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Cyclosquaramides as Kinase Inhibitors with Anticancer Activity

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We report the synthesis and biological evaluation of a new series of oligosquaramide-based macrocycles as anticancer agents. Compound **7**, considered as representative of this series, exhibited significant antiproliferative activity against the NCI-60 human tumor cell line panel, with IC_{50} values ranging from 1 to 10 μM. The results show that sensitivity to cyclosquaramides is clearly dependent on cell type, underscoring a degree of biological selectivity. The observed antiproliferative effects appear to be related to deregulation of protein phosphorylation, as compounds **7** and **8** are effective inhibitors of several important kinases such as ABL1, CDK4, CHK1, PKC, c-MET, and FGFR, among others. The corresponding acyclic oligosquaramides and smaller cyclosquaramides did not show antitumor activity, suggesting that a macrocyclic structure with minimal molecular size plays a key role in the observed antitumor activity.

Introduction

In recent years, squaramides have emerged as promising pharmacophores for drug design. The biological activity of squaramides relies on specific structural features; the squaramide[1] motif provides a planar aromatic[2] framework equipped with two adjacent carbonyl acceptor groups and two NH amide-type donor sites, capable of establishing multiple hydrogen bond interactions.[3–6] A number of squaramides are currently under investigation for biological effects such as bioisosteric substitution of phosphate groups in modified oligodeoxynucleotides[7,8] and of guanidines in compounds that show activity as potassium channel openers.[9–11] In addition, squaramide-based compounds target certain CXC-type chemokine receptors[12–14] and are involved in ligands designed for receptor-binding inhibition of enterotoxins.[15–17] Moreover, recent reports have also demonstrated the anticancer activity of certain squaramide compounds.[18,19]

Despite several structural similarities with secondary amides, squaramides have their own unique structural features. In solution, secondary squaramides show a similar, although less pronounced, preference for the *anti* isomer as do secondary amides. The free energy difference between the two rotamers is low (~0.25 kcal mol^{-1}). As in amides, the C–N bond of secondary squaramides has double bond character, and the rotational energy barrier (~12 kcal mol^{-1}) is also relatively low compared with that of secondary amides.[20] In fact, at sub-ambient temperatures both the *anti–anti* and *anti–syn* (*syn–anti*) isomers are detected by ^{1}H NMR spectroscopy.[21] We also demonstrated that the inclusion of tertiary amino groups as hydrogen bond donors in the vicinity of the squaramido NH groups induces formation of β-turn analogues in secondary squaramides, allowing the synthesis of oligomeric cyclosquaramides through hydrogen-bond-directed macrocyclization.[22]

Based on these precedents, we hypothesized that oligomeric cyclosquaramides might provide well-defined architectures with a certain degree of conformational pre-organization and flexibility very well suited for ligand binding on target surfaces with potential antitumor effects.[23] This compromise between pre-organization and flexibility is a key characteristic of macrocyclic cyclopeptides with known anticancer activity.[24–27] We anticipated that being nonpeptidic agents, cyclosquaramides could resist degradation. Herein we present a series of novel cyclosquaramides, and we report data that confirm the anticancer and kinase inhibitory activities of these compounds (Figure 1).

Results and Discussion

Synthesis

The synthesis of oligomeric cyclosquaramides **1**–**10** follows a modular approach. Macrocycles **4**–**9** were prepared as described previously by us,[22] obtaining linear squaramide compounds of various length (Scheme 1) and condensing them in a macrocyclization reaction either with one equivalent of diethyl squarate or one equivalent of the diethyl ester **12** to afford cyclosquaramides **4**–**9**, respectively (Scheme 2).

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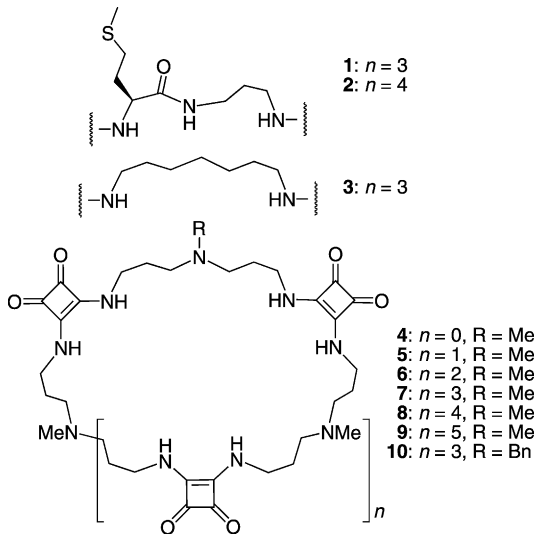
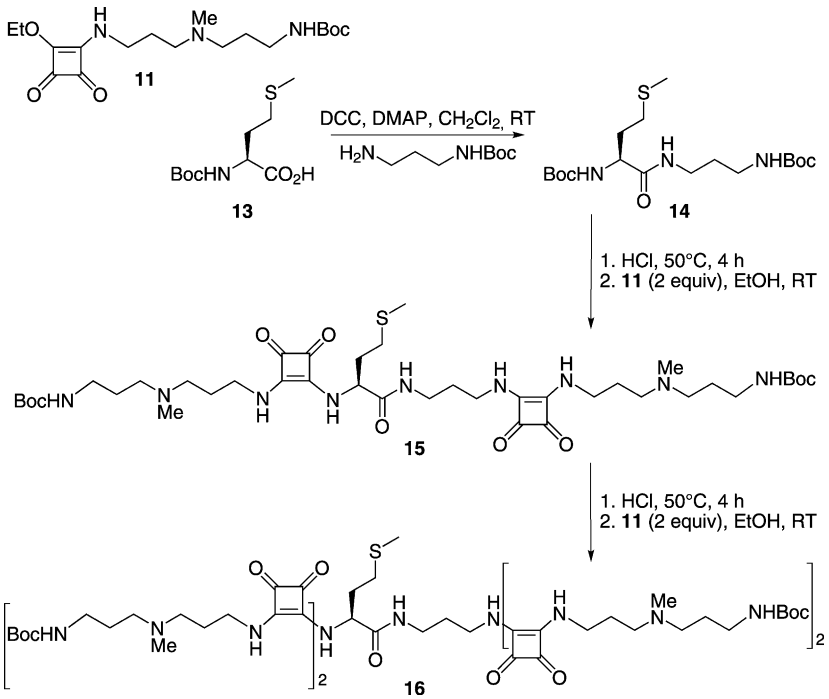


Figure 1. Structures of oligomeric cyclosquaramides.

Macrocycles **1**–**3** and **10** were synthesized according to this procedure with minor modifications using the corresponding diamines as starting material. As exemplified in Scheme 1, *N*-Boc-protected L-methionine **13** was condensed with *N*-Boc-1,3-diaminopropane via the agency of DCC–DMAP as coupling reagent. After deprotection of both Boc groups of **14** with aqueous acid, the resulting diamine was rapidly condensed with squaramide **11**, the growing module, to afford **15**. Repetition of a cycle of deprotection–condensation with **15** afforded the linear squaramide derivative **16**. Deprotection of **16** was followed by condensation with diethyl squarate or diethyl ester **12**, and subsequent intramolecular hydrogen-bond-directed macrocyclization to cyclosquaramides **1** and **2**, respectively (Scheme 2).

