Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2189–2194

BIOORGANIC & MEDICINAL CHEMISTRY

The Influence of Glutathione and Cysteine Levels on the Cytotoxicity of Helenanolide Type Sesquiterpene Lactones Against KB Cells

Jörg Heilmann,[a,*] Michael R. Wasescha[a] and Thomas J. Schmidt[b]

[a]*Departement für Angewandte Biowissenschaften, Institut für Pharmazeutische Wissenschaften, Eidgenössische Technische Hochschule (ETH) Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland*

[b]*Institut für Pharmazeutische Biologie der Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany*

Dedicated to Professor Dr. Otto Sticher, Institute of Pharmaceutical Sciences, ETH Zurich on the Occasion of his 65th Birthday

Received 3 March 2001; accepted 18 April 2001

Abstract—The biological activities of sesquiterpene lactones have been attributed to their reactivity with the cysteine residues of functional proteins forming covalent bonds via Michael type addition. In the present study we investigated the influence of different L-cysteine (cys) and glutathione (GSH) concentrations on the cytotoxicity of the sesquiterpene lactones (STLs) helenalin, 11α,13-dihydrohelenalin acetate and chamissonolide against KB cells. Due to the significantly higher reactivity of the α-methylene-γ-lactone (ML) towards cys as compared with the cyclopentenone (CP) site at physiological pH, addition of 20, 50 and 100 molar equivalents of cys decreased the cytotoxicity of helenalin and chamissonolide, whereas the cytotoxicity of 11α,13-dihydrohelenalin acetate remained unaffected. In contrast, the influence of GSH addition on the cytotoxicity of 11α,13-dihydrohelenalin acetate depends on the concentration of GSH added. Concentration–effect curves obtained for chamissonolide and GSH resembled the decline in cytotoxicity after cys addition. Helenalin showed a biphasic shape of the concentration–effect curve for the 100:1 GSH/helenalin ratio resembling at higher doses the chamissonolide and in lower doses the 11α,13-dihydrohelenalin acetate curve at 50-fold excess. These results can be explained by the different reactivity and equilibrium conditions for thiol addition of the two reactive centers of bifunctional STLs in cellular test systems and verified a clear correlation between the different reactivity of their electrophilic centers and the observed biological effects in in-vitro cell systems.

Introduction

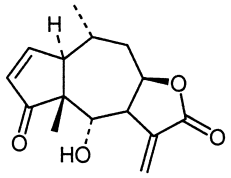
Sesquiterpene lactones (STL) are known to bind covalently to sulfhydryl groups of enzymes and other functional proteins by Michael type addition of their electrophilic α,β-unsaturated carbonyl structures. It is generally accepted that most of their biological effects, for example, the anti-inflammatory and anti-neoplastic activity, are due to this reaction with biological nucleophiles.[1,2,12]

It has been clearly demonstrated that the reaction kinetics of helenanolide type STLs with sulfhydryl reagents such as L-cysteine (cys) and glutathione (Gly-Cys-γ-Glu = GSH) depends on the number and type of the α,β-unsaturated carbonyl sites, the pH of the medium, the concentration of the thiol and the character of the sulfhydryl target structures.[3,4] Summarizing these results, a certain specificity of the reaction of STLs with biological thiols can be assumed.

A first investigation in a cellular test assay using the transcription factor NF-κB as target revealed that the isolated mono- and bisglutathionyl adducts of helenalin (**1**) showed similar inhibitory activity of NF-κB as helenalin itself.[4] Formation of glutathionyl adducts with the cyclopentenone (CP) structure of helenanolides is reversible at physiological pH and the biological activity of the GSH-adducts has been attributed to the fact that a fraction of free STL molecules will be available for reaction with protein targets even in the presence of excess GSH.[4]

*Corresponding author. Tel.: +41-1-635-6049; fax: +41-1-635-6882; e-mail: heilmann@pharma.ethz.ch

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00131-6



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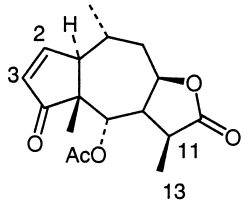

