cancer cell lines (KB, HeLa, MCF-7, A-549, Hep-G2, U-87, HL-60, HGC-27, etc.).[8–9] Numerous attempts have been made to develop analogues with higher biological activity. For example, compounds gained by dimerisation of quinine *via* ester bonds,[10] or functionalisation of quinine *via* copper-catalysed azide–alkyne cycloaddition (CuAAC)[11–14] exerted increased toxicity to both *Plasmodium falciparum* and cancer cells. However, conjugation of cinchona alkaloids with cytotoxic molecules such as salinomycin or monesin reduced the activity of these agents.[15] In a recent study, hydrogen-bond donor quinine-based thioureas and squaramides have also proved to be excellent Cl^- ion transporters selectively inducing cancer cell death through a caspase-dependent pathway.[16]

Effective treatment of cancer using chemotherapy is often hampered by multidrug resistance (MDR). MDR can develop by several mechanisms, including the extensively studied phenomenon linked to the overexpression of ATPase efflux pumps, such as P-glycoprotein (P-gp). P-gp uses the energy of ATP for the removal of cytotoxic drugs from inside the cell before they can elicit the desired therapeutic effects. Therefore, one possible method for overcoming MDR is finding compounds that can inhibit the P-gp efflux pump, or preferably, compounds that target MDR cancer cells via the activity of drug efflux transporters.[17] Since the introduction of the first P-gp inhibitors, such as verapamil, over 40 years ago, four generations of P-gp inhibitors have been developed.[18] Clinical trials have found a minor beneficial effect of quinine, when used as a multidrug resistance reversing agent in

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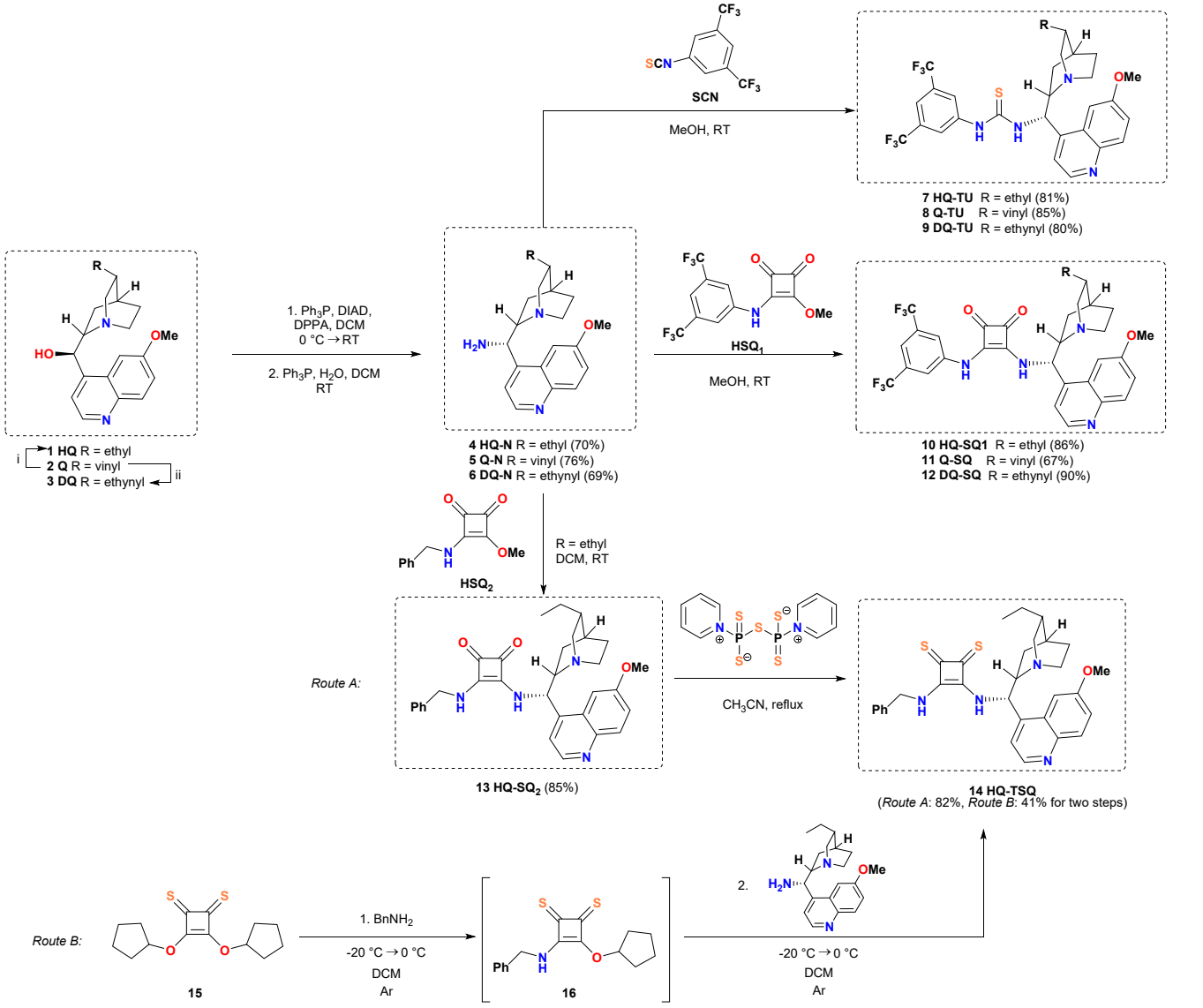
combination therapy with several chemotherapeutics, such as mitoxantrone, cytarabine, vincristine, paclitaxel and anthracyclines.[19–29] Despite several generations of compounds, intensive research is ongoing in pursuit of novel inhibitors and MDR-selective compounds that target and selectively eliminate the MDR cancer cells.[30]