Similarly, cyclosquaramide **10** was prepared as described for macrocycle **2**. The diamine N^1-(3-aminopropyl)-N^1-(phenylmethyl)-1,3-propanediamine was condensed with two equivalents of **11** to afford the di-oligosquaramide compound **18**. The deprotection and condensation cycle was repeated to afford the corresponding tetra-oligosquaramide compound **19**. Deprotection of **19** followed by condensation with diethyl squarate afforded cyclosquaramide **9**. Alternatively, cyclosquaramide **3** was prepared after deprotection of the tri-oligosquaramide **20** followed by its condensation with diethyl ester **17**. See the Supporting Information for an outline of the synthesis of cyclosquaramides **3** and **9**. For all cases studied thus far, the macrocycles were obtained in remarkably good yields without using high-dilution conditions.



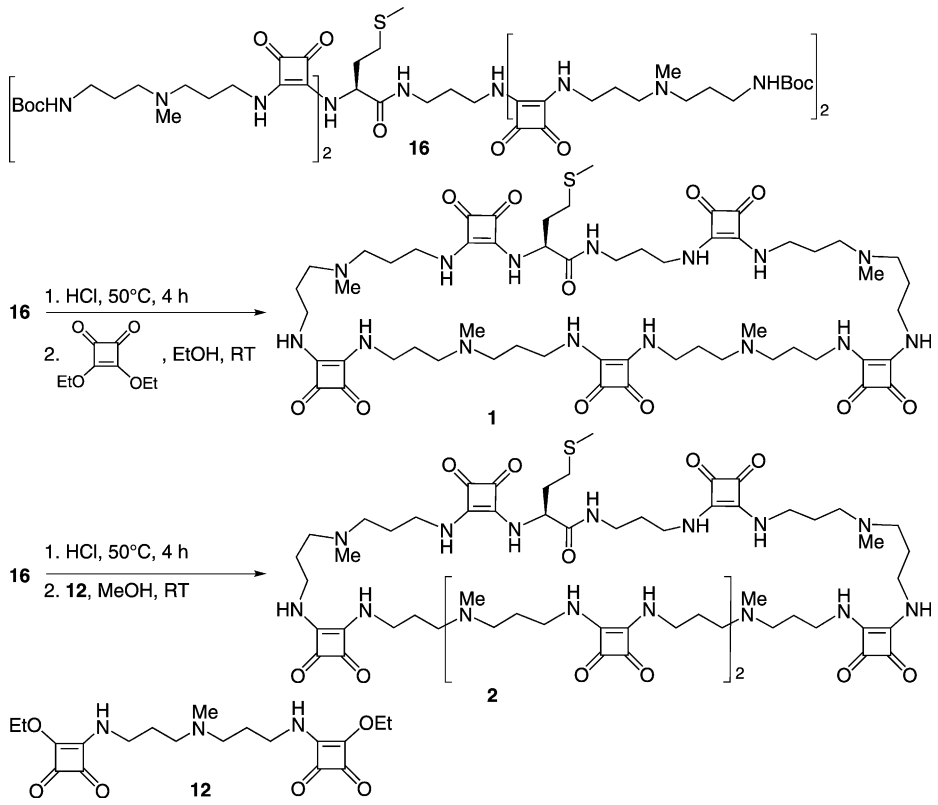
Scheme 1. Synthetic procedure for the preparation of oligomeric squaramides.

Biological evaluation

The anticancer activity of cyclosquaramides **4**–**9** was initially evaluated using the mantle cell lymphoma (MCL) cell lines Jeko-1 and Z-138. MCL is an aggressive subtype of non-Hodgkin lymphoma that is mostly incurable under current therapies; MCL patients have a median survival of about three years. It is characterized by the t(11;14)(q13;q32) chromosomal translocation, combined with deregulation of cell proliferation and survival pathways, chromosome instability, and disruption of the DNA damage response pathways.[28] To determine whether the synthesized oligomeric cyclosquaramides **4**–**9** could affect cancer cell proliferation or survival, MCL cell lines Jeko-1 and Z-138 were treated for 48 h and assayed for viability using a luminescence-based ATP assay (Cell Titer-Glo, Promega).

Data from dose–response studies listed in Table 1 reveal that cyclosquaramides display antiproliferative activity against both cell lines. However, there is clear evidence for the dependence of the observed antiproliferative activity on the size of the macrocycle. Whereas the smaller macrocycles **4**–**6**, respectively featuring two, three, and four squaramides, do not show significant activity against Jeko-1 cells ($IC_{50} > 200$ μM), the larger members of this series, namely cyclosquaramides **7** ($IC_{50} = 7.1$ μM), **8** ($IC_{50} = 10$ μM), and **9** ($IC_{50} = 17.9$ μM), display activities in the micromolar range.

This trend is also observed with the Z-138 cell line. Plots of the antiproliferative activity as a function of the number of squaramide units present in the macrocycles after treatment at



Scheme 2. Synthetic procedure for the macrocyclization reaction.

selectivity. Importantly, cyclosquaramide **7** is consistently the most active compound, even in the least sensitive cell lines.

To further investigate how structural modifications on the cyclosquaramide scaffold affect activity, we prepared some structurally different cyclosquaramides containing five and six squaramide units. The activities of compounds **1**–**3** and **10** were studied against Jeko-1 cells, which proved to be more structure-dependent than Z-138 cells regarding cyclosquaramide-induced antiproliferative activity (Figure 2). As shown in Table 1, modification of the aminoalkyl linker into alkyl, benzyl, or methionine fragments led to a moderate loss in biological activity; the activity of compound **7** is higher than that observed for modified cyclosquaramides **1**–**3** and **10**.

Table 1. In vitro activity profiles of cyclosquaramides **1**–**9** against Jeko-1 and Z-138 (MCL) cells.

Compd	Sq[a]	M_r [Da]	IC_{50} [μM][b]	
			Jeko-1	Z-138
4	2	446.5	>200	27.3±5
5	3	669.8	>200	21.2±7
6	4	893.1	>200	5.1±0.9
7	5	1116.3	7.1±1	4.7±0.9
8	6	1339.6	10±2	6.6±1
9	7	1584.9	18±2	7.0±1.3
1	5	1176.4	34±6	–
2	6	1199.7	24±4	–
3	5	1101.3	45±5	–
10	5	1192.4	27±5	–

[a] Number of squaramide units. [c] Data represent the mean±SD ($n=3$); all experiments were performed in triplicate.

