

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/277015743>

Discovery of Tricyclic Clerodane Diterpenes as Sarco/Endoplasmic Reticulum Ca²⁺-ATPase Inhibitors and Structure–Activity Relationships

ARTICLE in JOURNAL OF NATURAL PRODUCTS · MAY 2015

Impact Factor: 3.8 · DOI: 10.1021/acs.jnatprod.5b00062

READS

40

9 AUTHORS, INCLUDING:



Christian De Ford

University of Freiburg

5 PUBLICATIONS 3 CITATIONS

[SEE PROFILE](#)



Natalya U Fedosova

Aarhus University

59 PUBLICATIONS 831 CITATIONS

[SEE PROFILE](#)



Poul Nissen

Aarhus University

188 PUBLICATIONS 12,226 CITATIONS

[SEE PROFILE](#)

Discovery of Tricyclic Clerodane Diterpenes as Sarco/Endoplasmic Reticulum Ca^{2+} -ATPase Inhibitors and Structure–Activity Relationships

Christian De Ford,^{†,‡,§} Carlos Calderón,[#] Pankaj Sehgal,^{||,⊥} Natalya U. Fedosova,^{||,⊥} Renato Murillo,[#] Claus Olesen,^{||,⊥} Poul Nissen,^{||,⊥} Jesper V. Møller,^{||,⊥} and Irmgard Merfort*,^{†,‡,§}

[†]Department of Pharmaceutical Biology and Biotechnology, Albert Ludwigs University Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

[‡]Spemann Graduate School of Biology and Medicine (SGBM), Albert Ludwigs University Freiburg, Albertstrasse 19a, 79104 Freiburg, Germany

[§]Faculty of Chemistry and Pharmacy, Albert Ludwigs University Freiburg, Albertstrasse 25, 79104 Freiburg, Germany

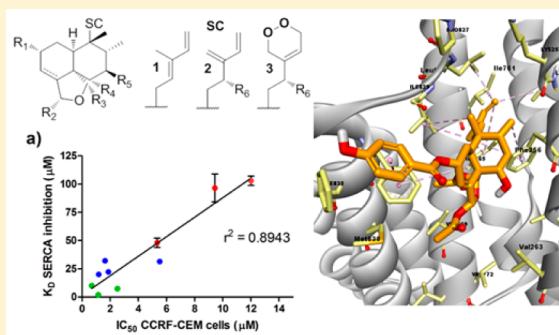
[#]Escuela de Química and CIPRONA, Universidad de Costa Rica, 2060 San José, Costa Rica

^{||}Department of Biomedicine, Aarhus University, Ole Worms Allé 3, DK-8000 Aarhus C, Denmark

[⊥]Centre for Membrane Pumps in Cells and Disease (PUMPkin), National Research Foundation, Aarhus, Denmark

S Supporting Information

ABSTRACT: Tricyclic clerodane diterpenes (TCDs) are natural compounds that often show potent cytotoxicity for cancer cells, but their mode of action remains elusive. A computationally based similarity search (CDRUG), combined with principal component analysis (ChemGPS-NP) and docking calculations (GOLD 5.2), suggested TCDs to be inhibitors of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump, which is also the target of the sesquiterpene lactone thapsigargin. Biochemical studies were performed with 11 TCDs on purified rabbit skeletal muscle sarcoplasmic reticulum membranes, which are highly enriched with the SERCA1a isoform. Casearborin D (**2**) exhibited the highest affinity, with a K_D value of 2 μM and giving rise to complete inhibition of SERCA1a activity. Structure–activity relationships revealed that functionalization of two acyl side chains (R_1 and R_4) and the hydrophobicity imparted by the aliphatic chain at C-9, as well as a C-3,C-4 double bond, play crucial roles for inhibitory activity. Docking studies also suggested that hydrophobic interactions in the binding site, especially with Phe256 and Phe834, may be important for a strong inhibitory activity of the TCDs. In conclusion, a novel class of SERCA inhibitory compounds is presented.



Cancer is a major public health problem, associated in particular with increased aging of populations.¹ Although mortality rates have declined, there is still a strong urgency to find new and effective anticancer drugs. Antibodies have an increasing impact in the market, but small-molecule natural product derived drugs constitute a large proportion of the chemotherapeutic agents used up to the present.^{2–4}

Previously, we have reported that clerodane diterpenes are highly effective against human acute lymphoblastic leukemia cells (CCRF-CEM), with a lesser influence on peripheral blood mononuclear cells from healthy human subjects.⁵ Although the number of reports on the cytotoxic activity of the clerodanes has considerably increased in recent years^{5–7} and, for example, Huang et al. suggested that cells treated with TCDs predominantly are killed by intrinsic apoptosis caused by down-regulation of Bcl-2 and Bcl-xL expression and up-regulation of Mcl-1S protein,⁸ the actual mode of action remains poorly understood.

Continuing studies on tricyclic clerodane diterpenes (TCDs), reported herein, are investigations to find the molecular target by which their cytotoxic activity can at least be partially explained. Using in silico approaches as proven tools in medicinal chemistry and drug discovery,^{2,9,10} the mechanism of action of TCDs is addressed. Web servers (e.g., CDRUG, ChemGPS-NP) as well as 3D modeling software enable such procedures, but experimental validation and refinement are required. The results are reported of a chemical structure similarity search combined with principal component analysis and docking calculations, which suggest that the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump may be an important target of TCDs, which were also confirmed by biochemical analysis. Furthermore, the effects of TCDs on SERCA activity correlated well with their effects on cell viability