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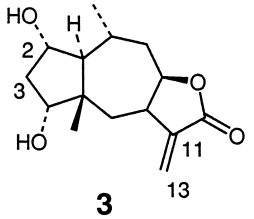

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It has been demonstrated that the strength of the biological effects of STLs can be decreased by addition of low molecular weight thiols, such as cys or GSH (e.g., refs 13–15). However, the influence of different levels of GSH and cys on the cytotoxicity or any other biological activity of STLs in cellular systems has not been studied systematically up to now. Different constitutive GSH levels in cells of different tissues or cell lines might be responsible for the observation that some cells are more susceptible to STL cytotoxicity than others (e.g., refs 8 and 15), since they might protect other sulfhydryl containing structures in the cell and thus decrease the rate of cell death. Therefore we investigated the influence of different GSH and free cys levels on the cytotoxicity of helenalin (**1**), 11α,13-dihydrohelenalin acetate (**2**) and chamissonolide (**3**) against the KB cell line (epithelial cells derived from a tumor, ATCC CCL17). The selection criteria for the compounds to be investigated were the possibility to compare the effects of the bifunctional helenalin (**1**), combining a cyclopentenone (CP) and an α-methylene-γ-lactone (ML) site in one molecule, with the effects of the monofunctional 11α,13-dihydrohelenalin acetate (**2**) and chamissonolide (**3**), containing only the CP *or* the ML site, respectively.

Results and Discussion

Initially, the IC_{50} for cytotoxicity of all three STLs, as well as of cys and GSH against the KB cells was determined (see Table 1). The STL concentrations chosen for the experiments conducted under cys and GSH addition include measured values in the slope as well as in the plateaus of the concentration–effect curves and it was shown that the thiols did not possess any cytotoxic effects themselves. Furthermore, the cytotoxic activity of the isolated C-2-mono-glutathionyl adducts of helenalin and 11α,13-dihydrohelenalin acetate was determined. In consistency with the above mentioned results in the NF-κB assay, both adducts were found to possess considerable cytotoxic activity (see Table 1). The finding that the adduct of dihydrohelenalin acetate—in spite of being devoid of potentially alkylating structure elements—is still cytotoxic is in agreement with the observation that alkylating structures are liberated to a certain extent at physiological pH due to the reversibility of GSH addition.[4]

The influence of three different cys concentrations, added in molar ratios of 20, 50 and 100:1, on the cytotoxicity of helenalin (**1,** IC_{50} 0.64 μM), 11α,13-dihydrohelenalin acetate (**2,** IC_{50} 6.3 μM) and chamissonolide (**3,** IC_{50} 2.3 μM) is depicted in Figure 1A–C. The cytotoxicity of chamissonolide (**3**) is most sensitive to the addition of cys resulting in a complete loss of cytotoxicity at the 50:1 ratio. At the same molar ratio of cys and helenalin (**1**) the cell viability is only about 70% compared to the untreated cells. Further experiments showed that a ratio of 500:1 (cys/helenalin) is required for an unaffected cell viability (data not shown). In contrast, the cytotoxicity of 11α,13-dihydrohelenalin acetate (**2**) is completely uneffected by cys addition. These results strongly suggested that the 'anti-cyto-

Table 1. Cytotoxicity of the investigated sesquiterpene lactones, thiols and sesquiterpene lactone adducts against KB cells (IC_{50} values given in μM, means ± SD, $n = 4$)

Compound	Cytotoxicity (IC_{50} in μM)
Helenalin (**1**)	0.64 ± 0.09
Dihydrohelenalin acetate (**2**)	6.3 ± 0.31
Chamissonolide (**3**)	2.3 ± 0.15
Cysteine	> 10,000
Glutathione	> 10,000
2β-*S*-Glutathionyl-2,3-dihydrohelenalin	1.2 ± 0.01
2β-*S*-Glutathionyl-2,3,11α,13-tetrahydrohelenalin acetate	6.5 ± 1
13-*S*-Glutathionyldihydrochamissonolide[a]	> 15
Podophyllotoxin	0.02 ± 0.001