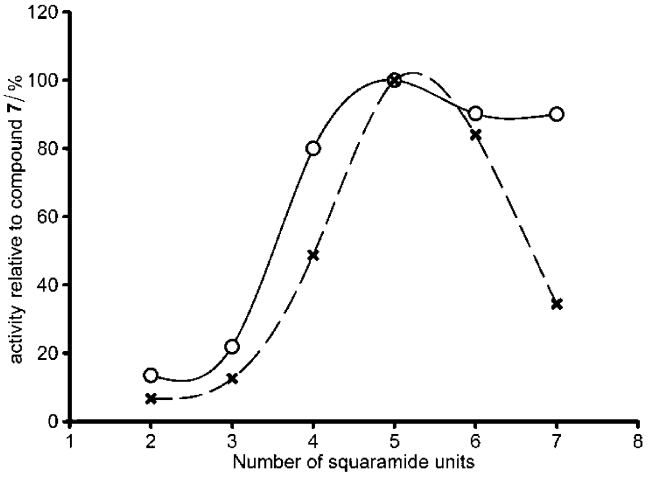


Figure 2. Antiproliferative activity toward Jeko-1 (×) and Z-138 (○) cells as a function of the number of squaramide units upon treatment with cyclosquaramides **4**–**9** (10 μM, 48 h).

a compound concentration of 10 μM for 48 h shows that cyclosquaramide **7**, formed by five squaramide units, is the most active molecule of the series against both cell lines (Figure 2).

To further investigate the influence of size on cyclosquaramide activity, additional MCL cell lines (Granta-519 and Rec-1), and cell lines of various tumor origin, such as glioblastoma (U87MG and LN229) and osteosarcoma (U2OS) cells, were treated with cyclosquaramides **7**–**9** at 10 μM for 48 h. As shown in Figure 3, sensitivity to cyclosquaramides is clearly dependent on cell type, which indicates a degree of biological

Among the physicochemical parameters that contribute to tumor cell growth inhibitory activity, molecular weight has proven to be one of the most important, together with rotatable bonds, low polar surface area, and total hydrogen bond count. From our data, it became clear that active cyclosquaramides are beyond the classical limitations used for assessing oral bioavailability of drug candidates. The molecular weights of the active compounds **1**–**3** and **7**–**10** are much higher than

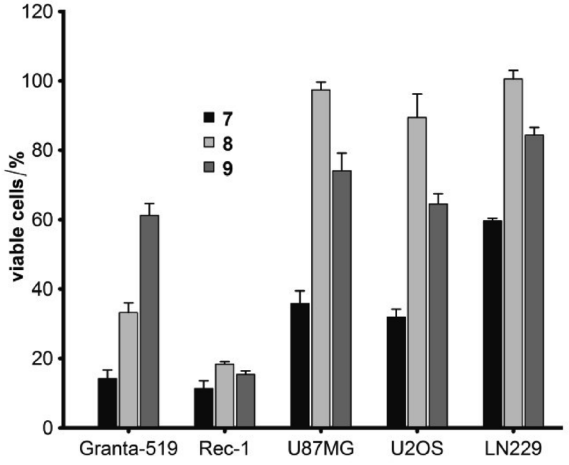


Figure 3. Percent cell viability upon treatment with cyclosquaramides **7**–**9** (10 μM) for 48 h relative to untreated cells: mantle cell lymphoma (Granta-519 and Rec-1), glioblastoma (U87MG and LN229), and osteosarcoma (U2OS); data represent the mean ± SD ($n = 3$ independent experiments).

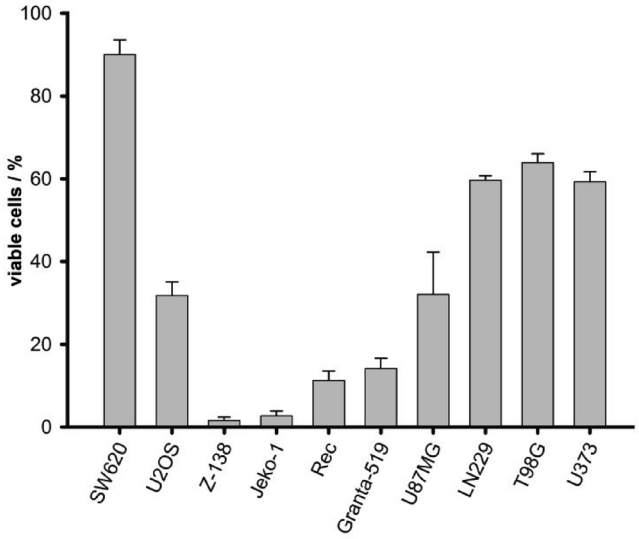


Figure 4. Percent cell viability upon treatment with cyclosquaramide **7** (10 μM, 48 h) relative to untreated cells. Cell lines of different tumor origins used: colorectal cancer (SW620), osteosarcoma (U2OS), mantle cell lymphoma (Z-138, Jeko-1, Rec-1, and Granta-519), glioblastoma (U87MG, LN229, T98G, and U373); data represent the mean ± SD ($n = 3$ independent experiments).

the $M_r < 500$ Da limit imposed by Lipinski rules. Higher M_r is usually considered detrimental to in vivo potency. However, these rules do not apply to many anticancer agents, of which a majority have $M_r > 500$ Da.[29] Therefore, new drugs can be found within the high-M_r range, provided that the number of rotatable bonds is minimized.[30]

Given our preliminary SAR data and considering that cyclosquaramide **7** is easily obtained at the multi-gram scale, we used it as our reference compound to further investigate the biological activity of cyclosquaramide compounds. The preliminary results obtained with cyclosquaramide **7** suggest the presence of some cell-type selectivity. This assumption was again confirmed by analyzing additional cell lines of various tumor origin (Figure 4).

The antitumor effect of cyclosquaramide **7** was also evaluated across the NCI-60 tumor cell line panel.[31] Compared with the conventional anticancer agent paclitaxel, compound **7** showed mid-range activity at the low-micromolar range of IC_{50} in the NCI-60 panel. This screen allowed us to detect certain selectivities. Thus, whereas a group of ovarian, lung, colon, melanoma, and renal cancer cell lines did not respond to compound **7** at the highest concentration tested (10 μM), other cell lines of the same tumor origin were sensitive to the action of **7** (Table 2). Moreover, compound **7** did not exhibit toxicity against control non-transformed cells, such as NIH3T3 fibroblasts (data not shown).

Because U87MG glioma cells, which are sensitive to compound **7** (Figures 3 and 4), spontaneously form multicellular tumor spheroids in culture,[32] we also investigated whether compound **7** could prevent spheroid formation. Whereas control U87MG cells formed high numbers of large and dense multicellular tumor spheroids, cells treated with compound **7** were largely resistant to spheroid formation (Figure 5). These observations confirm that this compound severely decreases glioma cell proliferation.