In this work, our aim was to improve the cytotoxicity of quinine. We modified its structure with hydrogen bond units, such as thiourea and (thio)squaramide.[31–34] We examined the cytotoxic activity of the synthesized cinchona analogues against the parental uterine sarcoma MES-SA cells and it's multidrug-resistant (MDR) derivative, Dx5. Moreover, we investigated the potential P-gp inhibitory effect of these quinine derivatives. To our knowledge, this is the first examination of the biological activity of thiosquaramides against an MDR cancer cell line.

2. Results and discussion

2.1. Synthesis of cinchona alkaloid derivatives

In our recent study of cinchona alkaloid organocatalysts, we synthesised several cinchona derivatives from the natural compound quinine.[35] Based on their structures, these can be classified into 4 groups: cinchona alcohols (**1**–**3**), cinchona amines (**4**–**6**), cinchona thioureas (**7**–**9**), and cinchona (thio)squaramides (**10**–**14**). Hydroquinine (**1**) was prepared by catalytic hydrogenation of the vinyl group of quinine (**2**), while didehydroquinine (**3**) was gained by bromine addition followed by double HBr eliminations from the dibromo derivative of quinine (**2**). Starting from these hydroxy derivatives, cinchona amino derivatives (**4**–**6**) were prepared by a one-pot reaction combining Mitsunobu and Staudinger reactions. Subsequently, cinchona thioureas **7–9** were synthesised by reacting the cinchona amines (**4**–**6**) with bis(trifluoromethyl)phenyl isothiocyanate (**SCN**). The reaction of cinchona amines **4**–**6** with the bis(trifluoromethyl)phenyl (**HSQ1**) or benzyl half-squaramide methyl ester (**HSQ2**) gave the corresponding cinchona squaramides (**10**–**13,** Scheme 1).[36] Finally, thiosquaramide **14** was obtained *via* two reaction routes. In route A, the benzyl-cinchona squaramide **13** was transformed to thiosquaramide **14** by using P_4S_{10}·pyridine complex as a thionating agent (Scheme 1, route A).[37] Alternatively, thiosquaramide **14** can be also prepared in a one-pot reaction following the general method of Rawal and co-workers (Scheme 1, route B).[38] To the best of our knowledge, this is the first time that unsymmetrical thiosquaramide has been synthesised by one-pot method.



Scheme 1. Synthesis of cinchona alkaloid derivatives from quinine[35–36,38] *i)* H_2, Pd/C, MeOH, RT, quant. yield *ii)* 1. Br_2, DCM, 0 °C → RT 2. KOH, TBAI, THF, 45 °C → RT, 85%; TBAI = tetrabutylammonium iodide, DIAD = diisopropyl azodicarboxylate, DPPA = diphenylphosphoryl azide.