Received: January 22, 2015

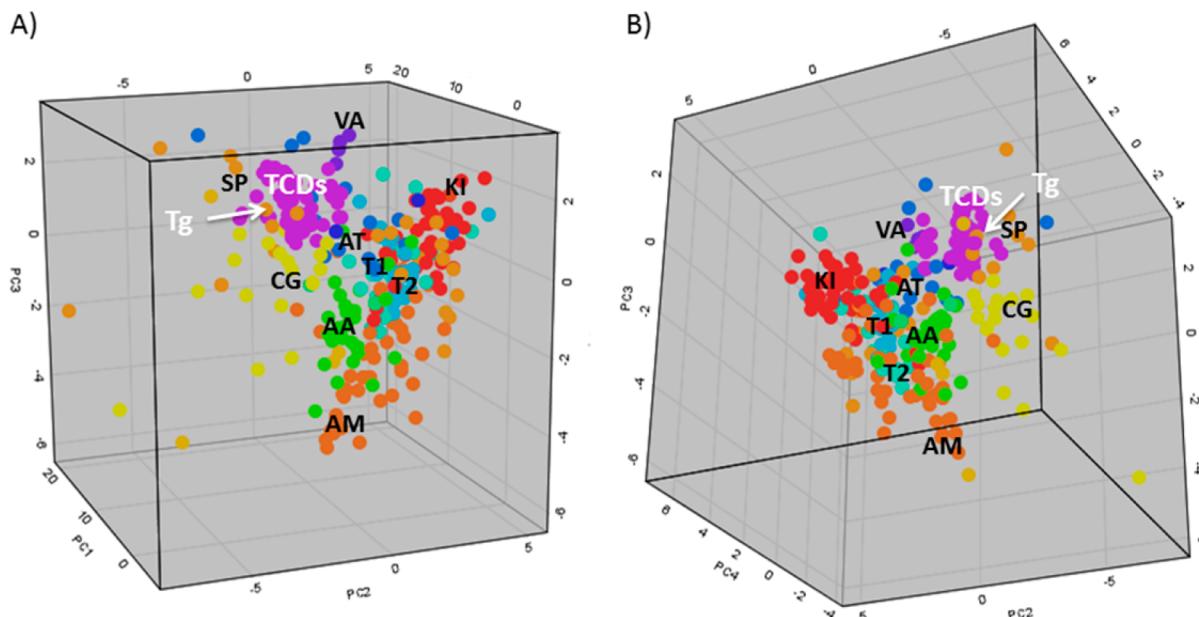


Figure 1. Score plot showing PC1 versus PC2 and PC3 (A) and PC2 versus PC3 and PC4 (B) based on the PCA with ChemGPS-NP and the used descriptors. Each color represents a different mechanism of action. AM: antimetabolites, AA: alkylating agents, T1: topoisomerase I inhibitors, T2: topoisomerase II inhibitors, AT: antitubulin agents, VA: vinca alkaloids, KI: tyrosine kinase inhibitors, CG: cardiac glycosides, SP: SERCA pump inhibitors, TCD: tricyclic clerodane diterpenes, Tg: thapsigargin. PC1 represents size, shape, and polarizability; PC2 shows aromaticity and conjugation; PC3 describes lipophilicity, polarity, and H-bond capacity; PC4 characterizes flexibility and rigidity.

of T-cell acute lymphoblastic leukemia cells (CCRF-CEM). Finally, structure–activity relationships (SARs) have pinpointed specific structural features of the TCDs that are important for the inhibition of the SERCA pump and consequently for the cytotoxic effect. Based on these SAR analyses, an optimized theoretical TCD is presented that may serve as a candidate for experimental testing and proposed starting point for future medicinal chemistry studies.

RESULTS AND DISCUSSION

Molecular Fingerprint Comparison and Similarity Search with CDRUG Identify Similarities with Thapsigargin. To obtain first insights into the mechanism of action, in silico studies were performed with CDRUG, which predicts anticancer activity of chemical compounds based on similarity of the input compounds to a database of known cytotoxic compounds.¹¹ Altogether, 95 TCDs were processed in SMILES format and compared (Table S2, Supporting Information). Three top-ranking matches were obtained, namely, thapsigargin, with the highest score, followed by oleandrin and yuanhuacacin (Figure S1, Supporting Information).

Thapsigargin, a sesquiterpene lactone, is a potent plant-derived inhibitor of the SERCA pump.¹² Thapsigargin serves as the lead compound for chemical synthesis of thapsigargin analogue prodrugs that are being explored against potential cancer targets.¹³ Inhibition of the SERCA calcium pump is believed to trigger cell death by depleting the endoplasmic reticulum (ER) stores of calcium. This causes an ER stress situation, often referred to as the “unfolded protein response”, which eventually causes cell death by intrinsic apoptosis^{14,15} and probably also by other cell death modalities. In addition to thapsigargin, other compounds such as the natural products alisol B¹⁶ and saikosaponin D¹⁷ are also SERCA pump inhibitors, but thapsigargin is by far the most potent inhibitor

identified to date with also cytotoxic and antineoplastic activities.¹⁸

Principal Component Analysis with ChemGPS-NP Confirms the SERCA Pump as a Possible Target of TCDs. To support the structural similarity of the TCDs to thapsigargin and SERCA as a possible target, a principal component analysis (PCA) and chemographic mapping with ChemGPS-NP was performed. This analysis served to characterize the chemical space occupied by clusters of chemotherapeutic compounds having the same or similar effects on cancer cells.¹⁹ Therefore, the chemical space of 95 TCDs was analyzed together with 228 chemotherapeutic compounds with a validated mechanism of action, including known SERCA pump inhibitors. The chemical space was set to have moderate flexibility (positive PC4 values), high lipophilicity, high hydrogen bond capacity (positive PC3 values), varied aromaticity (both positive and negative PC2), and a considerable size (PC1 contribution), according to the physicochemical properties of thapsigargin (Figure 1).

TCDs did not coincide with any of the predefined modes of action clusters represented by antimetabolites, alkylating agents, topoisomerase I and II inhibitors, tyrosine kinase inhibitors, tubulin-active agents, and Na^+/K^+ -ATPase inhibitors (cardiac glycosides). Instead, TCDs formed a cluster that resembles the SERCA pump inhibitor cluster. As can be depicted from Figure 1, those SERCA pump inhibitors that block Ca^{2+} transport in the E2 conformation by binding to an intramembranous binding site (e.g., thapsigargin, thapsigargin, saikosaponin D, and alisol B) are within the chemical space of the TCDs. On the other hand, the compounds that inhibit the SERCA pump in other conformations or binding sites are clustered elsewhere [e.g., cyclopiazonic acid (CPA), 2,5-di-*tert*-butyl-1,4-benzohydroquinone (BHQ), paxiline, galangin, among others]. Altogether, the relationship of the TCDs with the SERCA

pump inhibitor thapsigargin, shown by similarity search in CDRUG, was confirmed by this PCA with ChemGPS-NP.

TCDs Inhibit ATPase Activity of SERCA1a in an in Vitro Assay and Reduce Cell Viability in a Leukemia Cell Line. To confirm the SERCA pump as a target of TCDs, the ATPase activity of SERCA1a was tested in the presence of TCDs. Altogether 11 TCDs were studied, six of them (**4–6, 8–10**) were previously isolated from *Zuelania guidonia*,⁵ casearin J (**1**) was from *Casearia sylvestris*, casearborin D (**2**) and **3** were from *Casearia arborea*, and compound E (**7**) was from *Casearia nitida*. Compound **3**, the C-6-acetylated derivative of **2**, has not been published and was named casearborin F (manuscript in preparation, NMR data as well as the respective NMR spectra can be found in the Supporting Information). To include a compound without any acyl residues, TCD **1** was hydrolyzed (see Scheme S1, Supporting Information), and the structure of the obtained derivative (*7,18,19-deacylcasearin J, 11*) analyzed by NMR spectroscopy (Table 1) and HRMS. Thapsigargin¹²

Table 1. ^{13}C (100 MHz) and ^1H (400 MHz) NMR Data of **11** in CDCl_3 (δ in ppm)

position	δ_{C}	δ_{H} (J in Hz)
1 α	25.4 CH_2	1.70 m
1 β		2.00 m
2 β	72.9 CH	3.86 m
3	123.8 CH	6.17 brd (3.6)
4	145.9 C	
5	51.6 C	
6	76.6 CH	3.60 d (9.6)
7 α	73.8 CH_2	3.50 m
7 β		
8	42.2 CH	1.70 m
9	39.4 C	
10	35.6 CH	2.30 dd (14.3, 2.4)
11a	30.2 CH_2	1.60 m
11b		2.53 brdd (14.3, 10)
12	127.8 CH	5.50 m
13	132.8 C	
14	133.8 CH	6.74 dd (17.2, 10.0)
15a	114.6 CH_3	5.20 brd (17.2)
15b		5.08 brd (10.0)
16	20.0 CH_3	1.81 brs
17	11.2 CH_3	1.07 d (7.0)
18 α	97.5 CH	5.57 brs
19 α	97.8 CH	5.71 s
20	25.4 CH_3	0.85 s
C-2-OMe	57.0 CH_3	3.39 s

was used as a positive control for SERCA pump inhibition. The clerodane diterpenes were tested in a range of concentrations (1.25–100 μM) against the Ca^{2+} -ATPase in the E2 conformation (in the presence of 1 mM ethylene glycol tetraacetic acid, EGTA). Except for compound **11**, all TCDs rapidly inhibited the Ca^{2+} -ATPase activity of purified SERCA1a. Casearborin D (**2**) was the most potent inhibitor, with a K_D value of 2 μM , and the rank order of potency of TCDs was **2 > 9 > 1 > 3 > 4 > 6 > 10 > 5 > 8 > 7 > 11**. The top five TCDs exhibited lower K_D values than alisol B (27 μM)¹⁶ and saikosaponin D (50 μM).¹⁷ The onset of ATPase activity inhibition for all the TCDs was rapid and easily reversible by dilution, in contrast to that of thapsigargin, which binds with subnanomolar affinity. The results for all

compounds are given in Table 2. Figure S2 (Supporting Information) shows the dose–response curves of all the TCDs tested.

