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Cytotoxic and Trypanocidal Activities of Cinchona Alkaloid Derivatives

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Short running title: Cinchona alkaloid derivatives as drug candidates

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ABSTRACT

A series of 27 cinchona alkaloid derivatives (**1f-w**, **2a-e** and **3a-d**) were investigated for their cytotoxic and trypanocidal activities using seven different cancer cell lines (KB, HeLa, MCF-7, A-549, Hep-G2, U-87 and HL-60), two normal cell lines (HDF and CHO) and bloodstream forms of *Trypanosoma brucei brucei*, respectively. Four compounds (**1u**, **1w**, **2e** and **3d**) were identified with promising cytotoxic activity with 50% growth inhibition (GI_{50}) values below 10 µM. Two (**2e** and **3d**) of the four compounds also exhibited potent anti-trypanosomal activity with GI_{50} values of 0.3-0.4 µM. All four active compounds represented derivatives modified at their C-9 hydroxy group. With respect to anti-proliferative activity and selectivity, **2e** (*epi*-*N*-quinidyl-*N'*-bis(3,5-trifluoromethyl)phenylthiourea) proved to be the most promising derivative for both cancer cells and bloodstream forms of *T. b. brucei*. The cytotoxic activity of compounds **1u**, **1w**, **2e** and **3d** was attributed to their ability to induce apoptosis in cancer cells. The results demonstrate the potential of cinchona alkaloid derivatives as novel anti-cancer and anti-trypanosome drug candidates.

KEYWORDS

cinchona alkaloids, human cancer cells, *Trypanosoma brucei*, cytotoxicity, trypanotoxicity

1 INTRODUCTION

The bark of the cinchona tree (*Cinchona* sp.) contains many bioactive quinoline-type alkaloids. Among them, quinine is both the most important and best known alkaloid of this class of natural compounds. Historically, quinine was the sole cure for malaria for centuries until the introduction of the synthetic analogue chloroquine after World War II. Other major alkaloids found in the cinchona bark are the closely related compounds quinidine, cinchonine and cinchonidine, which together with quinine are collectively classified as 'cinchona alkaloids'.

Apart from their utilization as anti-malaria agents, cinchona alkaloids are also deployed to treat intestinal parasites, arrhythmia and other heart conditions, nocturnal leg cramps, and fever, and are used as a bitter digestive aid.[1,2] In addition, cinchona alkaloids have been reported to be inhibitors of the P-glycoprotein, a membrane transporter that is overexpressed on tumour cells and responsible for the process of efflux of chemotherapeutics. For example, cinchonine has been shown to improve the accumulation of doxorubicin in cancer cells.[3] Moreover, cinchonine and quinine were found to induce apoptosis in cancer cells and thus inhibit their proliferation.[4,5] Furthermore, cinchona alkaloids have been reported to display trypanocidal activity against bloodstream forms of *Trypanosoma brucei* and *T. congolense* with 50% growth inhibition (GI_{50}) values in the mid to low micromolar range.[6] These flagellated protozoan parasites are the causative agents of sleeping sickness in humans and nagana disease in cattle, and are transmitted by the bite of infected tsetse flies. African trypanosomiasis is one of the most neglected tropical diseases and throughout history, it has severely repressed the economic and cultural development of central Africa.[7] As the few currently available chemotherapies of sleeping sickness and nagana disease are unsatisfactory,[8] new and effective drugs are needed for treatment of African trypanosomiasis.

The previous findings that native cinchona alkaloids have anti-cancer and anti-trypanosomal properties prompted us to investigate whether the cytotoxic and trypanocidal activities of this class of natural products can be improved by their modification. In this study we report the cytotoxic and trypanocidal activities of 27 cinchona alkaloid derivatives (Table 1) of which 8 were commercially available (**1f-h**, **2a-e**) and 19 were synthesised (**1i-w**, **3a-d**) according to published methods.[9-16]

2 MATERIALS AND METHODS

2.1 Reagents

Unmodified cinchona alkaloids **1a-e** and their hydrogenated analogues **1f-h** and **1k** were purchased from Buchler GmbH (Braunschweig, Germany) or Sigma-Aldrich (Poznan, Poland). 9-Amino-9-*epi*-cinchona derivatives **2a-d** and the thiourea **2e** were bought from AdvaChemLab (Poznan, Poland). All other reagents required for organic synthesis and toxicity assays were obtained from Sigma-Aldrich (Poznan, Poland and Gillingham, U.K., respectively) and Fluorochem (Hadfield, U.K.) and used as received.

2.2 Chemistry

NMR spectra were recorded at 295 K on a Bruker Ascend 400 MHz spectrometer, operating at frequencies of 400 MHz for ^{1}H and 101 MHz for ^{13}C. All shifts were referenced to the internal tetramethylsilane. Mass spectra were determined on a Bruker Impact HD instrument. IR spectra were recorded using KBr pellets on a Brucker FT-IR IFS-66/s spectrometer.