[a]Mixture of 11α*H*- and 11β*H*-configurated adducts in a 20:80 ratio.

toxic' effect of cys is due to the reaction with the α-methylene-γ-lactone site of the STLs. This is in agreement with our previous findings[3] showing a dramatically increased reactivity of the ML reaction site towards the sulfhydryl group of free cys as compared with the CP group or with GSH addition to this center. The reaction half life ($t_{1/2}$) for binding of cys to C-13 (ML) is <5 min (20 mM STL and 40 mM cys) while binding of cys to C-2 (CP) under the same conditions showed a $t_{1/2}$ of about 1000 min [3], nearly a third of the experiment time for the cytotoxicity assay and therefore probably too slow to influence the cytotoxicity of 11α,13-dihydrohelenalin acetate (**2**).

The results after adding GSH in the same molar ratios, 20:1, 50:1 and 100:1, are shown in Figure 2A–C. The decrease in cytotoxicity of chamissonolide (**3**) after addition of GSH resembled the results with cys. In contrast with the result for free cys, a clear influence of GSH on the CP site mediated cytotoxicity of 11α,13-dihydrohelenalin acetate (**2**) could be observed. At a 20-fold excess of GSH its cytotoxicity is unaffected, but a 50-fold surplus influenced cytotoxicity incontinuously within the concentration–effect curve. Absolute concentrations lower than 7.5 μM 11α,13-dihydrohelenalin acetate (**2**) and 350 μM GSH showed nearly equal cytotoxicity compared to pure **2**, whereas at higher STL

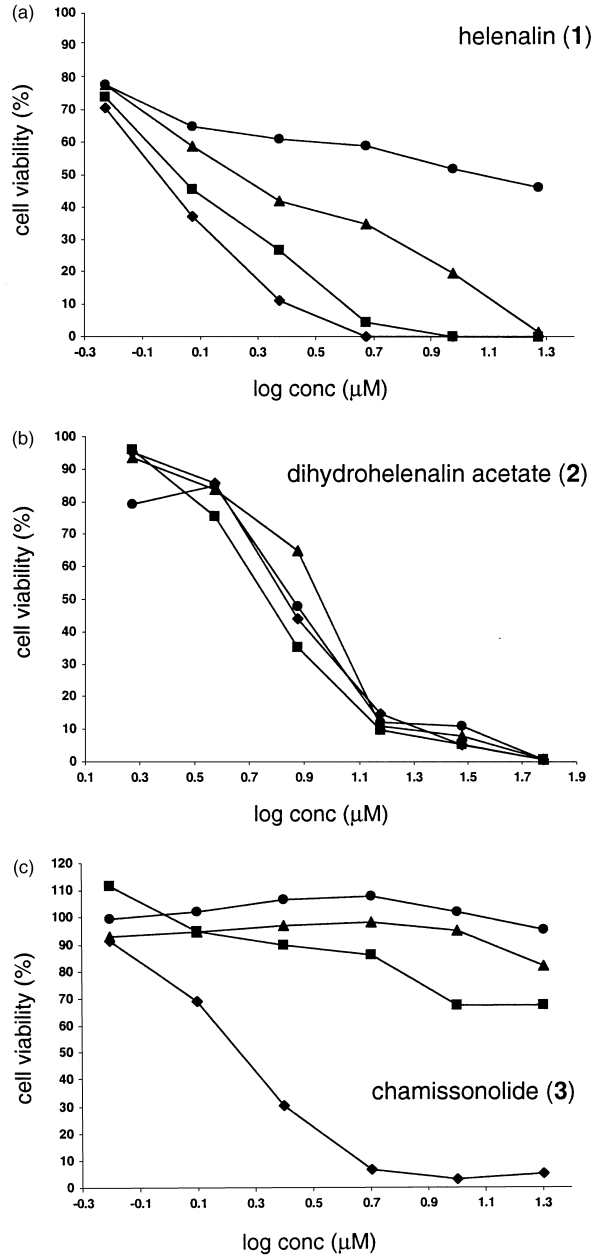

Figure 1. Cytotoxicity of the sesquiterpene lactones (STL) (a). helenalin (**1**), (b). 11α,13 dihydrohelenalin acetate (**2**) and (c). chamissonolide (**3**) under the influence of cystein addition. Symbols: rhombic, pure STL; square, STL + 20 mol equiv cys; triangle, STL + 50 mol equiv cys; circle, STL + 100 mol equiv cys. Standard deviations are omitted for clarity (see Experimental).