These results prompt the question of whether the inhibition of SERCA by TCDs also accounts for the observed cytotoxicity previously shown for TCDs **4–6** and **8–10**.⁵ To draw correlations between the binding affinity and the IC_{50} values obtained in the viability assay (MTT assay) with CCRF-CEM cells, TCDs **1–3** and **7** were also studied herein for their cytotoxicity in the same cell-based assay. All IC_{50} values are presented in Table 2.

Docking Experiments Place the Binding Site of TCDs at the Transmembrane Domain of SERCA. To evaluate the molecular interactions between the TCDs and SERCA1a and to correlate the scores with the experimental binding energies, computational docking studies were performed with GOLD 5.2. Docking results confirmed that TCDs bind in the transmembrane domain of the SERCA pump in the E2 conformation in the same binding site as thapsigargin, located between helices M3, M5, and M7 (Figure 2). Control docking experiments were carried out in the binding site of CPA, resulting in a divergence of solutions and low docking scores (data not shown).

Thus, the docking results were consistent with the similarity of TCDs with thapsigargin suggested by the PCA analysis. The interaction of TCDs with the SERCA pump is predominantly through nonpolar interactions with hydrophobic residues due to the low polarity of the binding site, in a similar way to thapsigargin. The most active compounds (**1, 2**, and **9**) showed strong hydrophobic interactions with both Phe256 and Phe834, as shown in Figure 2, and displayed a common binding mode with convergence of solutions (Figures S3 and S4, Supporting Information). Independent docking experiments showed consistency in the binding mode and reproducibility of the docking scores (Table 2). Moreover, compound **2** is the only TCD that showed π -stacking between the *p*-hydroxybenzoate group at R₁ and the phenyl ring of Phe834 that may be responsible for the high activity. Interestingly, structural analysis and mutagenesis studies have demonstrated that both phenylalanines are also important residues for thapsigargin binding and inhibition of the SERCA pump.²⁰ In contrast, TCDs with K_D values between 20 and 35 μM were found to possess strong interactions with Phe834 and moderate hydrophobic interactions with Phe256. The binding mode of this subset of compounds differed from that of the most active TCDs. Docking experiments were reproducible (see ASP scores in Table 2).

Furthermore, the hydrophobic contacts in the binding site for the least active TCDs ($K_D > 50 \mu\text{M}$) are less important, and Phe256 is poorly or totally unaffected (Figure 3c,e,f). In addition, these TCDs also showed a characteristic reproducible binding mode that does not resemble thapsigargin or the other TCDs and could explain the low inhibitory activity (Figure S4, Supporting Information). As a negative proof of concept, TCD **11**, which was inactive in the ATPase inhibition assay up to 100 μM , was also docked and the binding mode was analyzed. Compound **11** exhibited no interactions with Phe256 and had only one weak hydrophobic interaction with Phe834 (Figure 3h), which may not be sufficient to inhibit the Ca^{2+} -ATPase. Therefore, this study supports the concept that inhibition of the SERCA pump at the transmembrane domain requires crucial hydrophobic interactions, as previously reported for thapsigargin.^{21,22}

Table 2. Experimental and in Silico Results of TCDs, with the Binding Affinities on SERCA (K_D in μM) and the IC_{50} Values from the Cell Viability (MTT) Assay in CCRF-CEM Cells Presented

compound	binding affinity ^a (K_D , μM)	affinity ^a conv to free energy of binding (kcal/mol)	ASP docking score ^b	IC_{50} CCRF-CEM cells (μM)
casearin J (1)	10.1 ± 0.7	-6.9 ± 0.04	33.2 ± 1.0	0.7 ± 0.04
casearborin D (2)	2.0 ± 0.3	-7.8 ± 0.1	31.6 ± 0.8	1.1 ± 0.2
casearborin F (3)	20.1 ± 1.2	-6.4 ± 0.04	30.3 ± 2.2	1.2 ± 0.2
zuelaguidin E (4)	22.3 ± 1.1	-6.4 ± 0.03	29.6 ± 0.2	1.9 ± 0.1^c
zuelaguidin A (5)	48.1 ± 4.0	-5.9 ± 0.05	26.6 ± 1.5	5.3 ± 0.2^c
zuelaguidin B (6)	32.1 ± 0.2	-6.2 ± 0.02	29.3 ± 1.8	1.6 ± 0.3^c
compound E (7)	103 ± 4.1	-5.5 ± 0.1	22.6 ± 2.3	12.0 ± 1.1
esculentin A (8)	96.5 ± 12.4	-5.5 ± 0.02	22.2 ± 1.0	9.5 ± 1.2^c
zuelaguidin C (9)	7.5 ± 1.0	-7.0 ± 0.1	32.2 ± 1.0	2.5 ± 0.1^c
zuelaguidin D (10)	31.5 ± 2.2	-6.2 ± 0.04	29.5 ± 0.6	5.5 ± 1.2^c
7,18,19-deacyl-casearin J (11)	>100	nd	20.0 ± 1.1	4.3 ± 0.7
Tg	$0.2 \pm 0.1\text{nM}$	-13.2 ± 0.11	43.1 ± 2.8	nd
doxorubicin	nd	nd	nd	0.5 ± 0.03

^aExperimental values. ^bCalculated with GOLD 5.2. ^cData obtained from Calderón et al.⁵ nd: not determined. Tg: thapsigargin. conv: conversion.

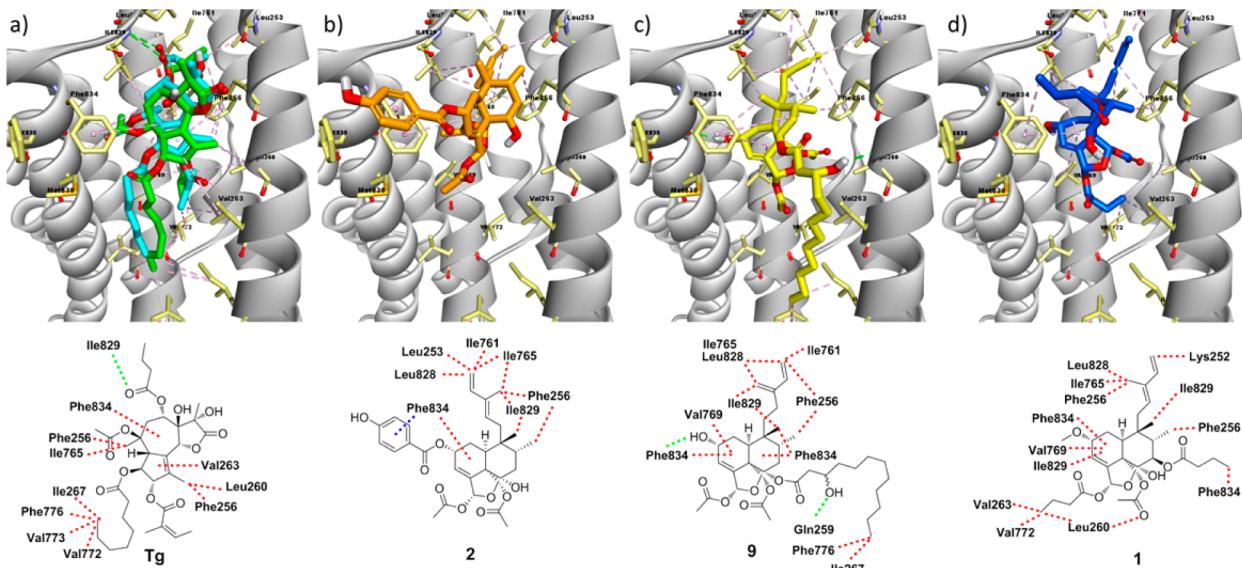


Figure 2. Docking of TCDs in the transmembrane domain of SERCA1a (PDB ID: 3NSK) with GOLD 5.2. (A) Docking validation, overlay of cocrystallized (green) and docked thapsigargin (light blue). (B) **2**. (C) **9**. (D) **1**. The binding modes show similarities to thapsigargin. The green lines represent hydrogen bonds, the red lines show hydrophobic interactions, and the blue lines show π -interactions.