2.2. Testing the cytotoxicity of cinchona alkaloid derivatives

Quinine and its analogues have been mostly characterized in non-toxic concentrations, as transporter inhibitors.[26,39] In this study, quinine (**2**) and the synthesised 13 cinchona alkaloid derivatives (**1**, **3**–**14**) were screened against the parental uterine sarcoma MES-SA and its doxorubicin-selected multidrug-resistant Dx5 cell lines. MES-SA and Dx5 cells were engineered to express mCherry (MES-SA) and eGFP (Dx5) proteins, respectively, to allow co-culture cytotoxicity tests.[40]

Cytotoxicity was determined by calculating the half-maximal growth inhibition values (IC_{50}) against MES-SA and Dx5 cells. In the case of cinchona alcohols (**1**–**3**), moderate toxicities (47–111 μM) were observed against both cell lines. Cinchona amines (**4**–**6**) exerted slightly higher toxicities on parental cells (29–81 μM), while they were remarkably less toxic (105–197 μM) against the multidrug- resistant cell line. A significant improvement of toxicity was achieved by using thiourea and squaramide hydrogen-bond donor units attached to the cinchona alkaloid skeleton. Thioureas (**7**–**9**) showed increased toxicities (1.3–4.2 μM) against both cell lines. Similarly, squaramides (**10**–**13**) also showed higher toxicities (3.1–4.2 μM) against MES-SA cells, however, they had lower cytotoxicity (11–21 μM) against Dx5 cells. Furthermore, for the thio analogue of **13** squaramide (**14**), lower activity (57 and 65 μM) was observed against both cell lines.

In general, the cytotoxicity (expressed as pIC_{50}) of the analogues had a linear, inverse correlation with log*P* (**14** was an outlier, Fig. S1). Next, we investigated the effects of saturation at the quinuclidine unit, which can influence log*P* values. Based on the drug-like property Ghose filter,[41] log*P* is preferred to be between −0.4 and 5.6 for sufficient membrane permeability. For most of our compounds, the log*P* values were in this region (1.9–7.1). In the case of cinchona alcohols (**1**–**3**) and cinchona thioureas (**7**–**9**), decreasing the degree of unsaturation results in increasing cytotoxicity, consistently with a change in predicted log*P* values. The difference may be due to the fact that cinchona alcohols (**1**–**3**) and cinchona thioureas (**7**–**9**) are better hydrogen-bond donors than amines **4**–**6**. Moreover, the amino group gives an extra basic character for the cinchona amines, which can also influence biological activity. Concerning cinchona squaramides (**10**–**12**), no connection was observed between log*P* and IC_{50} values regarding to the saturation at the quinuclidine unit.

For each examined compound, the resistance ratio (RR) was calculated as the ratio of the compound's IC_{50} against the MDR cell divided by its IC_{50} against the parental cell. The RR values were significant for cinchona alcohols **2** and **3** where RR < 1, and for cinchona amines (**4**–**6**), thiourea **8** and cinchona squaramides (**10**–**12**) where RR greater than 1. The latter group was less toxic against MDR cells which implies that they could be substrates of the P-gp efflux pump (Fig. 1.). All the other tested compounds were equally toxic to the two cell lines, thus evaded MDR. As controls, we used doxorubicin, a known P-gp substrate chemotherapeutic agent, and NSC57969, a robust MDR-selective compound.[42]

2.3. Investigation of the P-gp pump contribution to MDR with tariquidar

We tested several cinchona analogues in the presence of tariquidar, a third-generation P-gp inhibitor, to see if susceptibility/resistance is conveyed by the efflux pump (Fig. 2.). As expected, in the presence of tariquidar, the IC_{50} values against MES-SA did not change significantly for any of the tested analogues. For **2** and **3**, RR remained under 1, and even decreased slightly, thus the susceptibility of Dx5 cells to these two compounds was independent of the function of P-gp. This means that they cannot be considered as *bona fide* MDR-selective compounds such as NSC57969, whose selective toxicity is mediated by P-gp.[42] Resistance of Dx5 cells against **4**, **5** and **10**–**13** was eliminated by tariquidar. Interestingly, Dx5 remained resistant against **6** even in the presence of tariquidar, suggesting a mechanism independent from P-gp.