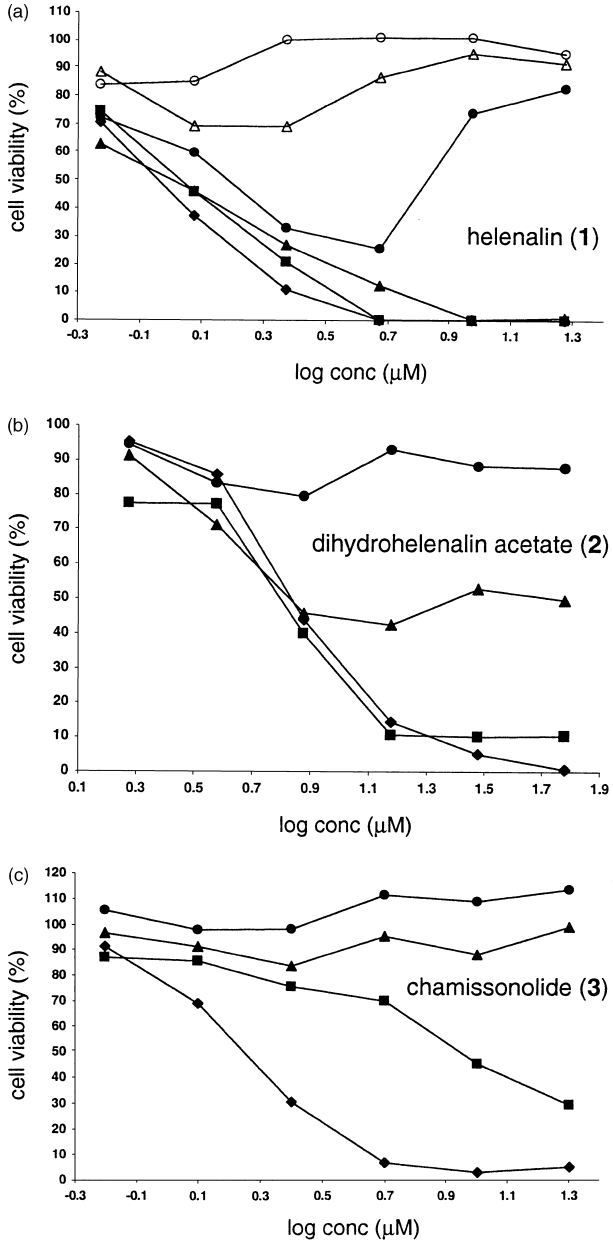

Figure 2. Cytotoxicity of the sesquiterpene lactones (STL) (a). helenalin (**1**), (b). 11α,13 dihydrohelenalin acetate (**2**) and (c). chamissonolide (**3**) under the influence of GSH addition. Symbols: rhombic, pure STL; square, STL + 20 mol equiv GSH; filled triangle, STL + 50 mol equiv GSH; filled circle, STL + 100 mol equiv GSH; open triangle + 200 mol equiv GSH; open circle, 500 mol equiv GSH. Standard deviations are omitted for clarity (see Experimental).

concentrations 50-fold excess GSH led to a significant decrease in cytotoxicity. One hundred-fold surplus of GSH resulted in almost normal cell growth within a broad concentration range. These observations can be rationalized if the previous finding is taken into consideration, that GSH binding to the CP site is reversible at physiological pH. The equilibrium constant $K = (= c(\text{Adduct})/[c(\text{STL})\cdot c(\text{GSH})])$ was determined as 0.93 at pH 8, indicating that the rate constant for the forward reaction is only marginally higher than that of the reverse reaction.[4] Thus, up to a certain threshold concentration, due to the low absolute concentrations of reactants, the amount of free STL molecules (or, in other words, the average time that an STL molecule spends in the unalkylated form) at equilibrium is obviously high enough to cause cytotoxicity to the same extent as the STL without GSH addition. Above this threshold, however, the amount of active STL is decreased to a level too low for an unaffected cytotoxic effect.