SERCA Inhibition of TCDs Correlates with ASP Docking Scores and Cytotoxicity against CCRF-CEM Cells. To evaluate whether cytotoxicity is related to inhibition of SERCA, the correlation was examined between their inhibitory effects in CCRF-CEM cells and the K_D values for binding to SERCA. As can be seen from Figure 4a, this analysis yielded a linear regression coefficient of 0.89, pointing to SERCA as an important cytotoxic target of the TCDs. The in silico docking scores were also subjected to the same kind of analysis. For this purpose, three types of scoring functions available in GOLD 5.2 were used, viz., the force field-based score (GoldScore), the empirical scores (ChemScore), and the knowledge-based Astex Statistical Potential (ASP) score. Among these three individual scoring functions, GoldScore and ChemScore only showed low correlations to the experimentally measured free energy of binding to SERCA ($r^2 = -0.5823$ and -0.4093 , respectively). The best results were obtained with the ASP knowledge-based scoring function, which showed a significant correlation ($r^2 = -0.8040$) (Figure

4b). ASP incorporates information on the distance distribution of protein–ligand interactions and is derived from a database of protein–ligand complexes allowing the inclusion of a statistical parameter in the scoring formula. ASP also incorporates some ChemScore terms.²³ The advantage of using the ASP scoring function with GOLD to study interactions with the SERCA pump has already been reported in a comparative study using several docking programs and score functions.²⁴ Altogether, the correlations obtained suggest that inhibition of the SERCA pump by the TCDs contributes to their cytotoxic effects and that GOLD/ASP can be used for the future design of new SERCA pump inhibitors.

TCDs Are Unable to Strongly Inhibit Other P-Type ATPases. P-type ATPases are a superfamily of pumps, transporting ions across biological membranes, including the sarco/endoplasmic reticulum Ca^{2+} -ATPase, Na^+ , K^+ -ATPase, and H^+ , K^+ -ATPase.²⁵ All members of this family are inhibited by phosphate analogues such as vanadate, MgF_x , and BeF_x . However, many inhibitors are specific to particular ATPases

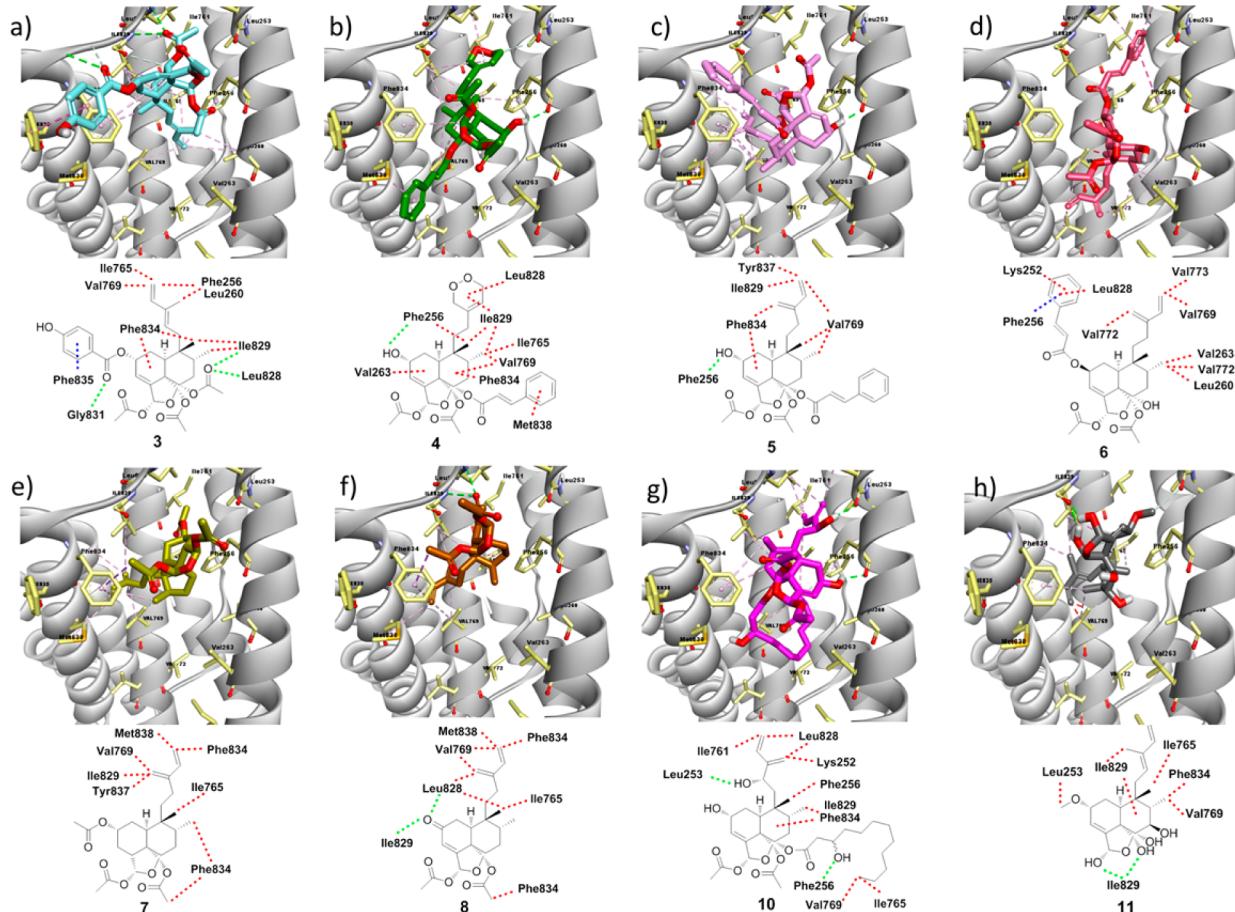


Figure 3. Docking of TCDs with K_D values higher than 20 μM in the transmembrane domain of SERCA1a (PDB ID: 3NSK): (A) 3, (B) 4, (C) 5, (D) 6, (E) 7, (F) 8, (G) 10, and (H) 11. The green lines represent hydrogen bonds, the red lines show hydrophobic interactions, and the blue lines show π -interactions.