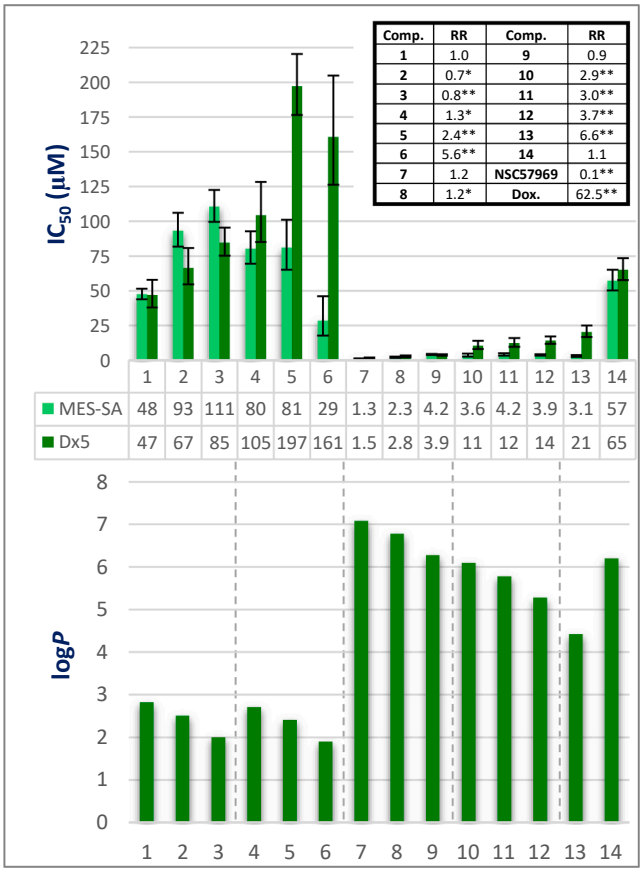


Fig. 1. IC_{50} and calculated log*P* values of the investigated compounds against MES-SA and Dx5 cell lines. Resistance ratios (RR = $IC_{50\ Dx5}/IC_{50\ MES\text{-}SA}$) of compounds are shown in the table (*= p-value < 0.05, **= p-value < 0.01), dox. = doxorubicin.

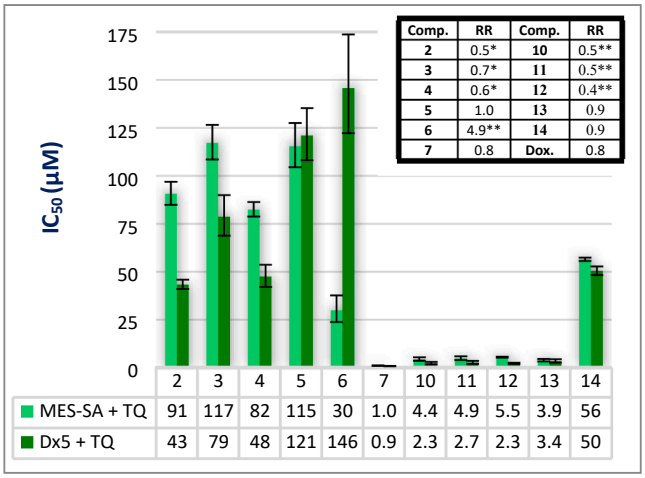


Fig. 2. The contribution of P-gp to drug susceptibility/resistance was investigated in the presence of the P-gp inhibitor tariquidar (TQ). Resistance ratios (RR = $IC_{50\ Dx5}/IC_{50\ MES\text{-}SA}$) of compounds are shown in the table (*= p-value < 0.05, **= p-value < 0.01), dox. = doxorubicin.

2.4. Calcein-AM efflux assay

Quinine and its close analogues are potent MDR-reversing agents: they inhibit the function of P-gp at physiologically relevant concentrations.[39,43–47] Therefore, as a preliminary experiment, we tested six cinchona analogues (**2**, **5**, **8**, **11**, **13** and **14**) in the calcein-AM efflux

assay. Compounds were added to the cells at 20 μM, which corresponds to the average plasma concentration of quinine after i.v. administration to leukemic patients.[46–47]

When tested on MES-SA parental cells, none of the compounds interfered with calcein accumulation (Fig. S2A and S2B). On Dx5 cell line, **2**, **5** and **8** inhibited P-gp with unimodal characteristics, while **11** and **14** (thio)squaramides with skewed distribution of the cells (Fig. 3A, Fig. 3B). Calcein accumulation in the presence of 20 μM **13** was apparently unique with a clear bimodal distribution (Fig. 3C), with distinct calcein positive and calcein negative populations (cf. the histogram was unimodal for the Mes-Sa parental line). The ratio of calcein positive Dx5 cells was 53.1% on average.

We repeated the calcein accumulation assay in 1 μM EDTA containing PBS (phosphate-buffered saline). At 20 μM, verapamil, **2**, **11** and **14**, similarly to the previous flow cytometry experiments, inhibited calcein–AM efflux (Fig. 3D, Supplementary information, Fig. S2C). However, when we tested **13**, calcein accumulation was almost as low as in the negative control, with unimodal distribution (Fig. 3D). As EDTA is a metal chelator, we hypothesized that the calcein-AM efflux inhibition by **13** requires a certain level of an intracellular ion (or ions), that is present only in part of the cells. Ion heterogeneity may be derived from the unsynchronized cell cycle of an in vitro culture. For example, during mitosis, intracellular free Ca^{2+} concentration can be dropped even by 50%.[48] More experiments are needed to delineate the role of ions; however, further investigation of this effect is out of the scope of the recent paper.