The pH in the test assay was not influenced by addition of higher GSH or cys concentrations, which excludes variations in binding half lives at the cyclopentenone site based on pH fluctuations. Due to the differences observed between the CP- and the ML-type alkylants, **2** and **3**, respectively, it could be hypothesized that the reaction of the ML structure of **3**—although much slower[3,4]—should be less readily reversible than that of the CP of **2**. This assumption could be proven experimentally by studying the tendency of the isolated GSH adduct of **3** to decompose at physiological pH, in an analogous way as previously reported for the CP-adduct of **2**.[4] When dissolved in phosphate buffer pH 7.4, no increase of UV-absorbance at 217 nm (λ_{max} of **3**) could be observed over a period of several hours, indicating that this adduct is much more stable than the CP adduct of **2**, or, in other words, that addition of GSH to **3** is irreversible under conditions where the analogous reaction of the CP of **2** is essentially reversible (data not shown). In accordance with this, the cytotoxicity of 13-*S*-glutathionyldihydrochamissonolide (mixture of 11α*H*- and 11β*H*-configurated adducts in a 20:80 ratio) against the KB cells showed a strong decrease to an IC_{50} higher than 15 μM (see Table 1).

The concentration–effect curves of helenalin (**1**) after addition of GSH are clearly distinct from the other STLs and also from its behaviour after addition of cys. Within the concentration–effect curves of the molar ratios 100:1 and 200:1, the cytotoxicity was not regularly decreased and, in a similar way as observed for **2**, depended on the employed absolute concentrations of helenalin (**1**) and GSH. Concentrations higher than 4.7 μM helenalin (**1**) and 470 μM GSH resulted in an over proportional decrease in cytotoxicity. The concentration–effect curves obtained with a lower (20- and 50-fold) or higher (500-fold) surplus of GSH lack such differential effects, that is cytotoxicity is more or less unaffected at 20- or 50-fold and completely abolished at 500-fold excess.

The conspicuous behaviour of **1** in the presence of 100- and 200-fold excess GSH can be explained by the fact that it possesses both reactive structure elements and by the different reactivity/reversibility of the reaction at these centres.

Assuming that each of these reactive sites contributes more or less independently to activity, it becomes clear that the curve for **1** at 100-fold GSH must be compared to those of **2** and **3** at 50-fold excess. In the low concentration range, up to 4.7 μM **1**, the effect is dominated by the reversible reaction of its CP site and the curve is congruent with the 50-fold excess curve of **2** for the same reasons as stated above. Although all molecules are probably transformed to the ML-monoadduct under these conditions, some fraction of the CP sites is still free and capable of damaging the cells. Above the threshold concentration, the curve resembles that of **3** at 50-fold excess. In consistency with the irreversibility of GSH addition to the ML site, in this range of concentrations the majority of the STL molecules are present in the form of the bis-adduct that does not possess any reactive centres capable of causing deleterious effects to cellular structures. Again, monitoring the pH excluded variations in binding half lives at the cyclopentenone or the α-methylene-γ-lactone site based on pH fluctuations.

It can therefore be concluded that the shape of the curve is characterized by the bifunctionality of helenalin (**1**) and a different ratio of free helenalin, mono- and bis-glutathionyl adducts due to the absolute concentration of reactants. To further confirm this assumption, investigation of other mono- and bifunctional STLs is in progress.

