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Synthesis of a High-Purity Chemical Library Reveals a Potent Inducer of Oxidative Stress

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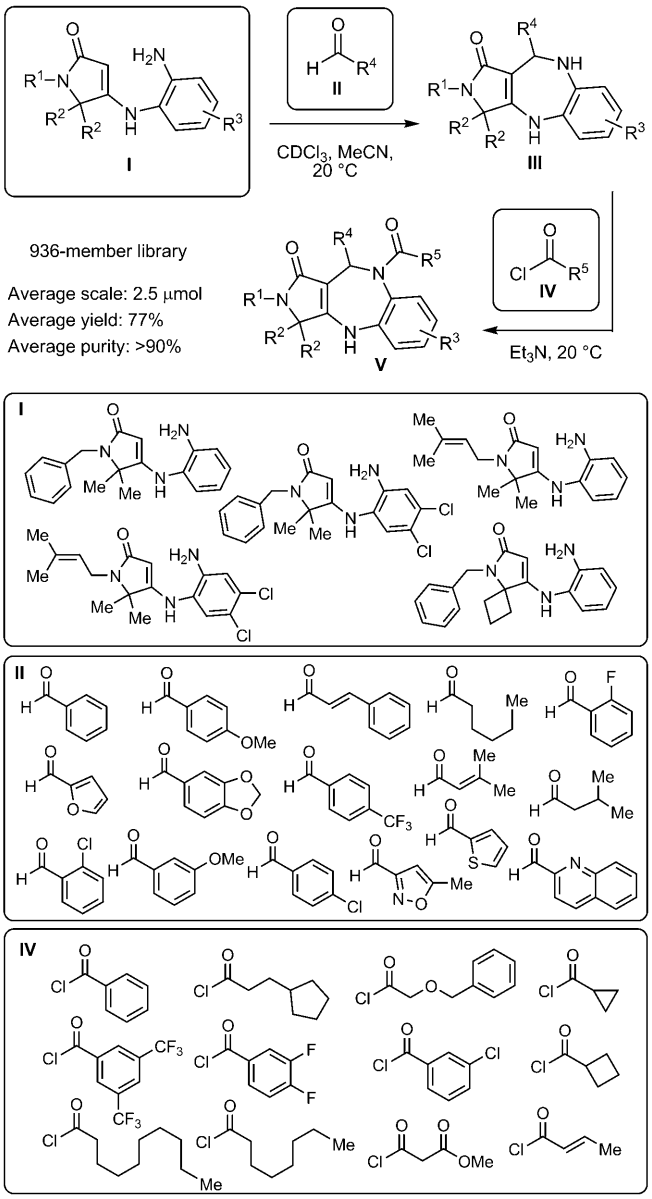
The high-throughput screening of chemical libraries plays an important role in biomedical research. The development of small-molecule libraries that would occupy the regions of vast chemical space that can be effectively recognized by biological targets remains challenging. One strategy is to assemble libraries that incorporate validated pharmacophores, also known as privileged structures,[1] in order to increase the probability of identifying potent bioactive chemotypes. We describe here the assembly of a 936-member small-molecule library that was designed to contain a fusion of benzodiazepine and tetramic acid subunits. The selection of this structural platform was guided by the prevalent biological activities of the two heterocyclic fragments,[1,2] as well as their favorable physicochemical properties. A cell-viability screen of this chemical library identified a small molecule that rapidly induced oxidative stress, produced substantial DNA fragmentation, and displayed an unusual mechanism of cell death initiation that entailed activation of both caspase-dependent and caspase-independent cellular pathways. The potent activity of this agent (IC_{50} = 0.16–1.0 μM) is remarkable given the relatively small number of compounds that were subjected to the primary screen, as well as the low toxicity of the library.

The parallel assembly process was based on a protocol originally described by Matsuo and Tanaka,[3] and entailed the condensation of five vinylogous ureas **I** with 16 aldehydes **II** to give 80 tricyclic amines **III**, followed by chemoselective N-acylation with 12 acid chlorides **IV** (Scheme 1). The resulting library **V** was prepared in solution on a 2.5 μmol scale (1.0–1.5 mg of final products) and was rapidly purified by parallel preparative thin-layer chromatography.[4] Subsequent analysis established that 936 out of 960 reactions proceeded successfully to deliver the final compounds in high chemical purity.[5] ^{1}H NMR analysis was employed to quantify the amount and purity of 120 randomly selected compounds.[5]

Evaluation of the proliferation of the A549 cell line in the presence of the library revealed that a single library member, which was termed triazatricyclamide (**1**) due to its polyheterocyclic architecture, suppressed cellular growth with an IC_{50} of 160 nM (Figure 1). Similarly potent activity of **1** was observed in several other cancer cell lines (Figure 1 and Figure S1 in the Supporting Information). Subsequent construction of a focused library (Figure 2) revealed that the cyclopropyl amide moiety (R^1) was crucial to the potency of **1**. *para*-substitution in the phenyl group (R^2) was well tolerated and resulted in increased activity of fluorine-substituted analogue. A disubstituted alkene to link the phenyl group to the tricyclic core, and an *N*-prenyl substituent (R^3) were both required for activity.