Dihydrocupreines **1i-j**, 10,11-didehydrocinchona alkaloids **1l-m**, cinchotenine derivatives **1n-p** and deoxyhydroquinidine **1q** were prepared as previously described.[9-12] Quinine esters **1r-s** and 9-*O*-carbamates **1t-w** were synthesised using general procedures.[13,14] Quaternary ammonium salts **3a-c** were prepared by reaction of quinine with 9-anthracenylmethyl chloride or appropriate benzyl halides as previously reported while the salt **3d** was obtained following a previous published protocol.[15,16] All compounds were properly characterized and their purity was >95%.

2.3 Cells

KB, HeLa, MCF-7, Hep-G2 and CHO cells were obtained from the European Collection of Cell Culture (ECACC) supplied by Sigma Aldrich (Poznan, Poland). A-549, U-87, HDF and HL-60 cells were purchased from the American Type Culture Collection (ATCC) through LGC Standards (Lomianki, Poland and Teddington, U.K., respectively). Bloodstream forms of *T. b. brucei* 427-221a were previously obtained from the Wellcome Centre for Molecular

Parasitology (Glasgow, U.K.). This subspecies is non-pathogenic to humans but morphological and biochemical indistinguishable from the human pathogenic subspecies *T. b. gambiense* and *T. b. rhodesiense*.

2.4 Cell culture and *in vitro* toxicity assay

2.4.1 Tumour and normal cells

KB, Hep-G2 and U-87 cells were cultured in EMEM medium while HeLa cells were grown in RPMI 1640 medium, A-549 and CHO cells in F-12K medium, MCF-7 cells in DMEM medium, U-87 in EMEM medium and HDF cells in Fibroblasts Growth Medium. Each medium was supplemented with 10% foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin solution (final concentrations 100 U/ml penicillin and 100 µg/ml streptomycin). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

For determining the cytotoxic activity of test compounds, the protein-staining sulforhodamine B (SRB) assay and/or the tetrazolium reduction (MTT) assay were employed. Both assays have been assessed by the National Cancer Institute (USA) as suitable for *in vitro* anti-tumour screening.[17] The SRB and the MTT assay estimate cell densities based on measurement of cellular protein content and activity of oxidoreductase enzymes, respectively. For the SRB assay, 100 µl of diluted cell suspension containing approximately 10^4 cells was added to the wells of 96-well plates. After 24 h, when a partial monolayer had formed, the supernatant was aspirated and 100 µl medium containing test compounds at six different concentrations (0.1, 0.2, 1, 2, 10 and 20 µg/ml) was added to the cells. Stock solutions of test compounds were prepared in DMSO and the concentration of DMSO in the assay did not exceed 0.1% which was found to be nontoxic to the different cell lines. After incubation for 72 h, 25 µl of 50% trichloroacetic acid was added to each well and the plates were incubated for 1 h at 4°C. Thereafter, the plates were washed with distilled water to remove traces of medium and air-dried. Then, the dried plates were stained with 100 µl 0.4% SRB (prepared in

1% acetic acid) for 30 min at room temperature. Unbound dye was removed by rapidly washing with 1% acetic acid and the plates were air-dried overnight. Finally, the protein-bound dye was dissolved in 100 µl of 10 mM unbuffered Tris and the absorbance read at 490 nm.

For the MTT assay, 180 µl of suspended cells (2×10^4/mL) was pipetted into the wells of 96-well plates. After 24 h incubation, 20 µl of test compounds was added (final concentrations of 0.1, 0.2, 1, 2, 10 and 20 µg/ml) and the plates were incubated for another 48 h. Then, 10 µl of 5 mg/ml MTT solution was added to each well and the plates incubated for additional 3 h. The formed formazan crystals were dissolved in 100 µl 10% sodium dodecyl sulphate (SDS) solution and the absorbance measured at 545 nm.

The concentration of a compound required to inhibit cell growth by 50% (half-maximal growth inhibition; GI_{50}) compared to the control was calculated using GraphPad Prism software.