In summary, the synthesised cinchona analogues **5**, **8**, **11** and **14** were capable of calcein–AM efflux inhibition at 20 μM to a certain degree, but they are assumed to be less potent than the natural quinine (**2**). Although we performed the calcein-AM efflux assay in serum-free medium, there is evidence in the literature that quinine and its analogues retain most of their activity even in serum-containing medium.[39]

2.5. Preliminary study of copper complexes of cinchona thiourea and thiosquaramide

Quinine (**2**) and its stereoisomer, quinidine are recognised not only as P-gp-inhibitors[23] but also as chelators of metals such as copper,[49–51] which, at least partly, can be responsible for their anticancer activity. In fact, metal complexes of chelators are getting more attention in anticancer research and therapy.[52,53] As an example, the thiosemicarbazone triapine, which also interacts with P-gp, entered already more than 30 clinical trials. Triapine, related to its copper and iron-chelating ability, was suggested to act in multiple ways, such as further exaggerating the already disturbed redox homeostasis of cancer cells,[54,55] or inhibiting the iron-containing ribonucleotide reductase enzyme.[56]

Another metal chelator called DpC (which completed a phase 1 trial[57]can possibly deliver copper and promote its irreversible binding to protein disulfide isomerase (PDI),[55] an endoplasmic reticulum enzyme that is important in protein processing, thus inducing cancer cell death.

To see if the cinchona analogues studied here are capable of copper-binding, we mixed compounds **8**, **11**, **14** with $Cu(OAc)_2$ (ratio: 1:1). In the case of the squaramide **11**, we did not observe colour change or precipitation which may indicate the formation of Cu(II)-complex. Cinchona thiourea **8** and cinchona thiosquaramide **14**, however, gave immediate reaction indicative of complexation.

The pre-formed Cu(II)-**8** exerted the same toxicity as **8**, suggesting that the complex was not stable. However, when we tested Cu(II)-**14**, we saw a remarkable increase in toxicity, especially against the MDR cell line Dx5 (20-fold, 65 μM vs 3.2 μM, Fig 1 and 4), while the complex was only 4.4-times more toxic to the parental line (57 μM vs. 13 μM). This selectivity was independent of P-gp function, as tariquidar did not change the RR value. Whether the Cu-dependent increase in toxicity is related to the (a) better solubility, (b) increased uptake, or (c) gain of function of the Cu(II)-**14** complex, will be investigated in our lab.

3. Conclusion

In conclusion, we have prepared thirteen cinchona organocatalysts and presented the first successful preparation of an unsymmetrical thiosquaramide by one-pot method. Moreover, we demonstrated the

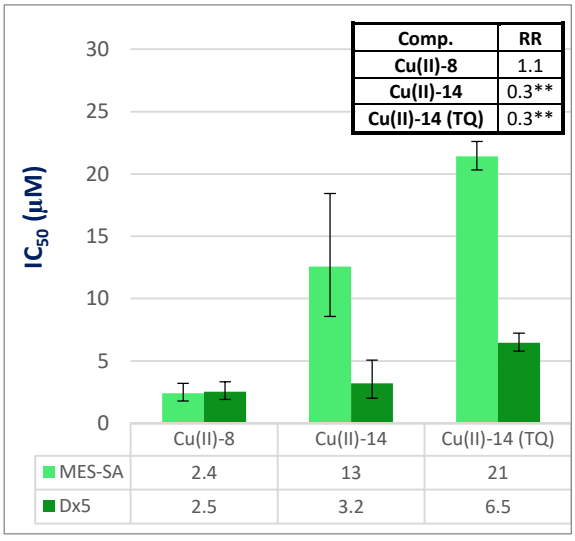


Fig. 4. IC_{50} of copper complexes of cinchona thiourea **8** and thiosquaramide **14** against MES-SA and Dx5 cell lines. Resistance ratios (RR = $IC_{50\ Dx5}/IC_{50\ MES\text{-}SA}$) of compounds are shown in the table (*= p-value < 0.05, **= p-value < 0.01); TQ: 0.4 μM tariquidar.