To determine if cyclosquaramide treatment induces changes in the cell-cycle profile, we examined cell-cycle phase distribution by flow cytometry (Figure 6). Treatment of the MCL cell lines Jeko-1 and JVM-2 with compound **7** (10 μM) for 24 h caused distinct effects on both cell lines. In Jeko-1 cells, **7** induced an increase in the sub-G_1 fraction from 12.1 to 27.3%, in parallel with a decrease in the S-phase fraction. Under the

Table 2. Cyclosquaramide **7** cytotoxicity determination against selected tumor cell lines.[a]

Cell line	Cell type	IC_{50} [μM][b]
OVCAR-3	ovarian carcinoma	5.07 ± 0.75
OVCAR-4	ovarian carcinoma	> 10
OVCAR-5	ovarian carcinoma	> 10
OVCAR-8	ovarian carcinoma	> 10
SK-OV-3	ovarian carcinoma	3.10 ± 0.12
IGR-OVI	ovarian carcinoma	2.38 ± 0.15
NCI-H460	lung carcinoma	6.88 ± 1.25
NCI-H522	lung carcinoma	> 10
HOP-92	lung carcinoma	3.53 ± 0.24
NCI-H226	lung carcinoma	1.53 ± 0.60
EKVX	lung carcinoma	2.75 ± 0.15
HCT-116	colon carcinoma	2.98 ± 0.15
HCT-15	colon carcinoma	> 10
HT-29	colon carcinoma	> 10
SW-480	colon carcinoma	1.80 ± 0.27
COLO-205	colon carcinoma	3.23 ± 0.18
SK-MEL-28	melanoma	> 10
LOX-IMVI	melanoma	> 10
SK-MEL-2	melanoma	3.29 ± 0.09
SK-MEL-5	melanoma	2.75 ± 0.03
UACC-62	melanoma	1.34 ± 0.18
786-O	renal carcinoma	> 10
TK-10	renal carcinoma	3.36 ± 1.00

[a] See the Supporting Information for a complete list of IC_{50} values. [b] Data represent the mean ± SD of three independent experiments performed in triplicate.

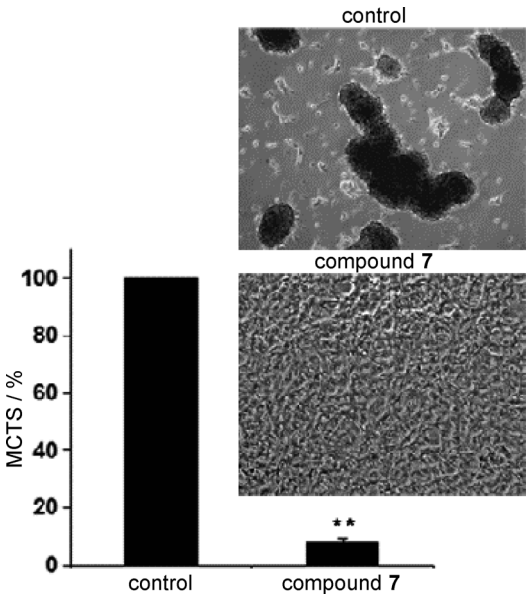


Figure 5. Representative phase-contrast micrographs of U87MG cells left for six days to allow formation of multicellular tumor spheroids (MCTS): untreated (control) or treated with 10 μM compound **7**. The bar chart indicates the values (mean ± SD) of MCTS formation from three independent experiments, each conducted in duplicate, expressed as the percentage relative to untreated cells. The differences between control and cyclosquaramide treatment are statistically significant (Student's *t*-test: $^{**}p < 0.01$).

same conditions, JVM-2 cells were drastically affected by **7**, with an increment of cells in the sub-G_1 population from 9.2 to 67.0%, indicating a dramatic induction of apoptosis.

Taken together, our results and the observed selectivities toward the NCI-60 panel are compatible with a semi-selective interaction of **7** with key biological targets. From this point of view, targeting protein kinases is of interest, as deregulated kinases are implicated in a variety of human diseases, and consequently, a number of protein kinase inhibitors are in clinical or preclinical evaluations as anticancer agents.[33,34] It is also known that squaramide-based anticancer agents can involve the inhibition of kinases. Examples include arylamidoaryl squaramides that inhibit KIT Tyr kinases,[18] and several heterocyclic squaramides that inhibit checkpoint kinases Chk1 and Chk2: two Ser/Thr regulatory kinases implicated in cell-cycle checkpoint control.[19]

To determine whether cyclosquaramides can inhibit protein kinases, the reference cyclosquaramide **7**, which is active against Jeko-1, JVM-2, and several cell lines within the NCI-60 panel, was initially tested at 10 μM in a single-point binding assay against a panel of kinases (ProQinase, Freiburg, Germany).[35] The inhibitory activity, expressed as a percentage of control values (PoC < 35), indicated that compound **7** inhibits 44 kinases over a panel of 210 representative kinases of the TK, TKL, STE, CK1, AGC, CAMK, and CMGC families of the human kinome (see Supporting Information). After this initial profile, we decided to determine if the enzyme inhibitory activity is related to the in vitro antiproliferative activity. To this end, we defined a subset of 25 kinases heavily affected by **7** in the initial profile. In these experiments, the inhibitory potency was determined against inactive cyclosquaramide **4** and active cyclosquaramides **7** and **8** at a single concentration of 10 μM (Table 3).

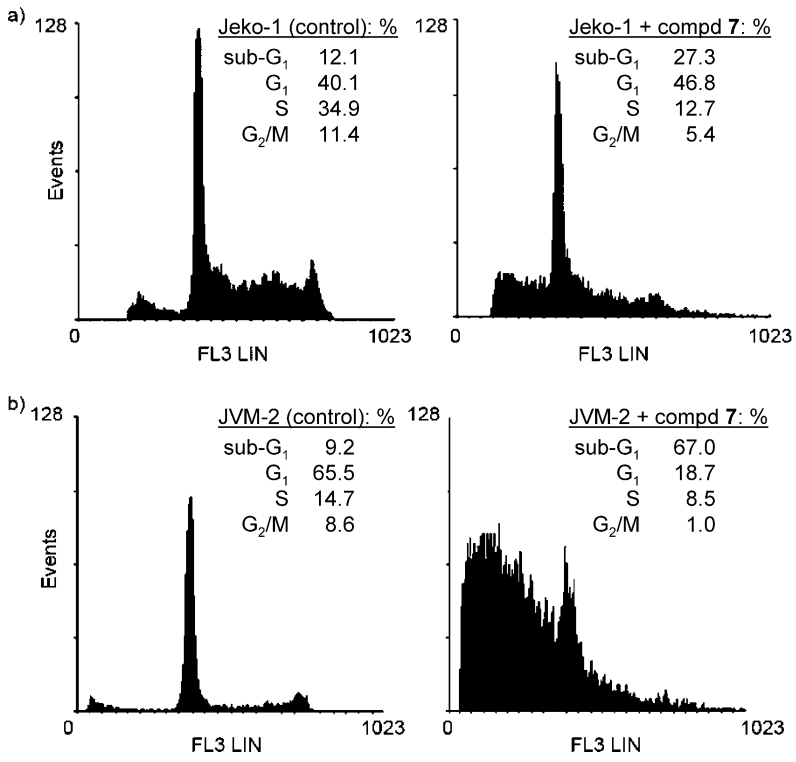


Figure 6. Effect of cyclosquaramide **7** treatment on cell-cycle distribution for a) Jeko-1 and b) JVM-2 cells, assessed by flow cytometry analysis upon propidium iodide staining. Representative histograms plotting cell events versus DNA content (FL3 LIN) after treatment with **7** at 10 μM for 24 h.