2.4.2 Trypanosomes and HL-60 cells

Bloodstream forms of the non-human pathogenic subspecies *T. b. brucei* (clone 427-221a[18]) and human myeloid leukaemia HL-60 cells[19] were grown in Baltz medium[20] supplemented with 16.7% heat-inactivated bovine serum at 37°C in a humidified atmosphere. Toxicity assays with trypanosomes and HL-60 cells were carried out as previously described[6] with some modifications. In brief, cells were seeded in 96-well plates in a final volume of 200 µl of Baltz medium containing test compounds serially diluted tenfold from 100 µM down to 100 pM and 1% DMSO. Wells just containing medium and 1% DMSO served as controls. The initial cell densities were 1×10^4/ml for trypanosomes and 5×10^4/ml for HL-60 cells. After 24 h incubation, 20 µl of a 0.5 mM resazurin solution prepared in sterile PBS was added and the cells were incubated for a further 48 h. Thereafter, the absorbance of wells was read at 570 nm (test wavelength) and 630 nm (reference wavelength). The GI_{50} value, i.e., the

concentration of a compound necessary to reduce the growth rate of cells by 50% compared to the control was determined by linear interpolation as described previously.[21] The minimum inhibitory concentration (MIC) values, i.e. the concentration of the drug at which all trypanosomes and human cells were killed, was determined microscopically.

2.5 Apoptosis Assay

The induction of apoptosis by test compounds was determined with the Cell Death Detection ELISA^{PLUS} from Roche Diagnostics GmbH (Mannheim, Germany). This is an enzyme immunoassay for the determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleotides) after induced cell death. Cells (10^4) were pipetted into wells of 96-well plates and test compounds added at final concentrations of 0.1, 1 and 10 µg/ml. Cells incubated in the absence of test compounds served as negative controls. After 4 h incubation, cells were harvested by centrifugation of the plates for 10 min at 200 × *g*. Supernatants were removed carefully and cell pellets re-suspended in 200 µl lysis buffer and incubated for 30 min at room temperature. After centrifugation of the plates (10 min, 200 × *g*), 20 µl of cell lysates were transferred in duplicate into wells of provided streptavidin-coated microplates. Then, 80 µl of the immunoreagent containing a mixture of anti-histone-biotin monoclonal antibody and anti-DNA-peroxidase monoclonal antibody was added to each well. The microplates were covered with a provided adhesive foil and incubated for 2 h at room temperature. Thereafter, the solution in the wells was removed by tapping the microplate. The wells were rinsed three times with incubation buffer and 100 µl of substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS) was added to each well. After 10-20 min, 100 µl of ABTS stop solution was pipetted to each well and the absorbance was read at 405 nm (test wavelength) and 490 nm (reference wavelength). The apoptotic index was calculated based on the enrichment of mono- and oligonucleosomes according to the following equation:

$$\text{apoptotic index} = [\{A_{405(\text{sample})} - A_{490(\text{sample})}\} \div \{A_{405(\text{negative control})} - A_{490(\text{negative control})}\}].$$

3 RESULTS AND DISCUSSION

3.1 Cytotoxicity of cinchona alkaloid derivatives

The cytotoxic activities of unmodified (**1a-e**) and derivatized (**1f-u**, **1w**, **2a-c**, **2e**, **3d**) cinchona alkaloids were evaluated with six human tumour cell lines (KB: cervix carcinoma derived from HeLa cells[a]; HeLa: cervix carcinoma; MCF-7: breast cancer; A-549: lung cancer; Hep-G2: liver cancer; U-87, glioblastoma) and two normal cell lines (HDF: human dermal fibroblast; CHO: Chinese hamster ovary cells) using the SRB and/or the MTT assay. The unmodified cinchona alkaloids **1a-c** were not very cytotoxic with GI_{50} (half-maximal growth inhibition) values ranging from 36.4 µM to 155 µM for tumour cells and from 111 µM to 397 µM for normal cells (Table 2). Similar cytotoxicity values have been previously reported for quinine and KB cells (GI_{50} = 118 µM; our study 55.5 µM) and for cinchonine and HeLa cells (GI_{50} = 180 µM; our study 155 µM).[4,5] Cinchona alkaloid molecules with an ethyl, an ethynyl or a carboxyl group (**1f-p**) instead of the vinyl group displayed similar cytotoxic activities as their unmodified counterparts (Table 2). This finding suggests the vinyl group to be non-essential to the cytotoxic action of cinchona alkaloids. On the other hand, some compounds with a modified C-9 hydroxy group showed improved cytotoxicities compared to the unmodified precursors. While compounds without a C-9 hydroxy group (**1q**) or with an esterified C-9 hydroxy group (**1r-s**) did not show increased cytotoxic activities, two 9-*O*-carbamates (**1u** and **1w**) displayed improved cytotoxicities with GI_{50} values in the low-micromolar range (Table 2). A 9-*O*-allyl cinchonidine derivative (**3d**) which was additionally quaternized at the quinuclidine nitrogen with a bulky 9-methylanthracene substituent (note that single modification of this position with a 9-methylanthracene substituent (**3a**) already increases the cytotoxic activity of cinchona alkaloids slightly; see below and Table 4) exhibited cytotoxic activities with GI_{50} values in the low-micromolar range as well (Table 2). Furthermore, compounds with a C-9 amino group of inverted absolute configuration instead of the native C-9 hydroxy group (**2a-c**) showed slightly improved cytotoxic activities (~4-fold lower GI_{50} value; Table 2). A 9-amino-9-*epi*-quinidine derivative in which the amino group was transformed into a 3,5-bis(trifluoromethyl)

phenylthiourea substituent (**2e**) exhibited further enhanced cytotoxicity with GI_{50} values in the low- and mid-micromolar range for tumour and normal cells, respectively (Table 2).