Cell-cycle analysis on HL-60 cells revealed that **1** induced a concentration-dependent increase in G0/G1 population and a



Scheme 1. Synthesis of 936-member small-molecule library.

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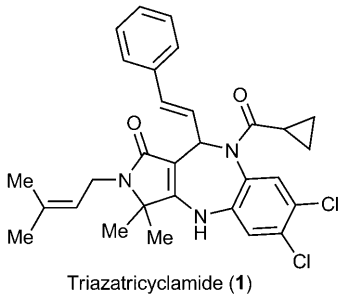

Cell line	IC_{50}
A549	0.16 μM
PC3	0.30 μM
MCF7	0.81 μM
HCT116	0.24 μM
HL-60	1.00 μM

Figure 1. Growth inhibitory constants (IC_{50}) of **1** in five cancer cell lines.

decrease in S population, while the G2 population was unaffected (Figure S2). Arrested growth was associated with substantial chromatin condensation (Figure 3) and DNA fragmentation detected by the standard Nicoletti method (Figure S3).[6,7] Since fragmented DNA and condensed chromatin are typical hallmarks of the apoptosis,[6] we next examined the activity of caspases that are involved in the execution of programmed cell death. Indeed, treatment of cells with **1** resulted in both dose- and time-dependent activation of caspases 3, 7 and 8 (Figures 4 and S4), with the dynamics of this process being distinctly different from that of a known apoptosis inducer, staurosporine (STS).[8]

Analysis of the dynamics of cell death induced by **1** revealed a concentration-dependent build-up of reactive oxygen species (ROS),[9] which was observed as early as three hours after initial incubation with **1** (Figures 5 and S6). This effect appeared to be dependent on the caspase activation, since treatment of cells with an established caspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate fluoromethyl ketone (z-VAD-fmk)[10] substantially suppressed the ROS generation induced by **1** (Figure S6). The absence of changes in mitochondrial membrane potential during the same incubation period (Figure S5) suggested that mitochondria were not directly involved in the early release of ROS; this pointed to an unusual mechanism of cell death initiation.

In addition to caspase activation, the presence of phosphatidyl serine (PS) on the outer leaflet of the membrane is highly indicative of early apoptotic cells.[11] We next analyzed the cellular exposure of PS, as well as the membrane integrity of HL-60 cells treated with **1**. Incubation of cells with **1** resulted in early (6–12 h after treatment) and caspase-dependent exposure of PS, as shown by the appearance of the annexin V-positive/propidium iodide-negative (AV+/PI−) population of cells and the ability of z-VAD-fmk to block its formation (Figures 6 and S7). The membrane integrity of cell, however, was progressively lost in caspase-independent manner during the first 24 h, since formation of cell population, which was positive in both annexin V (AV+) and propidium iodide (PI+), could not be inhibited by z-VAD-fmk (Figures 6 and S7). Additional results indicated that the cell death induced by **1** was independent of extrinsic and intrinsic apoptotic pathways. Similar levels of DNA fragmentation in response to treatment with **1** were observed Jurkat A3 (JA3) cells that lacked a Fas-associated protein with Death Domain (JA3 FADD−/−) or the apoptosis initiator caspase 8 (JA3 caspase8−/−) compared to JA3 parental cells (Figure 7), as well as MCF7-Fas cells transfected with Bcl-xL (MCF7-FB) compared to the same cells transfected only with vector control (MCF7-FV; Figure 8).[12]

Our initial analysis revealed that **1** activated both caspase-dependent and -independent cell-death pathways. The early