The finding that addition of different concentrations of cys or GSH can lead to differential effects on the cytotoxicity of STLs is of general importance. GSH is an extraordinarily important peptide occurring in high but variable concentrations (0.5–10 mM) in living cells.[7] Studies on the cytotoxicity of helenanolides against two cancer cell lines differing in their intracellular GSH levels (GLC_4 and COLO 320) showed a tendency to lower cytotoxicity against the cells with a higher GSH level (COLO 320) for helenalin and chamissonolide derivatives.[8] In contrast 11α,13-dihydrohelenalin revealed nearly the same cytotoxicity against GLC_4 and COLO 320 cells.[8] Due to our findings we assume that the GSH/STL ratio for this compound in both cell lines corresponded with the concentration–effect curve of **2** obtained for KB cells in lower concentrations (below the threshold concentration).

It must furthermore be noted as a consequence of the present results that for different cell types possessing different levels of GSH, different threshold concentrations must exist with respect to a particular STL, which will influence the STLs observed cytotoxicity. This may be one of the reasons for the observation that in many cases no clear correlation exists between the cytotoxic effects of a series of STLs against different cell lines (see comments on ref 15 in ref 12). While in one cell line, a certain STL concentration may lie below the critical

value, it may lie above such a threshold in another cell line with a different GSH level.

As mentioned above, a previous study[4] revealed that the inhibition of NF-κB required only slightly higher concentrations of the CP-mono- and the CP,ML-bis-glutathionyl adducts of helenalin (**1**) than of the free STL itself which was assumed to be caused by the reversibility of GSH addition leading to a fraction of free **1** capable of reacting with the target protein. In view of the present results with respect to the reversibility of GSH addition to the ML site, it may be concluded that the unexpectedly high activity of the bis-adduct is mediated mainly by the ML-monoadduct of **1**.

Summing up the results, clear correlations between the different reactivity of the electrophilic centers towards GSH and L-Cys and the in-vitro cytotoxicity of the STLs were proved. It can be concluded that the type of the α,β-unsaturated carbonyl site(s) present in the STL molecule, the concentration of the thiol and the character of the sulfhydryl target structure contribute significantly to the certain specificity of the effect of STLs on cellular biological systems.

A surplus of GSH significantly decreases the cytotoxic effects of helenanolide type STLs by reducing the number of free CP and ML sites. Due to the high GSH concentrations in living cells and the fact that STLs have recently been shown to exert their anti-inflammatory activity mainly by inhibiting the nuclear transcription factor NF-κB,[5,6] it would be of interest how this biological activity is influenced by different excess concentrations of GSH. Different concentration–effect curves could be one possible reason why the treatment of acute inflammations without cytotoxic effects is possible with STL preparations.

Experimental

Chemicals

The sesquiterpene lactones were isolated from *Arnica* species (see literature cited in ref 9). Glutathione and L-cysteine were purchased from Fluka Chemicals (Buchs, Switzerland). Glutathione adducts of **1** and **2** were prepared as described previously.[3,4] The glutathione addition to **3** was carried out as follows: To a solution of 10 mg **3** in 1.5 mL iPrOH, 50 mg GSH, dissolved in 5 mL phosphate buffer pH 7.4, were added. The reaction was monitored by TLC.[3,4] When no further increase of the product zone was observed (3 days), the solution was concentrated under reduced pressure to a volume of about 1 mL and separated on Sephadex LH-20/water. Eluates containing the product were combined and rechromatographed under the same conditions (3 times), yielding 7.9 mg of a mixture of the 11α and 11β configurated C-13-monoadducts of **3** (20:80), still containing a small impurity of glutathione disulfide.