Derivatives **1u**, **1w**, **2e** and **3d** were identified as the most toxic compounds for all cancer cells with GI_{50} values < 10 µM. As the reliability of cytotoxic assays are linked to the mode of action of the drug and the employed test methods, we also evaluated the cytotoxic activity of the four compounds with the MTT cell proliferation assay. While the MTT assay gave higher GI_{50} values for **1u**, **1w** and **3d**, the SRB assay gave higher GI_{50} values for **2e** (Table 2). As both methods (SRB and MTT) gave GI_{50} values of the same order of magnitude (1-10 µM), it can be concluded that the determined concentration range is the effective level of cytotoxicity of the four compounds.

In comparison to cytarabine and doxorubicin (reference drugs), two currently used conventional anti-cancer drugs, the four most cytotoxic compounds **1u**, **1w**, **2e** and **3d** displayed lower cytotoxicities (Table 2). Based on the results obtained by the MTT assay, only compound **2e** approximated the cytotoxic level of doxorubicin. However, a previous report showed that cytarabine displayed lower cytotoxic activity with GI_{50} values of 3-5 µM when determined with the SRB assay.[22] Based on this, it can be assumed that the four compounds exhibited similar cytotoxic activity levels as compared with cytarabine.

Overall, tumour cells were found to be more sensitive to all of the compounds tested than normal cells (Table 2). This observation indicates that cinchona alkaloid derivatives interfere with the replication of rapidly dividing cancer cells that are generally more susceptible to mitotic reagents than slow dividing normal cells. It is most likely that the compounds cause DNA damage that lead to cell death by apoptosis (see section 3.2). To determine the selectivity for the four most cytotoxic derivatives **1u**, **1w**, **2e** and **3d**, selectivity indices (SIs) were calculated as the ratio of the GI_{50} on the normal HDF cell line to the GI_{50} on the respective tumour cell line (Table 3). We calculated the SI only for **1u**, **1w**, **2e** and **3d** because these four compounds fulfil the pre-screen criterion for new anti-cancer agents

according to the National Cancer Institute (predetermined threshold inhibition concentration of 10 µM).[23] As cytotoxic compounds are usually not very selective, an SI of >3 is generally considered to be good.[24,25] According to the results of the SRB assay, the derivatives **1u** and **2e** displayed SI values that were close to or greater than 3 for the four tumour cell lines tested (Table 3) suggesting that both compounds could be interesting candidates for further drug development. Derivative **1w** had SI values less than 3 for the four tumour cell lines (Table 3) indicating that this compound exhibited some general toxicity. The quaternary ammonium salt **3d** had a surprisingly high SI value for the lung cancer cell line A-549 only (Table 3). The SI values calculated from the data of the MTT assay were generally lower. Only **2a** had an SI value of >2 for HeLa and A-59 cells (Table 2). On the other hand, the reference drugs cytarabine and doxorubicin showed SI values slightly higher than 2 for five tumour cell lines tested.

3.2 Induction of apoptosis by compounds 1u, 1w, 2e, and 3d

Since cinchona alkaloids have been shown to be able to trigger apoptosis in cancer cells,[5,26] we were wondering whether the cytotoxic action of the four most potent derivatives was due to the induction of programmed cell death. The ability of compounds **1u**, **1w**, **2e** and **3d** to induce apoptosis was investigated with five cancer cell lines (HeLa, KB, A-549, U-87 and Hep-G2). Cells were treated with three different concentrations of each compound (0.1 µg/ml, 1 µg/ml and 10 µg/ml) and fragmentation of DNA as indicator for apoptosis was determined using a cell death ELISA. All four compounds induced apoptosis to a similar extent in all five cancer cell lines (Figure 1). A significant increase in the apoptotic index was found for all compounds with increasing concentration indicating that the induction of apoptosis was dose-dependent. At the lower concentrations of 0.1 and 1 µg/ml, **3d** was the most effective compound in inducing apoptosis with mean apoptotic indices of 1.77 and 3.92, respectively. At the higher concentration of 10 µg/ml, **2e** proved to be the most potent apoptosis-inducing derivative with a mean apoptotic index of 6.97.