(e.g., cardiotonic steroids to Na^+,K^+ -ATPase, thapsigargin to SERCA, and omeprazole to H^+,K^+ -ATPase). Some organic molecules, such as ivermectin, have been shown to be nonselective inhibitors of mammalian P-type ATPases with similar IC_{50} values (6–17 μM).²⁶ To exclude that the TCDs are also nonselective inhibitors affecting several (or all) P-type ATPases, compounds **1**, **2**, **9**, and **11** were investigated for their ability to inhibit the ATPase activity of Na^+,K^+ -ATPase. Liberation of phosphate was measured as previously described.²⁷ The compounds were tested in a range of 1–50 μM . Compounds **1** and **11** showed a negligible inhibition and **2** and **9** a low inhibition of the Na^+,K^+ -ATPase at the very high concentration of 50 μM (Table S1, Supporting Information). These results demonstrated that TCDs are not nonselective ATPase inhibitors, but show a preference for the SERCA pump.

Structure–Activity Relationships with TCDs According to SERCA1a Inhibition. Based on the in vitro and in silico data obtained, SAR were established to pinpoint structural features, which play a crucial role for the inhibition of SERCA. The presence of the double bond between C-3 and C-4 of the *cis*-decalin ring seems to be vital for the inhibition of SERCA (Figure 5a). Although the binding mode of the TCDs does not appear to be congruent among all the compounds, the correct positioning of the functional groups is crucial to exhibit SERCA inhibition and the double bond prevents conformational changes in the *cis*-decalin ring, thereby imposing a certain rigidity to the backbone. Compound **7**, which lacks this double

bond, but otherwise presents favorable functionalities, such as acetates at R_2 and R_3 and no aromatic groups at R_4 , showed only a negligible affinity toward SERCA. The lack of inhibitory activity may be due to the rearrangement of the *cis*-decalin ring in a different conformation, altering the position of the functional groups of **7** in the binding pocket; thereby no clear interactions are created (see Figure 3e). Docking calculations with a theoretical analogue of **7** exhibiting the double bond show that the docking score increases from 22.5 to 28.4, accompanied by hydrophobic interactions with Phe256 and Phe834 (Figure S5, Supporting Information), corroborating the importance of the double bond between C-3 and C-4. According to the linear correlation, a K_D value of around 45 μM is predicted (to be compared with a K_D value of **7** of $103 \pm 4.1 \mu\text{M}$).

The two acyl moieties at R_2 and R_3 are further candidates as essential structural features for the inhibition of the SERCA pump. Removal of any of these groups results in a complete loss of inhibition of the ATPase activity, as shown for compound **11**, which is the hydrolysis product of **1** and additionally lacks the ester at R_5 . The importance of the acyl moieties was also shown by our docking calculations, as they interact in the binding site (see **1** in Figure 2). A hydroxy group at R_4 in combination with a functional group at R_1 (e.g., methoxy, *p*-hydroxybenzoate, cinnamate) enhances the inhibition of SERCA (**1**, **2**, and **6**). Consequently, compound **3**, with acyl moieties at R_1 and R_4 , is less active due to a slight change in

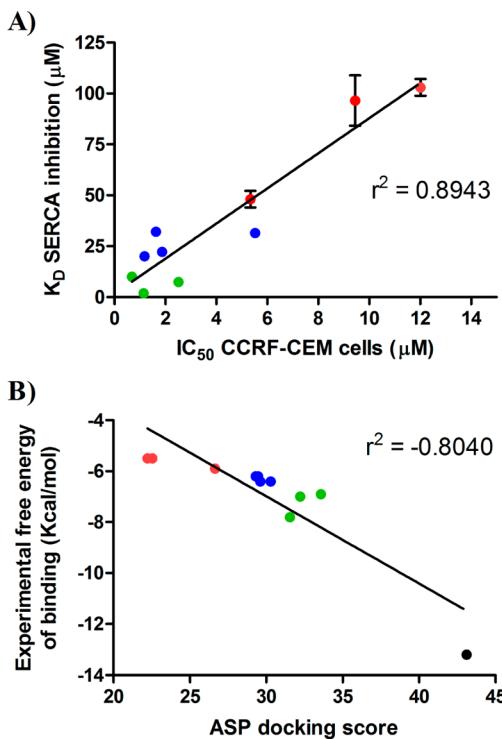


Figure 4. Correlation studies of TCDs with SERCA as target. Correlation of experimental binding affinities (K_D values) to the SERCA pump with IC_{50} values from the cell viability assay with human acute lymphoblastic leukemia CCRF-CEM cells comprising 10 TCDs (A) and correlation of the experimental free energy of binding of TCDs to SERCA and ASP docking scores from GOLD 5.2 (B). Data represent means \pm SD of three independent experiments (based on data from Table 2). Dots in green represent the TCDs with the best activity showing strong hydrophobic interactions with both Phe256 and Phe834; blue dots show TCDs with strong interactions with Phe834 and moderate interactions with Phe256; red dots show TCDs that interact through hydrophobic interactions only with Phe834. Thapsigargin is shown as a black dot in (B).

the binding mode that promotes a decrease in the hydrophobic interactions in the binding site. Acylation at R_4 with a long aliphatic chain, such as 3-hydroxylaureate (**9**), increases the inhibitory activity if R_1 is not esterified. However, if R_4 is esterified with an aromatic moiety, such as cinnamate, the inhibitory activity is negatively affected as observed with compound **5**. Aromaticity and structural rigidity at R_4 do not favor SERCA inhibition. The long aliphatic chain at this position resembles the octanoate group of thapsigargin and suggests that the lipophilicity and the accurate positioning of the side chains provided by the *cis*-decalin ring navigate the access to the binding site and consequently the inhibition of SERCA. This has already been shown for thapsigargin and its analogues.²¹

Moreover, the presence of an additional hydroxy group at R_6 led to a much lower inhibitory activity (compare TCDs **9** and **10**). Accordingly, docking results for **9** and **10** revealed a change in the binding mode that produces less interaction with Phe834, as shown by docking experiments. It can be argued that the side chain without a hydroxy group may provide the compounds with essential hydrophobicity, which enables a better interaction with the hydrophobic binding pocket. In the case of hydroxylation at R_6 the hydrophobicity is disrupted and the interaction is hindered with the binding site of the Ca^{2+} -ATPase. Furthermore, inhibition of SERCA also decreases if the acyl group (e.g., cinnamate) at R_1 is transferred to the R_4 position, indicating that aromaticity at R_1 is better tolerated than at R_4 (compare **6** and **5**). Interestingly, the presence of a 3,6-dihydro-1,2-dioxin moiety at the side chain attached to C-9 increases the inhibition of the Ca^{2+} -ATPase when compared to a butadiene moiety (**4** vs **5**). Additionally, a very poor inhibitory activity was observed when R_1 is oxidized to a ketone (**8**), suggesting that position 2 of the *cis*-decalin ring is of major importance for the inhibition of the Ca^{2+} -ATPase (Figure 5). A *p*-hydroxybenzoate seems to be optimal for the interaction in the binding site, whereas a smaller or a bigger group substantially decreases the activity (**2** compared to **6** and **8**). On the basis of our SAR results, an optimized theoretical structure was designed (Figure 5). Docking experiments showed an increase in the affinity of the optimized compound compared to **2** (ASP score of 35.3 and 31.5, respectively) due to strong intermolecular interactions with Phe834 and Phe256 (Figure 6).