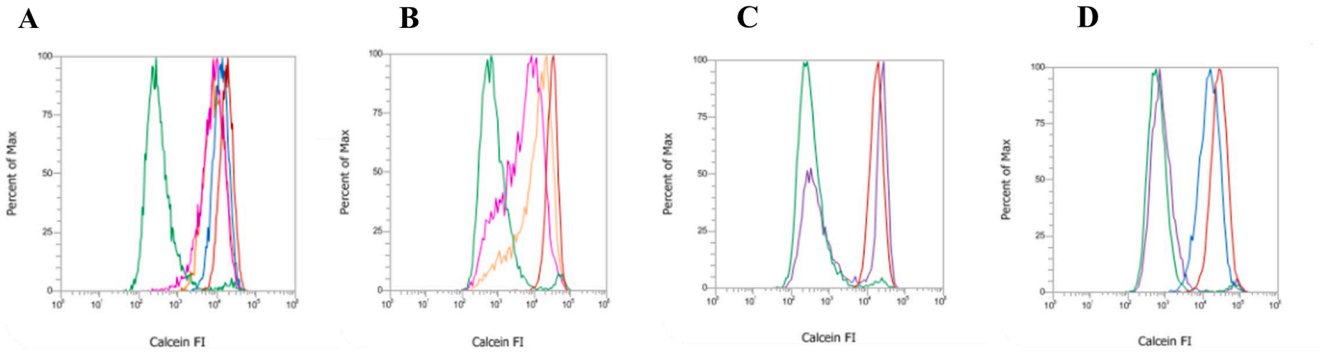


Fig. 3. Calcein accumulation assay in Dx5 cells. Panels A–D: Calcein fluorescence intensity (Calcein FI) in the absence of inhibitors (green) and in the presence of verapamil (red). Calcein FI in the presence of A) **2** (blue), **5** (orange), **8** (pink); B) **11** (pink), **14** (orange); C) **13** (purple). D) Calcein FI in PBS with 1 μM EDTA in the presence of **2** (blue) and **13** (purple). Concentration: 20 μM for each compound.

investigation of cinchona alkaloid organocatalysts for their potential anticancer activity against a multidrug-resistant cell line for the first time. Based on the cell viability assays, an increase of toxicity can be observed in case of cinchona thioureas, squaramides and thiosquaramide (IC_{50} = 1.3–65 μM) compared to cinchona alcohols and amines (IC_{50} = 29–197 μM) against MES-SA and Dx5 uterine sarcoma cell lines. According to the measured resistance ratios, Dx5 line was resistant to the cinchona squaramides and amines. Except for compound 6, resistance was mediated by P-gp's efflux function. On the other hand, the studied cinchona alcohols, and thioureas were able to overcome P-gp mediated multidrug-resistance. Based on results obtained in the calcein-AM efflux inhibition assay, cinchona derivatives proved to be potent P-gp inhibitors. Finally, the formation of a copper (II) complex of the cinchona thiosquaramide resulted in a significant (20-fold) increase in toxicity against the MDR cell line.

4. Materials and methods

4.1. General

Starting materials were purchased from commercially available sources (Sigma-Aldrich, Merck, and Alfa Aesar). Infrared spectra were recorded on a Bruker Alpha-T FT-IR spectrometer. Optical rotations were measured on a PerkinElmer 241 polarimeter that was calibrated by measuring the optical rotations of both enantiomers of menthol. Silica gel 60 F_{254} (Merck) and aluminium oxide 60 F_{254} neutral type E (Merck) plates were used for TLC. Aluminium oxide (neutral, activated, Brockman I) and silica gel 60 (70–230 mesh, Merck) were used for column chromatography. Ratios of solvents for the eluents are given in volumes (mL mL^{-1} Melting points were taken on a Boetius micro-melting point apparatus (VEB Dresden Analytik, Dresden, Germany), and they were uncorrected. NMR spectra were recorded at Directorate of Drug Substance Development, Egis Pharmaceuticals Plc., on a Bruker Avance III HD (at 600 MHz for ^1H and at 150 MHz for ^{13}C spectra) or at the Department of Inorganic & Analytical Chemistry, Budapest University of Technology and Economics, on a Bruker DRX-500 Avance spectrometer (at 500 MHz for ^1H and at 125 MHz for ^{13}C spectra) or on a Bruker 300 Avance spectrometer (at 300 MHz for ^1H and at 75 MHz for ^{13}C spectra) at temperatures given. The exact mass measurements were performed using Q-TOF Premier mass spectrometer (Waters Corporation, 34 Maple St, Milford, MA, USA) in positive electrospray ionisation mode. MarvinSketch was used for log*P* prediction, MarvinSketch 20.11, ChemAxon (https://www.chemaxon.com).