Although the underlying mechanisms of the derivatives in triggering apoptosis in cancer cells remains unknown, studies with their unmodified counterparts may provide some clues to possible modes of action. For example, cinchona alkaloids have been shown to intercalate into DNA which can trigger apoptosis.[26] Cinchonine has been reported to induce programmed cell death in cancer cells through competitive binding of TRAF6 leading to subsequent inhibition of AKT and TAK1 signalling pathways.[5] Quinine was found to trigger its apoptotic effect through the inhibition of the NF-κB signalling pathway via reduction of Bcl2 and mutant p53 and simultaneous up-regulation of caspase-3 and Bax.[27]

3.3 Trypanotoxicity of cinchona alkaloid derivatives

Since compounds that display anti-cancer activity often also exhibit trypanocidal activity,[28] we tested the cinchona alkaloid derivatives for their activity to inhibit the growth of bloodstream forms of *T. b. brucei* as well. The unmodified cinchona alkaloids **1a-d** (quinine, quinidine, cinchonine and cinchonidine, respectively) showed similar antitrypanosomal activities with GI_{50} values in the low to mid micromolar range and MIC values of 10 to 100 µM as previously reported (Table 4).[6,29] Most derivatives displayed similar or lower trypanocidal activity than their unmodified counterparts (Table 4). Exceptions were compounds **1n**, **1o** and **2a**, which showed no activity against trypanosomes with MIC and GI_{50} values >100 µM, and compounds **2e** and **3d**, which exhibited enhanced trypanocidal activity with MIC values below 10 µM and GI_{50} values in the sub-micromolar range (Table 4). When compared with the MIC value and the GI_{50} value of suramin (Table 4; reference drug), one of the drugs used in the treatment of sleeping sickness, compounds **2e** and **3d** were, however, about 10 times less trypanocidal. Interestingly, these two compounds were among the four derivatives that were highly active against the six cancer cell lines (see Table 2). The selectivity of the cinchona alkaloid derivatives was determined with human leukaemia HL-60 cells because their sensitivity for approved trypanocides is well established.[6,30] Most compounds including the unmodified cinchona alkaloids showed no cytotoxicity against HL-60 cells (Table 4). Only compounds **1s**, **1u**, **1v**, **1w**, **2e**, **3a** and **3d**

displayed different levels of cytotoxic activity against the leukaemia cells with GI_{50} values in the mid- to low-micromolar range. As the most trypanocidal compounds **2e** and **3d** were also quite cytotoxic for HL-60 cells, their SI were only moderate (just below 10) (Table 4). For comparison, drugs used for treatment of sleeping sickness have much higher SIs.[6,30] For example, the reference drug suramin displayed no cytotoxicity towards HL-60 cell and accordingly the MIC and GI_{50} ratios of this trypanocide were >1000 and >2941, respectively (Table 4). However, as HL-60 cells are cancer cells, the selectivity of the two compounds may be overestimated. In fact, the most trypanocidal compound **2e** was 1.5 to 6 times less cytotoxic towards non-malignant HDF cells than towards HL-60 cells (Table 2 and 4). Based on this, the selectivity index of compound **2e** would be 15-60. Hence, derivative **2e** almost fulfils the activity criteria for drug candidates for African trypanosomiaisis (GI_{50} < 1 μM; SI > 100).[33]

4 CONCLUSION

This investigation has shown that naturally occurring cinchona alkaloids can be considered as a source for an easy construction of derivatives with increased cytotoxic and trypanocidal activity. Of the four compounds (**1u**, **1w**, **2e** and **3d**) that displayed remarkable cytotoxicity, two derivatives (**2e** and **3d**) exhibited also strong antitrypanosomal activity. The common structural feature of all four compounds was the replacement of their C-9 hydroxy group to either *O*-carbamates (**1u** and **1w**), to thiourea (**2e**), or to *O*-allyl ether (**3d**). Thus, it seems that appropriate modification of the C-9 hydroxy group is crucial for improving the anti-proliferative activity of cinchona alkaloid derivatives. In addition, a particular stereochemistry of the alkaloid moiety is not a prerequisite for the activity as three (**1u**, **1w** and **3d**) out of the four compounds had *8S,9R* configuration (as in quinine or cinchonidine) but the 9-epimeric compound **2e** with *8S,9S* configuration displayed also high potency. Both 9-*O*-protected quaternary salts and 9-thiourea derivatives turned out to be the promising candidates for further activity optimization. Studies in this area are in progress and will be presented in due course.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

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FIGURE LEGENDS

Figure 1 Induction of apoptosis in cancer cells by cinchona alkaloid derivatives **1u** (A), **1w** (B), **2e** (C) and **3d** (D). Five cancer cell lines (KB, HeLa, A-549, Hep-G2 and U-87) were treated with the test compounds at concentrations of 0.1, 1 and 10 µg/ml for 4 h. Apoptosis was determined with a cell death ELISA. The apoptotic index refers to the ratio of nucleosome enrichment in treated cells to untreated (control) cells. Data shown are means ± SD from three independent experiments.