^{1}H NMR data (500 MHz, D_2O; δ): 11β*H*-13-*S*-glutathionyldihydrochamissonolide (major adduct): 5.03 (ddd, J = 3.5, 8.9, 12.3 Hz, H-8), 4.94 (ddd, J = 3.0, 8.0, 9.5 Hz, H-2), 4.63 (dd J = 5.4, 8.4 Hz, H-α-cys), 4.07 (d, J = 5.5 Hz, H-4), 3.95 (s, 2H, H_2-β-gly), 3.86 (d, J = 12 Hz, H-6), 3.82 (t, J = 6.4 Hz, H-α-glu), 3.23 (m*, H-13a), 3.15 (dd, J = 5.5, 14.0 Hz, H_2-β-cys(A)), 3.05 (m*, 2H, H-11, H-13b), 3.00 (m*, H-7), 2.97 (dd, J = 8.4, 14.2 Hz, H_2-β-cys(B)), 2.75 (ddd, J = 5.5, 9.3, 16.2 Hz, H-3β), 2.55 (m, 2H, H_2-γ-glu), 2.19 (m, 2H, H_2-β-glu), 2.13 (s, 3H, CH_3-ac), 2.15 (m**, H-1), 2.01 (m, 2H, H-10, H-9β), 1.78 (dd, J = 3.5, 12.8 Hz, H-9α), 1.47 (dd, J = 2.9, 16.2 Hz, H-3α), 1.09 (d, 3H, J = 7.1 Hz, CH_3-14), 0.988 (s, 3H, CH_3-15).

11α*H*-13-*S*-glutathionyldihydrochamissonolide (minor adduct): 4.97 (ddd, J = 2.7, 7.9, 11.0 Hz, H-8), 4.95 (m**, H-2), 4.63 (dd, J = 4.7, 9.0 Hz, H-α-cys), 4.07 (m**, H-6), 4.03 (d, J = 5.2 Hz, H-4), 3.93 (s, 2H, H_2-β-gly), 3.81 (m**, H-α-glu), 3.47 (ddd, J = 5.7, 7.3, 9.9 Hz, H-11(α)), 3.27 (ddd, J = 8.0, 10, 10 Hz, H-7), 3.21 (m**, H-13a), 3.15 (m**, H_2-β-cys(A)), 3.12 (m**, H-13b), 2.97 (m**, H_2-β-cys(B)), 2.74 (m**, H-3β), 2.55 (m**, H_2-γ-glu), 2.28 (dd, J = 7.9, 11.2 Hz, H-1), 2.19 (m**, H_2-β-glu), 2.13 (s, 3H, CH_3-ac), 2.07 (m**, H-9β), 2.01 (m**, H-10), 1.80 (m**, H-9α), 1.45 (dd, J = 2.8, 16.2 Hz, H-3α), 1.08 (d, 3H, J = 7 Hz, CH_3-14), 0.94 (s, 3H, CH_3-15).

* Non-first-order spin system, **multiplicity not determined due to signal overlap, shift values extracted from COSY spectrum.

To study the reversibility of the reaction[4], the UV absorbance of a 0.1 mM solution (phosphate buffer pH 7.4) of this adduct mixture was monitored at λ 217 nm over 3 h during which no significant increase of absorbance was observed. Similarly, at pH 8.3, only a marginal increase of absorbance was found over 10 h.

Cytotoxicity study using KB cells

The cytotoxicity of the compounds was determined using the KB cell line (ATCC CCL 17. The test was carried out with some modifications according to the screening technique of Swanson and Pezzuto[10] in 96-well plates (Falcon) with an inoculum of 2.5×10^4 cells/mL. Test solutions were made as stocks in ethanol. Test concentrations were freshly prepared by diluting the stock solution with water to the required concentration. Final ethanol concentration was 1% (v/v) or less. Total assay volume was 150 μL. STLs and GSH/L-cys were added simultaneously. For quantification of the cytotoxicity, 15 μL of an aqueous solution of methylthiazolyltetrazolium chloride (MTT, Fluka, 5 mg/mL in PBS) was added after 72 h.[11] During incubation at 37 °C for 4 h, the surviving cells metabolized MTT into an insoluble formazan dye. The culture medium was drawn off and the formazan dye was dissolved using 150 μL of 10% SDS (sodium dodecylsulfate) in water. After 24 h of incubation at room temperature, the optical density was measured at 540 nm using a microplate reader (MRX, Dynex Technologies, Embrach, Switzerland). For determination of the IC_{50} values, the optical density was plotted against the log concentration and six different concentrations have been tested. Every test was

performed at least in duplicates and all experiments have been repeated at least twice. Maximal observed standard deviation was about 20% (absolute). Positive control measurements were performed with podophyllotoxin.

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