4.2. Synthesis of benzyl-cinchona thiosquaramide (**14**, HQ-TSQ)

4.2.1. Route A:

To a solution of benzyl-cinchona squaramide (**13**, 100 mg, 0.196 mmol) in acetonitrile (1.2 mL) the pyridine complex of phosphorus pentasulfide (150 mg, 0.394 mmol) was added. The reaction mixture was stirred at 80 °C for 5 h. After the reaction was completed, it was cooled down to room temperature, and DCM (10 mL) was added. Next, the mixture was washed with water (10 mL). The organic phase was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (Al_2O_3; DCM → DCM:MeOH = 40:1). The obtained product was dissolved in DCM (0.5 mL) and added to hexane (10 mL) dropwise. The precipitated yellow crystals were filtered, washed with excess of hexane, and dried under reduced pressure to obtain the pure product (87 mg, 82%).

4.2.2. Route B:

To a solution of 3,4-bis(cyclopentyloxy)cyclobut-3-ene-1,2-dithione[38] (35 mg, 0.124 mmol) in dry DCM (1 mL), a solution of benzylamine (12 mg, 0.112 mmol) in dry DCM (1 mL) was added at −20 °C under argon atmosphere. The resulted solution was stirred for 15 mins at that temperature, then for 15 mins at 0 °C. After the reaction was completed, the reaction mixture was cooled down to −20 °C, and a solution of cinchona amine (44 mg, 0.135 mmol) in dry DCM (1 mL) was added. The reaction mixture was stirred for 30 min at −20 °C and then for 1 h at 0 °C. After the reaction was completed based on thin-layer chromatography, the solvent was removed under reduced pressure. The crude product was purified by preparative thin-layer chromatography (Al_2O_3; DCM:MeOH = 40:1). The obtained product was dissolved in DCM (0.5 mL) and added to hexane (10 mL) dropwise. The precipitated yellow crystals were filtered, washed with an excess of hexane, and dried under reduced pressure to obtain the pure product (28 mg, 41%).

TLC (aluminium oxide; chloroform:methanol:NH_3 = 40:1:0.01) R_f = 0.45; (SiO_2; chloroform:methanol:NH_3 = 40:1:0.01) R_f = 0.38. M.p. 211–213 °C, $[\alpha]_{25}^{D}$ −81.7 (c 1.00, $CHCl_3$) *IR (KBr):* ν_{max} 3431, 3165, 2958, 1761, 1702, 1621, 1562, 1509, 1475, 1433, 1357, 1241, 1146, 1096, 1028, 905, 850, 714, 699, 617, 543, 521, 491, 462 cm^{-1}.

^1H NMR (DMSO-d_6) δ: 9.90 (br, 1H), 9.50 (br, 1H), 8.89 (m, 1H), 8.00 (m, 1H), 7.99 (m, 1H), 7.98 (m, 1H), 7.49 (m,1H), 7.40 (m, 2H), 7.31 (m, 2H), 7.25 (m, 1H), 7.25 (m, 1H), 5.38 (br, 1H), 5.18 (m, 1H), 4.61 (br, 1H), 4.23 (br, 1H), 4.02 (s, 3H), 3.65 (m, 1H), 3.24 (br, 1H), 3.16 (br, 1H), 1.93 (m, 1H), 1.90 (m, 1H), 1.88 (m, 1H), 1.85 (m, 1H), 1.62 (br, 1H), 1.41 (m, 1H), 1.33 (m, 1H), 0.94 (m,1H), 0.80 (m, 3H) ppm.

^{13}C NMR (DMSO-d_6) δ: 205.4, 201.9, 170.7, 170.5, 158.4, 148.2, 144.5, 140.7, 137.3, 131.9, 128.8, 128.4, 127.9, 126.7, 122.8, 120.9, 102.0, 60.0, 56.5, 55.0, 51.3, 46.2, 41.5, 34.3, 25.6, 24.2, 23.9, 23.2, 11.6 ppm.

COSY: 8.89–7.99, 8.00–7.49, 7.40–7.31–7.25, (5.18, 5.38)–9.50, 4.61–7.25, (4.23, 3.24)–(1.93, 1.85), 3.65–3.16, 1.90–(3.65, 1.33), 0.94–(1.62, 1.88), 0.80–(1.41, 1.33).

HSQC (145 Hz): 8.89–148.16, 8.00–131.91, 7.99–120.90, 7.98–101.96, 7.49–122.84, 7.40–128.43, 7.31–128.84, 7.25–127.87, 7.25–51.29, (5.38, 5.18)–46.16, 4.61–59.95, (4.23, 3.24)–41.53, 4.02–56.47, (3.65, 3.16)–55.01, (1.93, 1.85)–24.15, 1.90–34.31, 1.88–23.90, (1.62, 0.94)–23.23, (1.41, 1.33)–25.55, 0.80–11.58.