These results strongly suggest a relationship between in vitro cellular antiproliferative activity and kinase inhibitory potency. Hence, compound **4**, which is inactive against Jeko-1 cells, showed high PoC values, indicating low inhibitory potency, whereas compounds **7** and **8** showed low PoC values. For example, **7** leads to PoC < 10 for CSK, MATK, NLK, PKC-γ, TRK-C and ZAP-70.

In a separate experiment, the inhibitory concentration (IC_{50}) was also calculated for reference compound **7** (Table 3). In general, the enzymatic IC_{50} value lies within the low-micromolar range, thus confirming the inhibitory activity of cyclosquara-

Table 3. Protein kinase affinity data for cyclosquaramides **4**, **6**, **7**, and **8**.

Kinase	Affinity [%][a]				IC_{50} [μM][b]
	4	**6**	**8**	**7**	**7**
ABL1	94	46	17	14	5.4 ± 0.75
ACK1	402	185	128	14	–[c]
CDK4/CycD3	100	90	58	90	5.3 ± 0.50
CDK5/p25NCK	81	65	38	18	9.2 ± 0.70
CDK7/CycH/Mat1	117	97	112	30	28.0[d]
CDK9/CycT	57	62	17	12	3.7 ± 0.71
CHK1	50	20	27	24	1.4 ± 0.15
CSK	66	42	37	3	7.2 ± 0.74
FGF-R1 VM	83	53	29	12	2.6 ± 0.58
FGF-R2	80	71	76	12	5.4 ± 0.50
MATK	186	157	117	0	–[c]
c-MET	91	66	21	13	3.7 ± 0.30
MUSK	96	44	15	14	1.5 ± 0.84
NLK	78	67	32	6	4.3 ± 0.65
PAK4	70	68	27	22	4.3 ± 0.49
PAK7	70	59	32	12	4.9 ± 0.73
PKC-α	93	58	17	13	2.3 ± 0.72
PKC-β1	85	55	44	14	7.6 ± 0.73
PKC-γ	101	77	11	4	4.9 ± 0.58
PLK1	86	53	49	36	26.0 ± 0.83
S6K	78	32	20	13	4.4 ± 0.47
TGFB-R1	91	41	37	10	3.3 ± 0.61
TRK-C	106	170	73	8	6.6 ± 0.26
VRK1	74	29	32	11	2.4 ± 0.55
ZAP70	434	413	286	5	3.7 ± 0.30

[a] Affinity data are given as a percent of control (PoC). [b] Data represent the mean ± SD; IC_{50} values were measured by testing 12 concentrations of the test compound in the range from 1×10^{-04} M to 3×10^{-11} M ($n=1$) in each kinase assay. [c] No valid IC_{50} value could be determined against this kinase. [d] Ambiguous fitting.

mides **7** and **8**. Notably, the enzyme inhibitory activity of **7**, in the low-micromolar range, is similar to its IC_{50} value obtained from in vitro cell viability assays in the NCI-60 panel. This strongly suggests that cyclosquaramide **7** can easily penetrate cellular membranes without being affected by the usual degradation pathways present in the cytoplasm.

To determine the mode of kinase inhibition by cyclosquaramide **7**, an ATP-competitive binding assay was performed. The kinase ABL1 was chosen for this experiment. For this purpose, the IC_{50} values of the compound were determined in the ABL1 assay at four different ATP concentrations (0.1, 1, 3, and 10 μM). The values obtained, ranging from 0.4 to 0.7 μM, were similar regardless of ATP concentration, clearly showing that increasing concentrations of ATP do not lead to an increase in IC_{50} value for the test compound in the ABL1 assay. On the contrary, the IC_{50} values showed a slight decrease with increasing amounts of ATP. Taken together, these results clearly indicate that the test compound does not behave as a mere ATP antagonist. For an ATP antagonist, an increase in IC_{50} value with increasing ATP concentration would be expected. In addition, the very steep IC_{50} curves (absolute values of Hill slopes $\gg 1$) suggest that the test compound binds at more than one site of the enzyme in a cooperative manner.

Conclusions

We have discovered cyclosquaramides as a novel class of kinase inhibitors with anticancer activity. The compounds tested so far showed significant (low-micromolar) inhibition of kinases implied in cell proliferation such as ABL1, CDK4, PKC, c-Met, and FGF-R, among others. Cyclosquaramide **7**, used as reference compound, also demonstrated antiproliferative activity, with IC_{50} values in the low-micromolar range in the NCI-60 screen and in two MCL cell lines. The observed IC_{50} values on the same order of magnitude in both the enzyme- and cell-based screens indicate that cyclosquaramides are cell-permeable and that degradation does not take place to a considerable extent. Although the biological target responsible for anticancer activity remains unknown at this point, the information obtained so far is compatible with a semi-selective interaction of cyclosquaramides with conserved parts of target proteins, presumably kinases. Further research to elucidate the mechanism of action and to determine the anticancer potential of cyclosquaramides is under way.

Experimental Section

Chemistry

General: All reagents and solvents were obtained from commercial sources and were used without further purification unless otherwise stated. Water used for preparing test samples was pure to a resistivity of 18.2 MΩ cm (Milli-Q). DMSO used to prepare test samples was purchased from Sigma–Aldrich (BioReagent). [D_6]DMSO and $CDCl_3$ (99.8% D) were stored on molecular sieves (3 Å). ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, at 23 °C unless otherwise specified. Chemicals shifts (δ) are reported in ppm referenced to the residual deuterium lock solvents. ^{13}C NMR spectra in D_2O were referenced to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. Coupling constants (J) are given in Hz. IR spectra were recorded on an FTIR Bruker IFS66 spectrophotometer. HRMS data were obtained on a MicroMass Autospec 3000 double-focusing magnetic sector mass spectrometer operating at 3000 m/z at a cone voltage of 4 V. Samples were introduced through an electrospray module (ESI) at 10^{-6} M in MeOH at a rate of 20 μL min^{-1}. The charge of the species corresponding to an observed ion rate was deduced directly from the spacing of the isotope peaks for masses <3000 m/z. Calibration was done with PER standards, giving closest peaks to the molecular ions under study. Analytical reversed-phase ion-pair HPLC was carried out on an Atlantis T3 C_{18} 5 μm 4.8×150 mm column, using mixtures of solution A (0.1 M NaOAc and 0.01 M sodium octanesulfonate in Milli-Q H_2O solution adjusted to pH 3.4 with acetic and formic acids) and solution B (CH_3CN) as mobile phase. Chromatography was carried out at a flow rate of 1 mL min^{-1} over 35 min starting from isocratic flux at 30% CH_3CN (solution B) + 70% solution A for 20 min and then gradient from 30% CH_3CN to 80% CH_3CN over 8 min. Luminescence was measured with a Hidex Chameleon 425-104 plate reader using 96-well ELISA microplates.