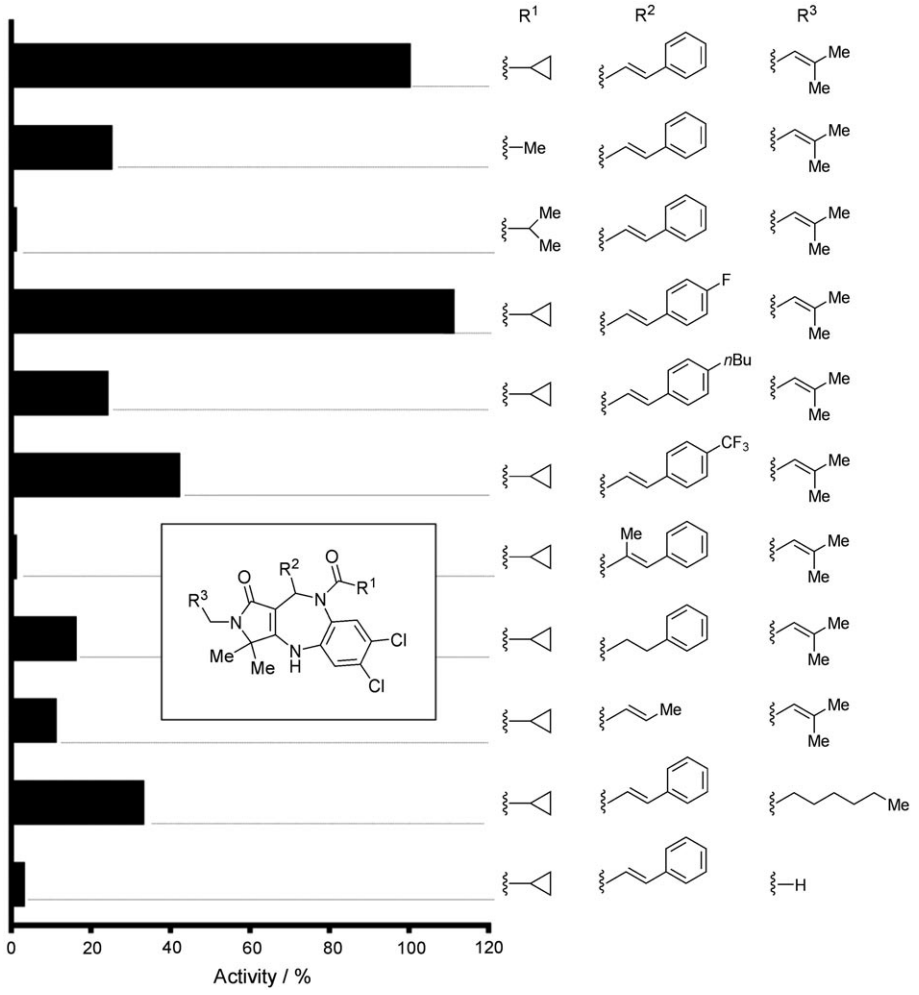

Figure 2. Structure–activity relationship of triazatricyclamides measured by their ability to inhibit the growth of A549 cells; compound **1** was assigned a relative activity of 100%, which corresponded to IC_{50} = 160 nM.

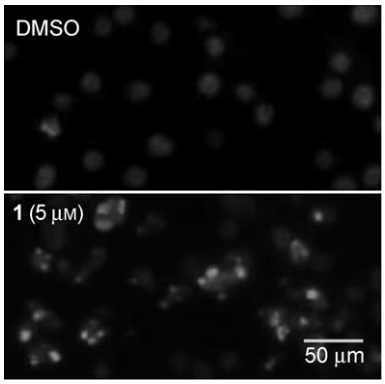

Figure 3. Chromatin condensation visualized by staining HL-60 cells with Hoechst 33342.

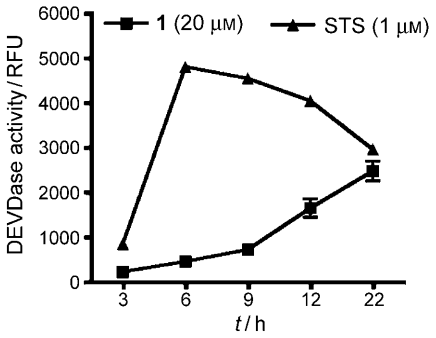

Figure 4. Time dependence of caspase-3/7 activation in HL-60 cells treated with **1** and staurosporine (STS). Error bars, standard deviation (SD); $n = 3$.