Previous studies on the cytotoxic activity of TCDs on cancer cells support the present SAR findings. Thus, TCDs with ethers or a dialdehyde instead of the hemiacetal ring turned out to be poor cytotoxic agents,^{28–30} supporting the importance of esters at R_2 and R_3 . In addition, it is proposed that the hydrophobicity of the side chain attached to C-9 is crucial for the cytotoxic activity, as hydroxylation at R_6 has a negative effect. The same behavior was observed with caseamembrols A and B³¹ and with argutins.³² Although Morita et al. reported that bulkiness at R_4 negatively affects the cytotoxic activity of TCDs,³³ this is in contrast with the present SAR analysis. On the basis of our results, it is proposed that an aliphatic chain at R_4 in conjunction with a hydroxy group at R_1 is favorable for cytotoxic activity. This suggestion is supported by the casearupestrins, which possess a 2Z,4E-decadienoate moiety at R_4 and a hydroxy group at R_1 and which also exhibit strong cytotoxic effects on leukemia cells.⁶

Despite the linear correlation between the inhibition of the SERCA1a and the cytotoxic effects of the tested TCDs on leukemia cells (Figure 4a), it should be pointed out that by comparing individual cases it was observed that low IC_{50} values in the MTT assay were not always accompanied by a high binding affinity to SERCA. Thus, the TCDs **1**, **2**, and **6** had similar IC_{50} values in the MTT assay, but not the same binding affinity to SERCA1a (K_D values of 10.1, 2.0, and 32.1 μM , respectively). Even more surprisingly, compound **11**, which lacks SERCA1a inhibitory activity, showed cytotoxic effects at the low micromolar range. Therefore, it can be concluded that these TCDs differently influence other, as yet unidentified targets besides SERCA, resulting in additive or synergistic effects in cell death. Studies are in progress to elucidate further mechanisms of action.

EXPERIMENTAL SECTION

General Experimental Procedures. All the chemicals used were reagent grade (Roth, Karlsruhe; Merck, and Sigma-Aldrich). Optical rotations were measured on a PerkinElmer model 341 polarimeter. UV spectra were recorded on an Amersham Biosciences Ultrospec 2100 Pro UV/visible spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Bruker DRX instrument at 400 MHz (1H) and 100 MHz (^{13}C). HRAPCIMS were measured with a ThermoExactive with an Orbitrap analyzer (ThermoFisher). Open-column liquid chromatography was carried out on silica gel 60 (Merck). Aluminum sheets with silica gel 60 F₂₅₄ (Merck) were used for TLC to test the purity of compounds **1–11**. TLC sheets were derivatized with anisaldehyde/

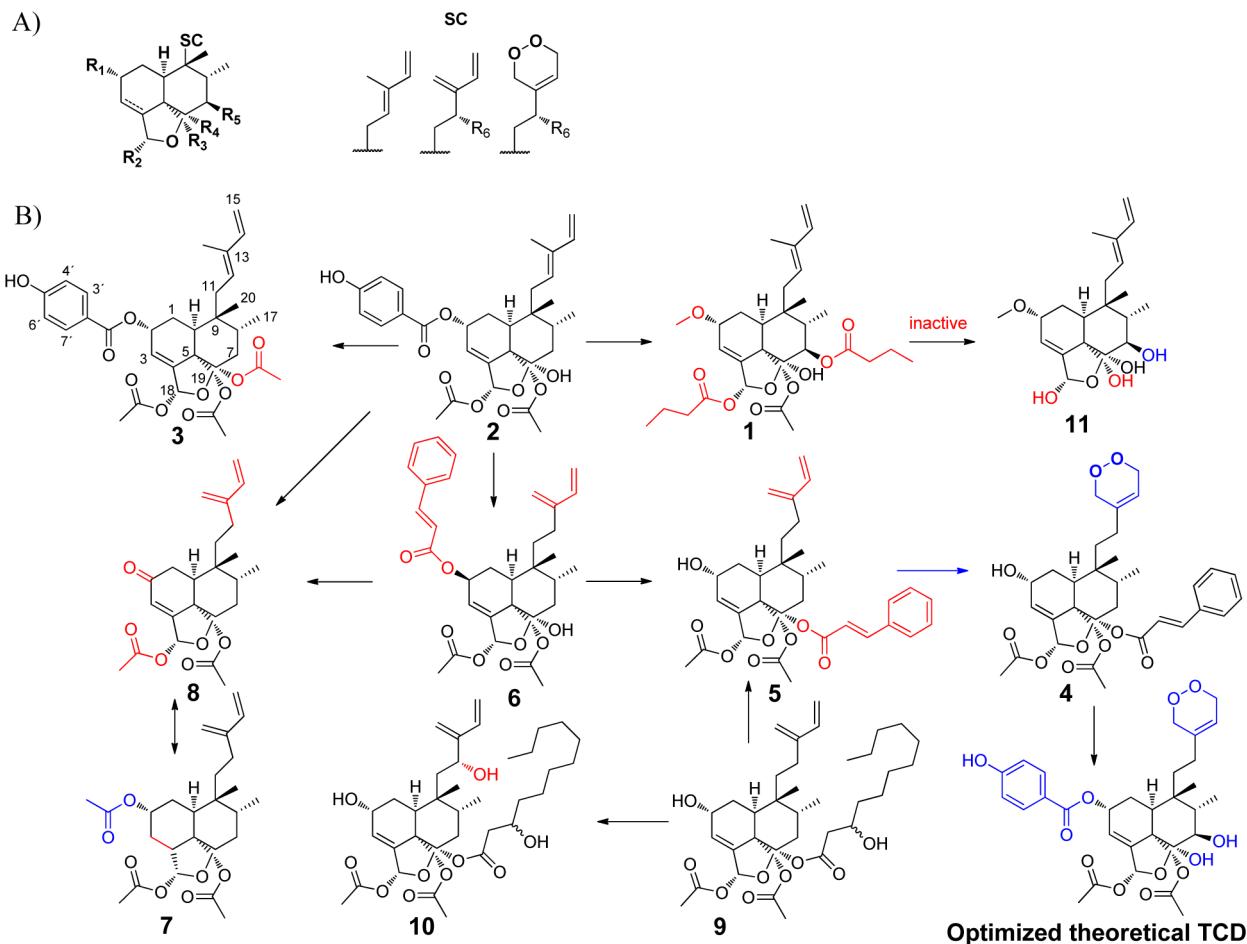


Figure 5. SAR of the TCDs tested on SERCA1a. (A) TCD scaffold. SC: side chain. (B) The functional groups in red reduce the inhibitory activity, and those in blue lead to an increase.

sulfuric acid as detection reagent, and the purity of all the compounds was at least 95%.

Preparation of Compound 11 from 1 by Hydrolysis.

Compound 1 (20 mg) was dissolved in MeOH containing 10% KOH w/v (3.5 mL) and stirred for 5 min at room temperature. The reaction was stopped by neutralizing with HCl solution. The resulting solution was filtered and washed with dichloromethane (CH_2Cl_2). The aqueous phase was extracted 3× with CH_2Cl_2 , and the organic-soluble phase was concentrated. The crude mixture was purified by open-column chromatography (CH_2Cl_2 –MeOH, 95:5) to yield 11 (10.4 mg) as a white, amorphous solid: $[\alpha]^{20}_{\text{D}} + 9.0$ (*c* 0.12, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 208 (3.96), 213 (4.05) nm; ^1H and ^{13}C NMR data, see Table 1; HRAPCIMS $[\text{M} + \text{NH}_4]^+$ *m/z* 380.2431 (calcd for $\text{C}_{21}\text{H}_{34}\text{NO}_5$, 380.2437).

NMR Data of Compound 3. Isolation and structure elucidation of compound 3 has not yet been published. NMR and MS data as well as NMR spectra are given in the Supporting Information.

Prediction of the Mechanism of Action with CDRUG.

CDRUG is a Web server for the prediction of anticancer activity (<http://bsb.kiz.ac.cn/CDRUG/>). Altogether, 95 TCDs were submitted in SMILES format and compared with a data set of cytotoxic compounds from the NCI-60 Developmental Therapeutics Program. This Web server uses a hybrid score to measure the compound similarity, giving as a result matches for the tested compounds.¹¹ Subsequently, these matches are examined to find a possible target.