Compounds **4**–**8**, **11**, **12**, and **17** were prepared as described previously.[21,22] Boc-methionine (2-(*tert*-butoxycarbonylamino)-4-methylsulfanylbutanoic acid) **13** was prepared as described elsewhere.[34]

Macrocycle 9: Linear oligosquaramide **20**, containing five squaramide units, was prepared as described elsewhere.[22] A solution of

HCl (3 M, 1.2 mL, 3.6 mmol) was added to **20** (350 mg, 0.24 mmol) in H_2O (10 mL). The solution was warmed at 50 °C for 6 h. The mixture was then cooled to room temperature and brought to pH 9–10 by adding solid Na_2CO_3. The solvent was removed, and the residue was suspended in MeOH (14 mL) and filtered. To this solution, solid Na_2CO_3 (106 mg) and **12** (95 mg, 0.24 mmol) in MeOH (6 mL) were added. The reaction was stirred at room temperature for 24 h. The solvent was then removed, and the crude was dissolved in HCl (3 M, 2 mL). NaOH (1 M) was added to pH 9–10. The precipitate was centrifuged, dried and extracted in CH_2Cl_2 (5 mL). Compound **9** was obtained as a pale-yellow solid (288 mg, 77%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.65$ (br, 28H, $CH_2CH_2CH_2$), 2.10 (s, 21H, NCH_3), 2.30 (br, 28H, CH_2N), 3.50 (br, 28H CH_2NHsq), 7.43 ppm (br, 14H, NH); ^{13}C NMR (75 MHz, D_2O + HCl): $\delta = 28.0$, 42.7, 43.8, 55.9, 171.7, 184.3 ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{77}H_{119}N_{21}NaO_{14}$: 1584.9138, found: 1584.9156.

(*S*)-*tert*-Butyl (3-(2-((1-(*tert*-butoxycarbonyl)amino)-4-(methylthio)butanamido)propyl)carbamate (14): Compound **13** (1.0 g, 4.01 mmol), *N*-*tert*-butoxycarbonyl-1,3-diaminopropane (0.7 g, 4.02 mmol), and DMAP (0.05 g, 0.41 mmol) in dry CH_2Cl_2 (30 mL) were added dropwise to a stirred solution of DCC (1.076 g, 5.2 mmol) in CH_2Cl_2 (10 mL) at 0 °C under inert (Ar) gas. The mixture was allowed to stand at 0 °C for 2 h and an additional 12 h at room temperature. The crude mixture was filtered. The solvent was removed, and the residue was suspended in Et_2O (10 mL) and filtered. The addition of hexane to the solution afforded **14** as a white solid (1.138 g, 70%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.44$ (s, 18H, $C(CH_3)_3$), 1.62 (m, 2H, $NHCH_2CH_2CH_2$), 1.92 (m, 1H, $CHCH_2CH_2S$), 2.11 (m, 1H, $CHCH_2CH_2S$), 2.11 (s, 3H, SCH_3), 2.55 (m, 2H, CH_2CH_2NHCOO), 3.15 (m, 2H, CH_2CH_2S), 3.31 (m, 2H, $CONHCH_2CH_2$), 4.20 (m, 1H, NHCHCONH), 4.90 (br, 1H, NHCOO), 5.2 (br, 1H, NHCOO), 6.7 ppm (br, 1H, CONH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 15.4$, 15.5, 28.5, 28.6, 30.4, 36.1, 37.1, 66.0, 77.4, 156.6, 157.2, 171.9 ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{18}H_{35}N_3O_5SNa$: 428.2195, found: 428.2198.

Oligosquaramide 15: Compound **14** (1.117 g, 2.05 mmol) was suspended in a solvent mixture of H_2O (30 mL) and MeOH (20 mL); HCl (3 M, 6.6 mL, 19.8 mmol) was then added until complete dissolution of **13**. The solution was stirred for 2 h at 50 °C and an additional 12 h at 40 °C. The cooled solution was then carefully brought to pH 9–10 with solid Na_2CO_3. The solvent was removed, and the residue was suspended in EtOH (10 mL) and filtered. The resulting solution was added dropwise to a suspension of **11** (1.59 g, 4.3 mmol) and Na_2CO_3 (2.12 g) in EtOH (20 mL). The reaction was stirred at room temperature for 24 h, then the solvent was removed, and the crude was suspended in CH_2Cl_2 (20 mL), washed with H_2O (2×10 mL), saturated $NaCl_{(aq)}$ (10 mL), and dried with Na_2SO_4. The solvent was removed, and the solid obtained was extracted in Et_2O (3×20 mL) to furnish **15** as a pale-yellow solid (1.473 g, 84%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (s, 18H, $C(CH_3)_3$), 1.64 (br, 4H, $NCH_2CH_2CH_2N$), 1.83 (br, 6H $NCH_2CH_2CH_2N$), 2.08 (s, 5H, $CH_2CH_2SMe + SCH_3$), 2.20 (s, 6H, NCH_3), 2.40–2.56 (br, 8H, CH_2NCH_3, $CHCONHCH_2$), 3.15 (br, 8H, CH2NHCO,CH2S),3.71 (br, 6H, CH_2NHCOO) 4.70 (br, 1H, NHCHCONH), 4.9–5.4 (br, 3H, NHCOO), 7.9 ppm (br, 4H, NHsq, NHCO); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 15.7$, 27.3, 28.6, 30.1, 39.2, 41.9, 43.3, 55.2, 77.3, 156.5, 167.6, 182.6 ppm; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{40}H_{70}N_9O_9S$: 852.5017, found: 852.5017.

Oligosquaramide 16: Compound **16** was prepared and purified as described above for **15** by starting from **15** (1.0 g, 1,17 mmol) and **11** (0.91 g, 2.46 mmol) and using 8 equiv HCl. The desired product **16** was obtained as a white solid (0.758 g, 50%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.33$ (s, 18H, $C(CH_3)_3$), 1.46 (m, 2H, CH_2CH_2S), 1.63 (br, 18H, $NCH_2CH_2CH_2N$), 2.00 (s, 3H, SCH_3), 2.08(s, 6H, NCH_3), 2.08 (s, 6H, NCH_3), 2.2–2.3 (m, 20H, $(CH_2)_2NCH_3$, $CH_2NHCOCH$, CH_2S), 2.89 (m, 4H, CH_2NHCOO), 3.47 (br, 14H, CH_2NHsq), 4.63 (br, 1H, NHCHCONH) 6.76 (br, 3H, $CHCONHCH_2$ + NHCO), 7.50 ppm (br, 8H, NHsq); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 14.6$, 27.0, 28.2, 28.5, 38.2, 41.6, 54.1, 54.1, 54.2, 54.6, 77.3, 155.5, 167.8, 182.3 ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{62}H_{103}N_{15}O_{13}SNa^+$: 1320.747, found: 1320.8105.