HMBC (8 Hz, 145 Hz): 8.00–158.44, 7.99–126.67, 7.49–144.48, 7.40–128.43, 7.31–(128.84, 137.28), 7.25–128.43, 5.18–128.43, 4.02–158.44, 0.80–(34.31, 25.55).

ROESY: 1.88–3.24, 4.02–(7.25, 7.98).

HRMS-ESI$^+$ (*m/z*): [M + H^+] calcd for $C_{31}H_{34}N_4OS_2$: 543.2252, found: 543.2255.

4.3. Cell lines and culture conditions

The human uterine sarcoma cell line MES-SA and its doxorubicin-selected multidrug-resistant phenotype, Dx5, were obtained from the American Type Culture Collection (ATCC). The cells expressing mCherry and eGFP fluorescent proteins were transduced with a second-generation lentiviral system and sorted for mCherry and eGFP positivity by flow cytometry.[40] To ensure P-glycoprotein overexpression, Dx5 cells were treated with 500 nM doxorubicin before the experiments. During the experiments, the cell passage number was not more than 20 after reviving cells from liquid nitrogen. Cell lines were cultivated in Dulbecco's modified eagle's medium (Merck) supplemented with 10% fetal bovine serum (Gibco), 5 mmol L^{-1} L-glutamine (Lonza), 50 unit mL^{-1} penicillin/streptomycin solution (Lonza) and maintained at 37 °C in a 5% CO_2 humified atmosphere.

4.4. Fluorescence-based in vitro cytotoxicity assay

For cytotoxicity assay, fluorescent cell lines were used. Both cell seeding and drug-addition steps were performed by an automated liquid handling workstation (Hamilton Robotics STAR Let). Prior to drug treatment, mixed suspensions of MES-SA mCherry and Dx5 eGFP cells were seeded into 384-well tissue culture plates with a density of 2500 cells/well (1250 cells from both) in 20 μL medium. Co-cultured cells

were allowed to settle overnight. The following day, serial dilutions of drug samples were carried out manually in a 96-well microplate and added to the cells. The final volume was 60 μL/well to achieve the required final concentration of the test compounds. Plates were then incubated for 144 h. The viability of cells was measured by detecting the respective fluorescence intensities with an EnSpire plate reader (Perkin Elmer). The excitation wavelength for mCherry was 585 nm, and fluorescence emission was detected at 610 nm. These values were 485 nm and 510 nm for eGFP, respectively. IC_{50} results were obtained from the spectrophotometric measurement files by automated data evaluation, which was performed by our custom program written in C#.[40] A two-sided unpaired Student's *t*-test was performed to determine statistical significance between the IC_{50} values, which are shown as *= $p < 0.05$, **= $p < 0.01$. In case of cell line-specific toxicity, we repeated the tests in the presence of 0.4 μM tariquidar, a potent inhibitor of P-gp, to address the function of the transporter.

4.5. FACS measurements

Calcein accumulation assay was carried out according to the protocol developed by Homolya and colleagues.[58] Briefly, 250 000 cells per tube were washed with serum-free DMEM, then pre-incubated for 5 min with the given concentrations of the cinchona alkaloids. Positive control was 20 μM verapamil, negative control was medium only. In the next step, calcein-AM solution was added at 250 nM final concentration, and the tubes were incubated for another 10 min. Incubations took place in a water bath at 37 °C with mild shaking. Reaction was stopped with an excess amount of ice-cold PBS, and tubes were kept on ice until measuring by an Attune NxT flow cytometer. We used TO-PRO-3 dye to distinguish live/dead cells. Experiments in the presence of EDTA were performed in the same way, but every step from pre-incubation until measurement were made in 1 μM EDTA PBS. For the FACS measurements, we applied non-fluorescent MES-SA and Dx5 cells.

CRediT authorship contribution statement

Szonja Polett Pósa: Investigation, Data curation, Formal analysis. **Gyula Dargó:** Investigation, Formal analysis, Writing – original draft. **Sándor Nagy:** Investigation, Writing – review & editing. **Péter Kisszékelyi:** Writing – review & editing. **Zsófia Garádi:** Data curation, Formal analysis. **Lilla Hámori:** Investigation. **Gergely Szakács:** Supervision, Funding acquisition, Project administration, Writing – review & editing. **József Kupai:** Conceptualization, Methodology, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Szilárd Tóth:** Methodology, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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