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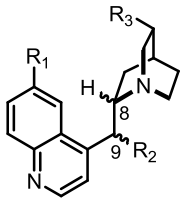
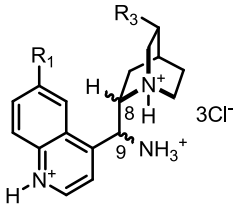
N O T E S

[a]Originally, the KB cell line was derived from an epidermal carcinoma of the mouth of an adult male Caucasian, but was subsequently contaminated with HeLa cells. As KB cells are indistinguishable from HeLa cells by DNA fingerprinting, KB cells should be considered as derived from HeLa cells.

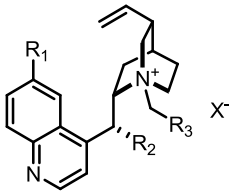
TABLE 1 Chemical structure of cinchona alkaloids (**1a-e**) and their derivatives (**1f-w**, **2a-e**, **3a-d**)



1a-w, 2e



2a-d



3a-d

Compound	Configuration at C-8 and C-9	R_1	R_2	R_3	X^-
Unmodified cinchona alkaloids					
1a	*8S,9R*	OMe	OH	vinyl	-
1b	*8R,9S*	OMe	OH	vinyl	-
1c	*8R,9S*	H	OH	vinyl	-
1d	*8S,9R*	H	OH	vinyl	-
1e	*8R,9R*	OMe	OH	vinyl	-
Modification of vinyl group					
1f	*8S,9R*	OMe	OH	ethyl	-
1g	*8R,9S*	OMe	OH	ethyl	-
1h	*8R,9R*	OMe	OH	ethyl	-
1i	*8S,9R*	OH	OH	ethyl	-
1j	*8R,9S*	OH	OH	ethyl	-
1k	*8R,9S*	H	OH	ethyl	-
1l	*8S,9R*	OMe	OH	ethynyl	-
1m	*8R,9S*	OMe	OH	ethynyl	-
1n	*8R,9S*	H	OH	COOH	-
1o	*8S,9R*	H	OH	COOH	-
1p	*8S,9R*	H	OH	COOEt	-
Modification of 9-hydroxy group					
1q	*8R*	OMe	H	ethyl	-
1r	*8S,9R*	OMe	OAc	vinyl	-

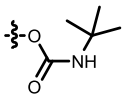
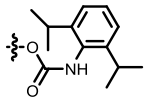
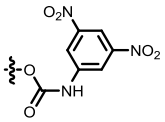
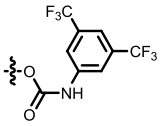
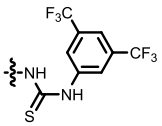
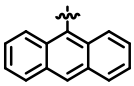
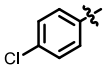
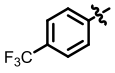
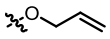
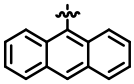
1s	*8S,9R*	OMe	OBz	vinyl	-
1t	*8S,9R*	OMe		vinyl	-
1u	*8S,9R*	OMe		vinyl	-
1v	*8S,9R*	OMe		vinyl	-
1w	*8S,9R*	OMe		vinyl	-
9-Amino-9-*epi*-alkaloids and their derivatives					
2a	*8S,9S*	OMe	-	vinyl	-
2b	*8R,9R*	OMe	-	vinyl	-
2c	*8S,9S*	H	-	vinyl	-
2d	*8S,9S*	OMe	-	ethynyl	-
2e	*8R,9R*	OMe		vinyl	-
Quaternary ammonium salt derivatives					
3a	*8S,9R*	OMe	OH		Cl^-
3b	*8S,9R*	OMe	OH		Br^-
3c	*8S,9R*	OMe	OH		Br^-
3d	*8S,9R*	H			Br^-

TABLE 2 *In vitro* activity of cinchona alkaloid derivatives against tumour and normal cells[a]