Macrocycle 1: A solution of HCl (3 M, 1 mL, 3 mmol) was added to **15** (0.314 g, 0.24 mmol) in H_2O (15 mL). The solution was warmed at 50 °C for 12 h. The mixture was then cooled to room temperature and brought to pH 9–10 by adding solid Na_2CO_3. The solvent was removed, and the residue was suspended in EtOH (10 mL) and filtered. To this solution, solid Na_2CO_3 (106 mg) and diethyl squarate (0.045 g, 0.26 mmol) in EtOH (1 mL) were added. The reaction was stirred at room temperature for 24 h. The solvent was then removed, and the crude was dissolved in HCl (3 M, 2 mL). NaOH (1 M) was added until pH 9–10. The precipitate was centrifuged, dried and extracted in CH_2Cl_2 (5 mL). Compound **1** was obtained as a pale-yellow solid (0.1 g, 35%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta =$ 1.62 (br, 18H, $CH_2CH_2CH_2$ + 1H $CHCH_aH_bCH_2S$), 1.99 (br, 3H, SCH_3 + 1H$CHCH_aH_bCH_2S$), 2.07 (s, 12H, NCH_3), 2.30 (br, 16H, CH_2N), 3.11 (br, 2H, CH_2CH_2S), 3.48 (br, 18H, CH_2NH(sq) + 2HCH_2NHCO), 4.59 (br, 1H, NHCHCONH), 8,12 (br, 10H, NH) 8.2 ppm (br, 1H, NHCO); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 17.7$, 31.6, 32.0, 33.9, 39.0, 44.6, 57.2, 58.0, 171.0, 185.3 ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{56}H_{85}N_{15}NaO_{11}S$: 1198.6166, found: 1198.6171.

Macrocycle 2: Compound **2** was prepared and purified as described above for **1** by starting from **16** (0.4 g, 0.31 mmol) in MeOH and diester **12** (0.133 g, 0.34 mmol). Purification of the crude product by washing with THF (2×5 mL) and acetone (2×5 mL) afforded **2** as a pale-yellow solid (0.176 g, 41%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.63$ (br, 22H $CH_2CH_2CH_2$ + 1H$CHCH_aH_bCH_2S$), 1.99 (s, 3H, SCH_3 + 1H$CHCH_aH_bCH_2S$), 2.09 (s, 15H, NCH_3), 2.29 (br, 20H, CH_2N), 3.12 (br, 2H, CH_2CH_2S), 3.48 (br, 22H, CH_2NH(sq) + 2HCH_2NHCO), 4.61 (br, 1H, NHCHCONH), 7.42 ppm (br, 13H, NH); ^{13}C NMR (75 MHz, D_2O-HCl): $\delta = 16.9$, 28.0, 31.8, 39.2, 42.7, 43.8, 55.9, 170.7, 175.3 184.3 ppm; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{67}H_{103}N_{18}O_{13}S$: 1399.7667, found: 1399.7673.

Macrocycle 3: Compound **3** was prepared in 30% yield as described for compound **7** from the trimeric aminosquaramide and diester **17**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.27$ (br, 6H, $NHCH_2CH_2(CH_2)_3$), 1.48 (br, 4H, $NHCH_2CH_2(CH_2)_3$), 1.64 (br, 16H, $NCH_2CH_2CH_2N$), 2.09 (s, 12H, NCH_3), 2.29 (br, 16H, CH_2NCH_2), 3.48 (br, 20H, CH_2NH(sq)), 7.47 ppm (br, 10H, NH); ^{13}C NMR (75 MHz, D_2O-HCl): $\delta = 27.9$, 32.6, 42.8, 43.9, 47.1, 55.9, 170.3, 170.7, 183.4, 184.1 ppm; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{55}H_{85}N_{14}O_{10}$: 1101.6568, found: 1101.6580.

Oligosquaramide 18: Compound **18** was prepared as described above for compound **15** from N^1-(3-aminopropyl)-N^1-benzylpropane-1,3-diamine (445 mg, 2.01 mmol) and compound **11** (1.78 g, 4,82 mmol) to give **18** as a pale-yellow solid (1.32 g, 74%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.36$ (s, 18H, $C(CH_3)_3$), 1.49 (m, 4H, $NCH_2CH_2CH_2N$), 1.66 (m, 8H, $NCH_2CH_2CH_2N$), 2.10 (s, 6H, CH_3), 2,27(m, 8H, $NCH_2CH_2CH_2N$), 2.42 (m, 4H, $NCH_2CH_2CH_2N$), 2.91 (m, 4H, CH_2NHCOO), 3.52 (m, 10H, NCH_2Ar), 6.76 (br, 2H, CH_2NHCOO), 7.10–7.60 ppm (m, 5H, Ar + 4HNH); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 27.7$, 29.0, 39.0, 42.3, 42.4, 50.9, 55.0, 55.3, 58.4, 78.1, 127.5, 128.8, 129.4, 140.1, 156.4, 168.7, 183.1 ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{45}H_{73}N_9NaO_8^+$: 890.5474, found: 890.5441.

Oligosquaramide 19: Compound **19** was prepared and purified as described above for **16** by starting from **18** (486 mg, 0.60 mmol) and **11** (454 mg, 1.23 mmol). The desired product was obtained as a white solid (477 mg, 60%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta =$ 1.35 (s, 18H, C(CH$_3$)$_3$), 1.48 (m, 4H, NCH$_2$*CH*$_2$CH$_2$N), 1.64 (m, 16H, NCH$_2$*CH*$_2$CH$_2$N), 2.10 (s, 12H, CH$_3$), 2,24 (br, 16H, NCH$_2$CH$_2$*CH*$_2$N), 2.40 (m, 4H, N*CH*$_2$CH$_2$CH$_2$N), 2.90 (m, 4H, *CH*$_2$NHCOO), 3.49 (br, 2H, N*CH*$_2$Ar + 16HC*H*$_2$NH(sq)), 6.76 (br, 2H, CH$_2$N*H*COO), 7.00–7.80 ppm (m, 5H Ar + 8HNH); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta =$ 27.2, 28.7, 37.9, 42.0, 42.1, 50.4, 54.5, 54.9, 58.0, 78.2, 78.5, 127.2, 128.5, 129.2, 139.5, 156.3, 168.1, 182.5 ppm; HRMS (ESI): *m/z* [*M* + Na]$^+$ calcd for $C_{67}H_{107}N_{15}NaO_{12}^+$: 1336.8116, found: 1336.8148.