Exploration of Chemical Space of the TCDs with ChemGPS-NP. In order to explore the chemical space of the TCDs and compare the mechanism of action with other chemotherapeutic compounds, a principal component analysis with ChemGPS-NP (<http://chemgps.bmc.uu.se>) was carried out.³⁴ In total, 95 TCDs in SMILES format

were submitted to ChemGPS-NP and compared with the chemical space of 228 cancer chemotherapeutic compounds including known SERCA inhibitors. The set of chemotherapeutic compounds comprised 42 tyrosine kinase inhibitors, 46 antimetabolites, 24 SERCA pump inhibitors, 18 cardiac glycosides, 34 alkylating agents, 19 topoisomerase II inhibitors, 26 topoisomerase I inhibitors, and 19 tubulin-active agents. On the basis of the analysis of 35 molecular descriptors that are subdivided into eight principal components (PCs) with physicochemical properties considered (e.g., size, aromaticity, polarity, lipophilicity, flexibility), new molecules are mapped into the chemical space according to the score prediction of the PCA analysis. The list of the compounds as well as the values of the eight PCs is available in Table S2, Supporting Information. Since PC1–4 are responsible for 77% of the data variance, they were analyzed as already published.^{34,35} The 3D graphs were created with the Erl-Wood Cheminformatics 2D/3D scatterplot node for Knime, and the cluster analysis was done with CheS-Mapper³⁶ taking into account the eight PC dimensions obtained with the PCA. This tool detects clusters within a data set and can be used to analyze possible structure–activity relationships.³⁶

Ca²⁺-ATPase Activity. SERCA1a (Ca²⁺-ATPase) was purified from sarcoplasmic reticulum vesicles prepared from rabbit skeletal muscles as previously described.³⁷ The effects of the TCDs on Ca²⁺-ATPase activity were tested using the methodology described by Winther et al. with slight modifications.²¹ Briefly, the protein (1 mg/mL) was equilibrated in the E2 conformation in a buffer containing 1 mM EGTA, 1 mM Mg²⁺, 10 mM Tes (pH 7.5), and 100 mM KCl. The equilibrated protein (10 μg) and the compounds were added to a cuvette with 3 mL of an ATP-regenerating assay medium containing basically the same electrolyte composition, but additionally 0.1 mM

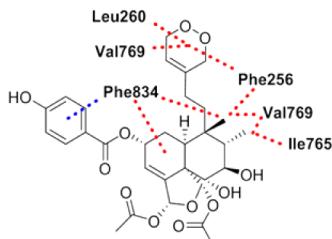
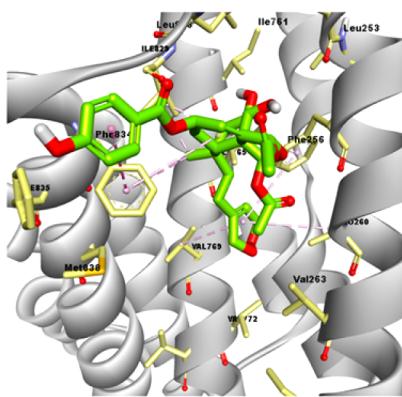
**New optimized TCD**

Figure 6. Docking of the optimized theoretical TCD in the transmembrane domain of SERCA1a (PDB ID: 3NSK). The red lines show hydrophobic interactions, and the blue lines π -interactions.

Ca^{2+} instead of EGTA, 1 mM phosphoenolpyruvate, 65 units pyruvate kinase, 82.5 units lactate dehydrogenase, and 0.175 mM NADH and incubated for 5 min. The reaction was initiated by addition of 5 mM Mg-ATP to the reaction cuvette after the incubation of the protein with the compounds at 23 °C. Activities were measured spectrophotometrically at 340 nm over a 6 min period. The experiments were repeated three times.

Na⁺,K⁺-ATPase Activity. The Na⁺,K⁺-ATPase membranes from the pig kidney outer medulla were purified as described earlier.³⁸ The steady-state Na⁺,K⁺-ATPase activity was assayed at 37 °C in an assay medium containing 130 mM NaCl, 20 mM KCl, 4 mM MgCl₂, 3 mM ATP, and 20 mM histidine (pH 7.4).²⁷ Ouabain (1 mM) was used as blank for nonspecific ATPase activity.²⁷

Docking Calculations with GOLD 5.2. The crystal structure of SERCA1a was obtained from the protein data bank³⁹ (PDB ID: 3NSK)²² and subjected to molecular docking using GOLD 5.2 software (CCDC, Cambridge, UK). Docking of all the tested ligands was performed in three independent experiments with the default docking settings producing a total of 30 genetic algorithm runs per compound each time. To speed up the calculations, the docking was terminated when the top three solutions were within 1.5 Å RMSD. All other values were set to the default. The active site radius was set at a distance of 13 Å from Phe256. Before generating these models, the protocols were validated with cocrystallized thapsigargin. The ligand was removed from the active site and redocked. The best docking result was compared further with the cocrystallized structure, and the intermolecular interactions in the binding site were described using Discovery Studio 4.0 (Accelrys Inc., San Diego, CA, USA).

Cytotoxicity Assay. The cytotoxicity of the TCDs 1–3 and 7 was tested against human acute lymphoblastic leukemia CCRF-CEM cells using the MTT assay as described in Calderon et al.⁵ Briefly, cells were seeded in 96-well plates at a density of 4×10^4 cells/well. The cells were incubated for 24 h with various concentrations of TCD. Doxorubicin was used as the positive controls, and 0.1% DMSO was the solvent control. The IC₅₀ values were obtained by nonlinear regression using the GraphPad Prism 5 program (Intuitive Software for Science, San Diego, CA, USA). The data are expressed as means \pm SD of three independent experiments.

ASSOCIATED CONTENT

Supporting Information

A table with the 95 TCDs used for the in silico analysis, CDRUG results, further docking figures, the values of PC1–8 obtained with ChemGPS-NP, a reaction scheme, and NMR spectra of compounds 3 and 11. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00062.

AUTHOR INFORMATION

Corresponding Author

*Tel: +49-761-203-8373. Fax: +49-761-203-8383. E-mail: irmgard.merfort@pharmazie.uni-freiburg.de.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful to Prof. T. Efferth, Department of Pharmaceutical Biology, Johannes Gutenberg University, Mainz, Germany, for providing CCRF-CEM cells as a gift, Dr. J. Wörth and C. Warth at the Institute of Organic Chemistry, University of Freiburg, for the HRMS data, and M. Wagner for measuring the optical rotation and S. Ferlaino for taking the NMR spectra, both of the Department of Pharmaceutical and Medicinal Chemistry, University of Freiburg. C.D.F. is grateful for a DAAD doctoral fellowship. This research was partly supported by the Excellence Initiative of the German Research Foundation (GSC-4, Speemann Graduate School). The authors are also grateful to F. Binns and R. Arce for the isolation of some diterpenes.