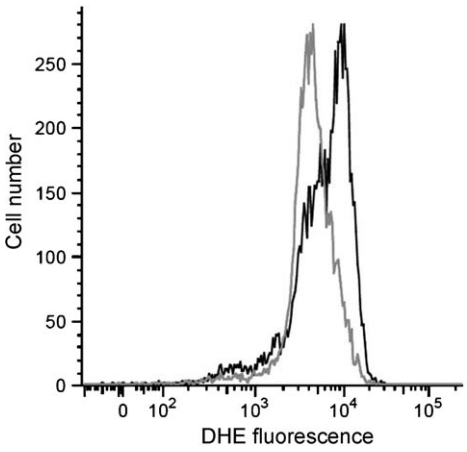

Figure 5. Release of ROS in HL-60 cells in response to **1** (20 μM) for 3 h (shown in black) compared to DMSO control (shown in gray) measured by monitoring dihydroethidium (DHE) fluorescence.

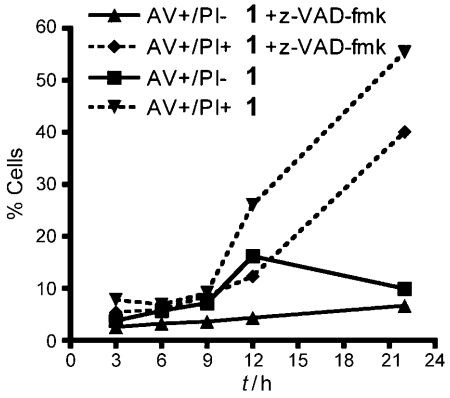

Figure 6. Effect of z-VAD-fmk on PS exposure and membrane integrity in HL-60 cells treated with **1**.

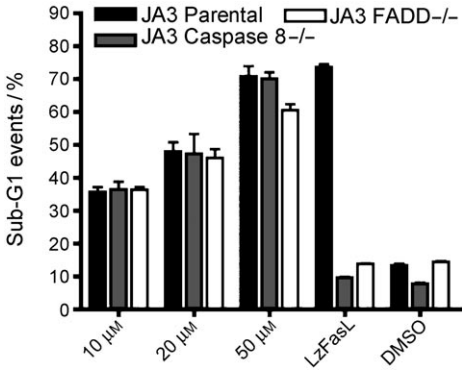

Figure 7. DNA fragmentation in Jurkat A3 cells lacking key components of the extrinsic apoptotic pathway upon treatment with **1**, leucine zipper-tagged Fas ligand (LzFasL) and DMSO for 24 h. Error bars, SD; $n = 3$.

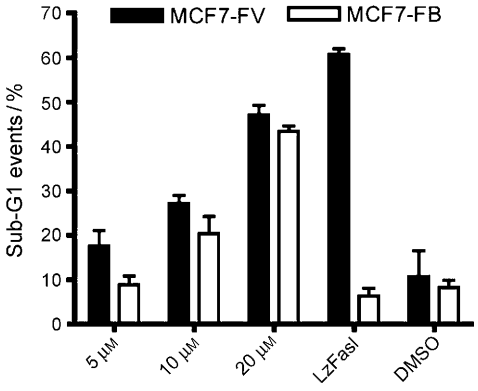

Figure 8. DNA fragmentation in MCF7-Fas cells overexpressing Bcl-xL upon treatment with **1**, LzFasL and DMSO for 24 h. Error bars, SD; $n = 3$.

events, such as ROS generation and PS exposure, were found to be highly dependent on caspase activation. It appears, however, that at some point DNA degradation and cell death induced by **1** become independent of caspase activity. A number of caspase-independent forms of cell death have been described.[13] Most of them involve the release of mitochodrial proteins such as apoptosis-inducing factor (AIF), high-temperature requirement protein (HtrA2/OMI), or endonuclease G (ENDO G), which can cause nuclear changes independently of caspase activation.[13] However, the release of such factors would be inhibited by Bcl-xL. The fact that Bcl-xL did not prevent cell death, at least not in MCF7 cells, seems to suggest that **1** activated a pathway that was independent of mitochondria.[14] To our knowledge, this activity profile does not correlate to any of the existing agents that are capable of inducing either caspase-dependent or -independent cell death.[15]

In closing, we have demonstrated that the high-throughput synthesis of a chemical library based on the fusion of two privileged biogenic subunits resulted in the identification of a potent chemical agent that impaired cellular viability by an unusual mechanism involving both caspase-dependent and -independent cell-death pathways. Detailed investigation of the mechanism of action and elucidation of the cellular target of triazatricyclamide are under investigation.