Macrocycle 10: Compound **10** was prepared as described above for compound **7** by starting from oligosquaramide **19** and diethyl squarate. In a typical procedure, **19** (477 mg, 0.363 mmol) was dissolved in H$_2$O (15 mL) and HCl (3 M, 1.1 mL, 3.3 mmol) and stirred for 5 h at 50 °C. The resulting solution was cooled to room temperature, and solid Na$_2$CO$_3$ was carefully added to pH 9. The solvent was evaporated, and the crude residue was dissolved in DMSO (2 mL) and MeOH (40 mL). The solution was filtered to eliminate salts prior to the addition of diethyl squarate (54 μL, 0.365 mmol) in MeOH (10 mL). The reaction mixture was stirred at room temperature for 72 h. The solvent was removed, and the crude was dissolved in aqueous Na$_2$CO$_3$ (5%, 20 mL) and extracted with CHCl$_3$ (4×15 mL) and CHCl$_3$/*t*BuOH 50:50 *v/v* (2×15 mL). The organic layers were dried over anhydrous Na$_2$SO$_4$, and the solvent was removed. The solid residue was crystallized from CH$_2$Cl$_2$/Et$_2$O (30:70) to yield **10** as a pale-yellow solid (303 mg, 70%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta =$ 1.67 (br, 20H, CH$_2$), 2.14 (br, 12H, NCH$_3$), 2.38 (br, 20H, CH$_2$), 3.03 (br, 2H, CH$_2$), 3.50 (br, 20H, CH$_2$), 8.2–7.2 ppm (br, 15H, NH + C$_6$H$_5$); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta =$ 27.8, 42.7, 44.0, 52.5, 55.9, 131.4, 131.9, 132.6, 133.4, 170.3, 182.9 ppm; HRMS (ESI): *m/z* [*M* + H]$^+$ calcd for $C_{61}H_{90}N_{15}O_{10}$: 1192.6995, found: 1192.6969.

Biology

Cell culture: The human MCL cell lines Jeko-1, JVM-2, Granta-519, Rec-1, and Z-138 were maintained in RPMI 1640 medium. Human glioblastoma cell lines T98G, U373, U87MG, and LN229, human osteosarcoma U2OS cells, and human colorectal cancer SW620 cells were maintained in DMEM. All media were supplemented with 10% heat-inactivated fetal bovine serum and 100 U mL^{-1} penicillin/streptomycin. All cell lines were grown under humidified air containing 5% CO$_2$ at 37 °C.

*In vitro viability assays with compounds **1**–**10***: The number of viable cells in culture was determined based on quantification of ATP, which signals the presence of metabolically active cells, using the Cell Titer-Glo luminescent assay kit (Promega, Madison, WI, USA) by following the manufacturer's instructions. Briefly, cells (4×10^5 cells mL^{-1}) were treated with the compound under assay (**1**–**9**) for 48 h followed by the addition of Cell Titer-Glo Reagent. Twelve final concentrations were tested: 200, 50, 20, 8, 3, 1, 0.3, 0.1, 0.05, and 0.01 μM. Moreover, DMSO was added as a negative control (0.01% *v/v*). Luminescence was detected using a multi-well scanning spectrophotometer (Plate Chameleon, Hidex, Finland). Cell viability is represented as a percentage relative to vehicle-treated cells, and data are the mean of three independent determinations performed in duplicate. IC$_{50}$ is defined as the compound concentration required to produce 50% inhibition of growth relative to vehicle-treated control. IC$_{50}$ values were calculated from sigmoidal analysis of dose–response curves using GraphPad Prism v.5 for MacOS (GraphPad Software Inc., La Jolla, CA, USA).

Multicellular tumor spheroid formation assays: To monitor the formation of multicellular tumor spheroids in culture, 4×10^5 U87MG cells were plated in six-well plates and treated as indicated in the figure legends. After 4–6 days, cells were stained with 0.5% (*w/v*) crystal violet in 70% EtOH, and the number of multicellular tumor spheroids from representative fields (>10) were counted under a light microscope.

Flow cytometry: Cell-cycle analysis was performed using propidium iodide staining. Briefly, Jeko-1 and JVM-2 cells were treated with cyclosquaramide **7** for 24 h, and were then washed in PBS and fixed in 90% EtOH. Fixed cells were washed twice in PBS and stained in 50 μM propidium iodide containing 5 μg mL^{-1} DNase-free RNase for 1 h, then processed by flow cytometry using a FACScan (Coulter Epics XL-MSL, Beckman Coulter, Fullerton, CA, USA) and analyzed with winMDI software.

Kinase inhibition assays: These assays were performed by ProQinase GMBH, Freiburg (Germany), according to the procedure described by ProQinase.[35] A radiometric protein kinase assay (PanQinaseActivityAssay) was used for measuring the kinase activity of the 25 protein kinases. All kinase assays were performed in 96-well FlashPlates from PerkinElmer (Boston, MA, USA) in a 50 μL reaction volume. The reaction cocktail was pipetted in four steps in the following order: 15 μL ATP solution (in H$_2$O), 20 μL assay buffer (see below), 5 μL test sample in 10% DMSO, and 10 μL enzyme/substrate mixture (in storage buffer). The assay for all enzymes contained 60 mM HEPES·NaOH (pH 7.5), 3 mM MgCl$_2$, 3 mM MnCl$_2$, 3 mM Na$_3$VO$_4$, 1.2 mM DTT, 50 μg mL^{-1} PEG 20000, 1 μM [γ-^{33}P]ATP (~6×10^5 cpm per well), protein kinase (variable amounts; see table S1 in the Supporting Information), and substrate (variable amounts; see table S1).

Evaluation of the mode of ABL1 inhibition: A radiometric filter-binding assay was used for measuring the kinase activity under various conditions. All kinase assays were performed in 96-well polypropylene microtiter plates in a 50 μL reaction volume. The reaction cocktail was pipetted in five steps in the following order: 20 μL assay buffer (standard buffer), 5 μL test compound (in 10% DMSO), 10 μL enzyme solution, 10 μL substrate solution, and 5 μL ATP solution (in H$_2$O). The assay contained 60 mM HEPES·NaOH (pH 7.5), 3 mM MgCl$_2$, 3 mM MnCl$_2$, 3 μM Na$_3$VO$_4$, 1.2 mM DTT, 50 μg mL^{-1} PEG 20000, and various amounts of [γ-^{33}P]ATP (~1.5×10^6 cpm per well).

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Keywords: antitumor agents · biological activity · inhibitors · macrocycles · squaramides

[1] Throughout this paper the term "squaramide" is used to designate 3,4-diamino derivatives of 3,4-dihydroxycyclobut-3-ene-1,2-dione (squaric

acid). We coined the term "cyclosquaramide" to designate cyclic oligomeric aminoalkyl-squaramide compounds.
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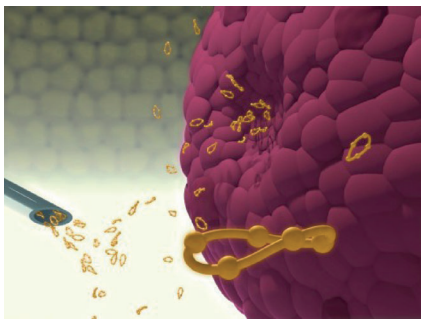
Cyclosquaramides as Kinase Inhibitors with Anticancer Activity



It's hip to be square: A new series of oligosquaramide macrocycles were synthesized and evaluated as anticancer agents. Sensitivity to cyclosquaramides clearly depends on cell type, indicating a degree of biological selectivity. The macrocyclic structure with minimal molecular size plays a key role in the observed antitumor activity, and the antiproliferative activity is related to the effective inhibition of several important kinases.