	Tumour cells						Normal cells	
Compound	**KB**	**HeLa**	**MCF-7**	**A-549**	**Hep-G2**	**U-87**	**HDF**	**CHO**
Unmodified cinchona alkaloids								
1a	55.5±0.1	56.4±1.2	55.5±0.1	n.d.[b]	n.d.	n.d.	160±1	n.d.
1b	38.3±0.3	37.0±0.2	36.4±0.1	n.d.	n.d.	n.d.	151±1	n.d.
1c	109±1	155±1	129±1	n.d.	n.d.	n.d.	397±1	n.d.
1d	67.6±0.1	65.9±0.3	96.8±0.7	n.d.	n.d.	n.d.	236±1	n.d.
1e	67.2±0.5	64.7±0.2	64.7±1.4	67.5±0.1	n.d.	n.d.	120±1	111±3
Modification of vinyl group								
1f	83.6±0.1	82.7±0.6	82.7±1.0	66.8±0.1	n.d.	n.d.	123±1	89.1±0.1
1g	67.7±0.9	69.8±0.2	75.1±1.7	55.8±0.1	n.d.	n.d.	184±1	211±2
1h	59.4±0.5	58.5±1.2	58.2±0.4	58.2±0.9	n.d.	n.d.	85.8±0.1	65.9±1.5
1i	76.8±0.2	73.3±0.2	70.7±0.1	52.5±0.9	n.d.	n.d.	89.6±0.4	102±1
1j	40.3±0.4	38.4±1.6	38.4±0.2	25.9±1.1	n.d.	n.d.	61.8±0.3	54.4±1.0
1k	229±1	169±1	194±1	173±2	n.d.	n.d.	273±1	293±1
1l	52.4±0.2	49.6±2.6	49.6±0.4	n.d.	n.d.	n.d.	436±1	n.d.
1m	149±1	140±1	153±1	n.d.	n.d.	n.d.	270±1	n.d.
1n	86.4±0.1	92.8±2.6	92.8±0.5	75.6±0.2	n.d.	n.d.	101±4	102±1
1o	122±1	102±1	112±1	112±1	n.d.	n.d.	193±1	195±1
1p	55.8±0.8	56.7±0.3	55.8±0.1	32.3±0.1	n.d.	n.d.	61.7±1.8	76.7±0.1
Modification of 9-hydroxy group								
1q	145±1	158±1	137±1	110±1	n.d.	n.d.	284±1	263±1
1r	60.3±1.7	73.7±1.1	59.8±0.2	35.2±0.1	n.d.	n.d.	131±1	114±1
1s	94.7±1.5	105±1	107±1	74.9±0.4	n.d.	n.d.	145±1	145±1
1t	73.2±0.8	72.2±0.1	59.5±1.8	50.5±0.3	n.d.	n.d.	89.7±0.1	89.7±0.1

1u	3.98±0.02	3.79±2.07	3.22±0.16	0.95±0.03	n.d.	n.d.	11.6±0.1	11.4±0.4
1u (MTT)	5.65±0.06	5.80±2.03	n.d	5.39±0.23	4.06±0.21	5.20±0.63	7.36±0.17	n.d.
1w	3.11±0.12	1.90±0.05	2.24±0.01	2.24±0.31	n.d.	n.d.	4.31±0.05	4.83±0.91
1w (MTT)	7.44±0.07	7.06±0.02	n.d.	7.56±0.12	7.11±0.03	8.47±0.76	10.5±0.7	n.d.
9-Amino-9-*epi*-alkaloids and their derivatives								
2a	15.7±1.0	13.9±0.4	15.5±1.6	13.2±0.5	n.d.	n.d.	25.4±0.1	27.1±0.8
2b	18.5±0.4	18.5±0.9	18.5±0.8	16.9±0.4	n.d.	n.d.	38.1±0.1	41.6±0.1
2c	15.1±0.3	14.9±0.5	13.2±0.1	12.4±0.1	n.d.	n.d.	21.1±2.6	21.1±0.9
2e	4.88±0.6	2.86±0.23	3.20±0.03	2.86±0.03	n.d.	n.d.	19.3±0.8	18.5±0.1
2e (MTT)	2.91±0.07	1.79±0.05	n.d.	1.98±1.40	3.13±0.25	1.96±1.19	4.76±0.13	n.d.
Quaternary ammonium salt derivatives								
3d	1.32±0.02	1.32±0.08	1.32±0.01	0.20±0.11	n.d.	n.d.	2.64±0.15	1.82±0.49
3d (MTT)	6.62±1.27	6.92±0.10	n.d.	8.01±0.03	7.61±0.02	6.89±0.81	11.8±0.1	n.d.
Reference drugs								
cytarabine (MTT)	0.62±0.04	0.66±0.12	n.d.	0.45±0.70	0.53±0.25	0.78±0.37	1.32±0.49	n.d.
doxorubicin (MTT)	1.25±0.13	1.14±0.29	n.d.	1.31±0.02	1.34±0.24	1.42±0.04	2.93±0.09	n.d.

[a]The cytotoxic activity of test compounds was determined using the SRB assay or the MTT assay (indicated). Results are mean values of three independent experiments and expressed as GI_{50} values ± SD in µM. KB, human cervix carcinoma derived from HeLa cells; HeLa, human cervix carcinoma; MCF-7, human breast cancer; A-549, human lung cancer; HepG2, human hepatocyte carcinoma; U-87, human glioblastoma: HDF, human dermal fibroblast; CHO, Chinese hamster ovary cells.

[b]n.d., not determined.