Experimental Section

Library synthesis: The following procedure represents the synthesis of the first set of 96 compounds. One of the five vinylogous ureas **I** (256 μmol) was dissolved in acetonitrile (1.92 mL) and $CDCl_3$ (3.2 mL). The resulting solution was divided into eight equal batches (640 μL each) that were treated individually with eight aldehydes **II** (40–60 μmol each). Following subsequent treatment of each reaction mixture with 12 N aqueous HCl (1 μL), the solutions were left at 20 °C until the starting material had been completely consumed. Each reaction mixture was treated with Et_3N (15 μL) and divided into twelve equal batches (each 50 μL batch containing 2.5 μmol of one benzodiazepine **III**), which were transferred into a polypropylene 96-well PCR plate. Each well was treated with 12 acid chlorides **IV** (0.60–1.2 μL) according to the plate map shown in the Supporting Information. Upon complete consumption of diamines **III**, each well was treated with a solution of 2 N aq. NaOH/MeOH (1:4, 15 μL). The reaction mixtures were transferred onto preparative TLC plates by using a multichannel pipettor with adjustable gaps. The plates were developed in acetone/dichloromethane (1:6), containing 1% of NEt_3. The products were detected under UV light and removed from TLC plates as circular silica gel pellets. Each compound was eluted from the silica gel with MeOH (0.6 mL). The solvent was removed in vacuo, and 12 randomly selected compounds were dissolved in CD_3OD (0.5 mL) and analyzed by ^1H NMR. The amount of material in each sample was determined by integration using residual MeOH as a precalibrated internal standard.

Triazatricyclamide (1): ^1H NMR (400 MHz, CD_3OD): $\delta = 7.54$ (s, 1H), 7.43 (s, 1H), 7.16–7.24 (m, 5H), 6.47 (d, $J = 3.6$ Hz, 1H), 6.13–6.15 (m, 2H), 5.20 (t, $J = 6.6$ Hz, 1H), 4.01 (d, $J = 6.4$ Hz, 2H), 1.76 (s, 3H), 1.71 (s, 3H), 1.52 (s, 3H), 1.42 (s, 3H), 1.30–1.32 (m, 1H), 0.94–0.96 (m, 1H), 0.77–0.87 (m, 2H), 0.62–0.64 (m, 1H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 171.40$, 167.61, 157.14, 140.02, 135.91, 133.26, 132.56, 132.17, 130.47, 129.03, 128.98, 127.74, 126.36, 123.27, 122.51, 121.24,102.75, 61.20, 52.52, 35.89, 25.41, 24.09, 23.51, 17.62, 12.61, 8.39, 8.18.; MS (APCI) calcd for $C_{30}H_{31}Cl_2N_3O_2$: 535.18 $[M]^+$, found 536.1 $[M+H]^+$.

Cellular growth inhibition: All assays were performed in at least three replicate wells for each compound concentration tested. Threefold serial dilutions in DMSO were performed for each active compound by using stock DMSO solutions with NMR-calibrated concentrations. We seeded cells in 96-well white plates at a density of 1000 cells per well (A549, PC3, HCT116, MCF7 cell lines) or 3000 cells per well (HL-60 cell line) in the appropriate cell culture medium (100 μL). Adherent A549, PC3, HCT116 and MCF7 cells were allowed to attach and grow for 24 h before treatment with serially diluted compound solutions, and incubation for a further 48 h. HL-60 cells were treated with the compound solutions at the time of seeding and incubated for 48 h. After incubation, the number of cells was determined by using CellTiter-Glo (Promega). Compound concentrations that gave a 50% reduction in cell growth when compared with vehicle control cells were calculated from sigmoidal plots of cell viability versus the logarithm of drug concentration.

Chromatin condensation: HL-60 cells were harvested by centrifugation (250 *g*, 5 min) and resuspended in complete growth medium. Cells were counted by using a hemacytometer. For each sample, approximately 1×10^6 cells were transferred into an untreated polystyrene culture dish. Complete growth media containing various concentrations of drug as well as DMSO vector only were added (6 mL total volume, 0.1% DMSO). After incubation for 24 h, the cells were harvested by centrifugation (250 *g*, 5 min) and washed with phosphate-buffered saline (PBS, 1 mL). The cells were then resuspended in PBS (1 mL) and treated with a solution of Hoechst 33342 (1 μL, 5 mg mL^{-1}). The samples were protected from light and incubated on ice for 30 min before analysis on an Olympus IX81 fluorescence microscope.

ROS production: HL-60 cells (0.25×10^6 cells in 1 mL of medium) were seeded into 12-well plates and treated with desired stimuli (0.2% DMSO in all samples). After the indicated incubation time, cells were harvested by centrifugation, washed with PBS (1 mL) and resuspended in a solution of dihydroethidium (DHE) in PBS (0.5 mL, 10 μM). The cells were protected from light and incubated at 37 °C for 15 min, followed by analysis on a BD FACSCanto flow cytometer.

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