REFERENCES

- (1) Siegel, R. *CA Cancer J. Clin.* 2013, 63, 11–30.
- (2) Collins, I.; Workman, P. *Nat. Chem. Biol.* 2006, 2, 689–700.
- (3) Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V. *Nat. Prod. Rep.* 2007, 24, 1225–1244.
- (4) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* 2012, 75, 311–335.
- (5) Calderón, C.; De Ford, C.; Castro, V.; Merfort, I.; Murillo, R. *J. Nat. Prod.* 2014, 77, 455–463.
- (6) Vieira-Júnior, G. M.; Dutra, L. A.; Ferreira, P. M. P.; de Moraes, M. O.; Costa Lotufo, L. V.; Pessoa, C. D. O.; Torres, R. B.; Boralle, N.; Bolzani, V. D. S.; Cavalheiro, A. J. *J. Nat. Prod.* 2011, 74, 776–781.
- (7) Ferreira, P. M. P.; Santos, A. G.; Tininis, A. G.; Costa, P. M.; Cavalheiro, A. J.; Bolzani, V. S.; Moraes, M. O.; Costa-Lotufo, L. V.; Montenegro, R. C.; Pessoa, C. *Chem. Biol. Interact.* 2010, 188, 497–504.
- (8) Huang, D.-M.; Shen, Y.-C.; Wu, C.; Huang, Y.-T.; Kung, F.-L.; Teng, C.-M.; Guh, J.-H. *Eur. J. Pharmacol.* 2004, 503, 17–24.
- (9) Ekins, S.; Mestres, J.; Testa, B. *Br. J. Pharmacol.* 2007, 152, 21–37.
- (10) Yang, S.-Y. *Drug Discovery Today* 2010, 15, 444–450.
- (11) Li, G.-H.; Huang, J.-F. *Bioinformatics* 2012, 28, 3334–3335.
- (12) Thastrup, O.; Cullen, P. J.; Drebbal, B. K.; Hanley, M. R.; Dawson, A. P. *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87, 2466–2470.
- (13) Doan, N. T. Q.; Paulsen, E. S.; Sehgal, P.; Möller, J. V.; Nissen, P.; Denmeade, S. R.; Isaacs, J. T.; Dionne, C. A.; Christensen, S. B. *Steroids* 2015, 97, 2–7.
- (14) Denmeade, S. R.; Isaacs, J. T. *Cancer Biol. Ther.* 2005, 4, 14–22.
- (15) Dubois, C.; Vanden Abeele, F.; Sehgal, P.; Olesen, C.; Junker, S.; Christensen, S. B.; Prevarskaya, N.; Möller, J. V. *FEBS J.* 2013, 280, S430–S440.
- (16) Law, B. Y. K.; Wang, M.; Ma, D.-L.; Al-Mousa, F.; Michelangeli, F.; Cheng, S.-H.; Ng, M. H. L.; To, K.-F.; Mok, A. Y. F.; Ko, R. Y. Y.; Lam, S. K.; Chen, F.; Che, C.-M.; Chiu, P.; Ko, B. C. B. *Mol. Cancer Ther.* 2010, 9, 718–730.

- (17) Wong, V. K. W.; Li, T.; Law, B. Y. K.; Ma, E. D. L.; Yip, N. C.; Michelangeli, F.; Law, C. K. M.; Zhang, M. M.; Lam, K. Y. C.; Chan, P. L.; Liu, L. *Cell Death Dis.* **2013**, *4*, 1–13.
- (18) Sohoel, H.; Jensen, A.-M. L.; Møller, J. V.; Nissen, P.; Denmeade, S. R.; Isaacs, J. T.; Olsen, C. E.; Christensen, S. B. *Bioorg. Med. Chem.* **2006**, *14*, 2810–2815.
- (19) Rosén, J.; Rickardson, L.; Backlund, A.; Gullbo, J.; Bohlin, L.; Larsson, R.; Gottfries, J. *QSAR Comb. Sci.* **2009**, *28*, 436–446.
- (20) Wootton, L. L.; Michelangeli, F. *J. Biol. Chem.* **2006**, *281*, 6970–6976.
- (21) Winther, A.-M. L.; Liu, H.; Sonntag, Y.; Olesen, C.; le Maire, M.; Soehoel, H.; Olsen, C.-E.; Christensen, S. B.; Nissen, P.; Møller, J. *V. J. Biol. Chem.* **2010**, *285*, 28883–2892.
- (22) Paulsen, E. S.; Villadsen, J.; Tenori, E.; Liu, H.; Bonde, D. F.; Lie, M. A.; Bublitz, M.; Olesen, C.; Autzen, H. E.; Dach, I.; Sehgal, P.; Nissen, P.; Møller, J. V.; Schiøtt, B.; Christensen, S. B. *J. Med. Chem.* **2013**, *56*, 3609–3619.
- (23) Mooij, W. T. M.; Verdonk, M. L. *Proteins* **2005**, *61*, 272–287.
- (24) Lape, M.; Elam, C.; Paula, S. *Biophys. Chem.* **2010**, *150*, 88–97.
- (25) Yatime, L.; Buch-Pedersen, M. J.; Musgaard, M.; Morth, J. P.; Winther, A.-M. L.; Pedersen, B. P.; Olesen, C.; Andersen, J. P.; Vilsen, B.; Schiøtt, B. *Biochim. Biophys. Acta - Bioenerg.* **2009**, *1787*, 207–220.
- (26) Pimenta, P. H. C.; Silva, C. L. M.; Noël, F. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **2010**, *381*, 147–152.
- (27) Esman, M. *Methods Enzymol.* **1988**, *156*, 105–115.
- (28) Ferreira, P. M. P.; Costa-Lotufo, L. V.; Moraes, M. O.; Barros, F. W. A.; Martins, A. M. A.; Cavalheiro, A. J.; Bolzani, V. S.; Santos, A. G.; Pessoa, C. *An. Acad. Bras. Cienc.* **2011**, *83*, 1373–1384.
- (29) Beutler, J. A.; McCall, K. L.; Herbert, K.; Johnson, T.; Shoemaker, R. H.; Boyd, M. R. *Phytochemistry* **2000**, *55*, 233–236.
- (30) Dos Santos, A. G.; Ferreira, P. M. P.; Vieira Júnior, G. M.; Perez, C. C.; Gomes Tininis, A.; Silva, G. H.; Bolzani, V. D. S.; Costa-Lotufo, L. V.; Pessoa, C. D. O.; Cavalheiro, A. J. *Chem. Biodiversity* **2010**, *7*, 205–215.
- (31) Shen, Y.-C.; Cheng, Y.-B.; Ahmed, A. F.; Lee, C. L.; Chen, S.-Y.; Chien, C.-T.; Kuo, Y.-H.; Tzeng, G.-L. *J. Nat. Prod.* **2005**, *68*, 1665–1668.
- (32) Whitson, E. L.; Thomas, C. L.; Henrich, C. J.; Sayers, T. J.; McMahon, J. B.; McKee, T. C. *J. Nat. Prod.* **2010**, *73*, 2013–2018.
- (33) Morita, H.; Nakayama, M. *Chem. Pharm. Bull.* **1991**, *39*, 693–697.
- (34) Rosén, J.; Lövgren, A.; Kogej, T.; Muresan, S.; Gottfries, J.; Backlund, A. *J. Comput.-Aided. Mol. Des.* **2009**, *23*, 253–259.
- (35) Felth, J.; Rickardson, L.; Rosén, J.; Wickström, M.; Fryknäs, M.; Lindskog, M.; Bohlin, L.; Gullbo, J. *J. Nat. Prod.* **2009**, *72*, 1969–1974.
- (36) Gütlein, M.; Karwath, A.; Kramer, S. *J. Cheminform.* **2012**, *4*, 1–16.
- (37) Møller, J.; Lind, K.; Andersen, J. *J. Biol. Chem.* **1980**, *255*, 1912–1920.
- (38) Klodos, I.; Esman, M.; Post, R. L. *Kidney Int.* **2002**, *62*, 2097–2100.
- (39) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235–242.