TABLE 3 Selectivity indices for the most active compounds (with GI_{50} values <10 µM)

	SI[a]					
Compound	**KB**	**HeLa**	**MCF-7**	**A-549**	**Hep-G2**	**U-87**
1u	2.9	3.1	3.6	12.2	n.d.[b]	n.d.
1u (MTT)	1.3	1.3	n.d.	1.4	1.8	1.4
1w	1.4	2.3	1.9	1.9	n.d.	n.d.
1w (MTT)	1.4	1.5	n.d.	1.4	1.5	1.2
2e	4.0	6.7	6.0	6.7	n.d.	n.d.
2e (MTT)	1.6	2.8	n.d.	2.4	1.5	2.4
3d	2.0	2.0	2.0	13.2	n.d.	n.d.
3d (MTT)	1.8	1.7	n.d.	1.5	1.6	1.7
cytarabine (MTT)	2.1	2.0	n.d.	2.9	2.5	1.7
doxorubicin (MTT)	2.3	2.6	n.d.	2.2	2.2	2.1

[a]The selectivity index (SI) is defined as the ratio of the GI_{50} on the normal cell line HDF to the GI_{50} on the respective cancer cell line and were calculated from GI_{50} values shown in Table 1. An SI >1.0 indicates that the compound has a greater activity against tumour cells than against normal cells.

[b]n.d., not determined.

TABLE 4 *In vitro* activity of cinchona alkaloid derivatives against *T. b. brucei* and HL-60 cells

	T. b. brucei		**HL-60 cells**		**Selectivity**	
Compound	**MIC[a]**	**GI_{50}[b]**	**MIC[a]**	**GI_{50}[b]**	**MIC ratio[c]**	**GI_{50} ratio[d]**
Unmodified cinchona alkaloids						
1a	10	3.26±0.32	>100	>100	>10	>30
1b	10	2.62±0.26	>100	>100	>10	>38
1c	10	3.61±0.29	>100	>100	>10	>27
1d	100	23.8±10.1	>100	>100	>1	>4.2
1e	>100	44.1±9.1	>100	>100	n.a.[e]	>2.2
Modification of vinyl group						
1f	10-100 (40)	3.75±0.93	>100	>100	>1-10	>26
1g	10	2.72±0.35	>100	>100	>10	>36
1h	100	24.8±3.2	>100	>100	>1	>4.0
1i	100	26.2±4.7	>100	>100	>1	>3.8
1j	10	5.58±3.05	>100	>100	>10	>17
1k	10-100 (40)	4.98±0.88	>100	>100	>1-10	>20
1l	100	22.3±6.2	>100	>100	>1	>4.4
1m	100	29.4±13.6	>100	>100	>1	>3.4
1n	>100	>100	>100	>100	n.a.	n.a.
1o	>100	>100	>100	>100	n.a.	n.a.
1p	100	34.5±6.4	>100	>100	>1	>2.8
Modification of 9-hydroxy group						
1q	100	27.2±6.4	>100	>100	>1	>3.6
1r	100	16.5±8.7	>100	>100	>1	>6.0
1s	10-100 (40)	3.44±0.72	100	35.7±2.6	1-10	10.4
1t	100	22.2±7.7	>100	>100	>1	>4.5
1u	100	26.7±4.7	100	37.6±1.7	1	1.4
1v	10	3.24±1.44	100	35.5±1.2	10	11.0

1w	10	3.09±0.42	100	35.3±1.7	10	11.4
9-Amino-9-*epi*-alkaloids and their derivatives						
2a	>100	>100	>100	>100	n.a.	n.a.
2b	100	30.6±5.0	>100	>100	>1	>3.2
2c	100	26.5±6.8	>100	>100	>1	>3.7
2d	>100	48.1±21.6	>100	>100	n.a.	>2.0
2e	1	0.33±0.04	10	3.16±0.10	10	9.6
Quaternary ammonium salt derivatives						
3a	10	3.22±0.22	100	31.9±1.2	10	9.9
3b	100	38.5±1.9	>100	>100	>1	>2.5
3c	100	43.5±5.2	>100	>100	>1	>2.2
3d	1-10 (7)	0.41±0.17	10	3.97±0.43	1-10	9.7
Reference drug						
suramin	0.1	0.034±0.002	>100	>100	>1000	>2941

[a]MIC values are expressed in μM. In case that not all three experiments gave the same MIC value, a range from the lowest to the highest MIC values observed is instead provided with the average MIC value in parenthesis.

[b]GI_{50} values are expressed as mean values ± SD in μM.

[c]Defined as $MIC_{(HL\text{-}60)}/MIC_{(T.\ b.\ brucei)}$.

[d]Defined as $GI_{50(HL\text{-}60)}/GI_{50(T.\ b.\ brucei)}$.

[e]n.a., not assignable (since values for both HL-60 cell and *T. b. brucei* are >100 μM).