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

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Abstract

Synthesis of high-purity biogenic heterocyclic library enabled identification of a small molecule, which potently inhibited proliferation of several cancer cell lines and induces rapid oxidative stress. This agent elicited unusual mechanism of cell death induction, which entailed activation of both caspase-dependent and independent pathways.

Keywords

chemical libraries; cell death; cytotoxicity; reactive oxygen species; caspase

High-throughput screening of chemical libraries plays an important role in biomedical research. Development of small-molecule libraries that would occupy the regions of vast chemical space that can be effectively recognized by biological targets remains to be challenging. One strategy is to assemble libraries that would 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, which 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. Cell viability screen of this chemical library resulted in identification of a small molecule, which rapidly induced oxidative stress, produced substantial DNA fragmentation, and displayed an unusual mechanism of cell death initiation, which entailed activation of both caspase-dependent and caspase-independent cellular pathways. The potent activity of this agent (IC₅₀ 0.16–0.1 μM) is remarkable taking into account a relatively small number of compounds, which was 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 5 vinylogous ureas **I** with 16 aldehydes **II** to give 80 tricyclic amines **III**, followed by chemoselective N-acylation using 12 acid chlorides **IV** (Figure 1A). The resulting library **V** was prepared in solution on 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

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successfully to deliver the final compounds in high chemical purity.^[5] ¹H NMR analysis was employed to quantify the amount and purity of 120 randomly selected compounds.^[5]

Evaluation of proliferation of A549 cell line in the presence of chemical library **V** revealed that a single library member, which was termed triazatricyclamide (**1**) due to its polyheterocyclic architecture, suppressed cellular growth with an IC₅₀ of 160 nM (Figure 1B). Similarly potent activity of **1** was observed in several other cancer cell lines (Figure 1B and Figure S1). Subsequent construction of a focused library (Figure 1C) revealed that cyclopropyl amide moiety (R₁) was crucial for potent activity of **1**. Para-substitution in the phenyl group (in R₂) was well tolerated and resulted in the increased activity of fluorine-substituted analog. Disubstituted alkene, which links the phenyl group to the tricyclic core, as well as the N-prenyl substituent (R₃) were both required for activity.

Cell cycle analysis using HL-60 cells revealed that **1** induced concentration-dependent increase in G0/G1 population, decrease in S population while the G2 population was unaffected (Figure S2). The growth arrest was associated with substantial chromatin condensation (Figure 1D) and DNA fragmentation detected by the standard Nicoletti method (Figure S3).^[6,7] Since fragmented DNA and condensed chromatin represent typical hallmarks of apoptosis,^[6] we next examined activity of caspases that are involved in 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 (Figure 1E and Figure S4) with dynamics of this process being distinctly different from a known apoptosis inducer, staurosporine (STS).^[8]

Analysis of the dynamics of cell death induced by **1** revealed concentration-dependent build-up of reactive oxygen species (ROS),^[9] which was observed as early as three hours after the initial incubation with **1** (Figure 1G and Figure 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 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, which 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 hours after treatment) and caspase-dependent exposure of PS, which was evident by the appearance of the annexin V-positive/propidium iodide-negative (AV+/PI–) population of cells and ability of z-VAD-fmk to block its formation (Figure 1H and Figure S7). The membrane integrity of cell, however, was progressively lost in caspase-independent manner during the first 24 hours, 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 (Figure 1H and Figure 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 lacking a Fas-associated protein with Death Domain (JA3 FADD–/–) or the apoptosis initiator caspase 8 (JA3 caspase8–/–) compared to JA3 parental cells (Figure 1F), as well as MCF7-Fas cells transfected with Bcl-xL (MCF7-FB) compared to the same cells (MCF7-FV) transfected only with vector control (Figure 1I).^[12]

Our initial analysis revealed that **1** activated both caspase-dependent and caspase-independent cell death pathways. The early events, such as ROS generation and PS

exposure, were found to be highly dependent on caspase activation. It appears, however, that DNA degradation and cell death induced by **1** become at some point independent of caspase activity. A number of caspase independent forms of cell death have been described.^[13] Most of them involve release of mitochondrial 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] This activity profile, to our knowledge, does not correlate to any of the existing agents that are capable of inducing either caspase-dependent or caspase-independent cell death.^[15]

In closing, we demonstrated that high-throughput synthesis of a chemical library, which is based on fusion of two privileged biogenic subunits, resulted in identification of a potent chemical agent that impaired cellular viability by an unusual mechanism involving both caspase-dependent and caspase-independent cell death pathways. Detailed investigation of the mechanism of action and elucidation of the cellular target of triazatricyclamide is under active current 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 μ mol) was dissolved in acetonitrile (1.92 mL) and CDCl_3 (3.2 mL). The resulting solution was divided into 8 equal batches (640 μ L each) and treated individually with 8 aldehydes **II** (40–60 μ mol each). Following subsequent treatment of each reaction mixture with 12N aqueous HCl (1 μ L), the solutions were left at 20 °C until complete consumption of starting material. Each reaction mixture was treated with Et_3N (15 μ L) and divided into 12 equal batches (each 50 μ L batch containing 2.5 μ mol 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 μ L) according to the plate map shown in Supplementary Information. Upon complete consumption of diamines **III**, each well was treated with 15 μ L of a solution containing 2N aq. NaOH and MeOH (1:4). The reaction mixtures were transferred onto preparative TLC plates using a multichannel pipettor with adjustable gaps. The plates were developed using acetone: dichloromethane = 1:6, containing 1% of NEt_3 . The products were detected using UV light and removed from TLC plates as circular silica gel pellets. Each compound was eluted from silica gel with 0.6 mL of MeOH. 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 pre-calibrated internal standard.

Triazatricyclamide (**1**)

^1H NMR (400 MHz, CD_3OD)TM 7.54 (s, 1H), 7.43 (s, 1H), 7.16–7.24 (m, 5H), 6.47 (d, 1H, J = 3.6 Hz), 6.13–6.15 (m, 2H), 5.20 (t, 1H, J = 6.6 Hz), 4.01 (d, 2H, J = 6.4 Hz), 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)TM 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) calculated for $\text{C}_{30}\text{H}_{31}\text{Cl}_2\text{N}_3\text{O}_2$ 535.18 (M⁺), found 536.1 (M+H).

Cellular Growth Inhibition

All assays were performed using at least three replicate wells for each compound concentration tested. Threefold serial dilutions in DMSO were performed for each active compound using stock DMSO solutions with NMR-calibrated concentrations. We seeded cells in 96-well white plates at the density of 1,000 cells/well (A549, PC3, HCT116, MCF7 cell lines) or 3,000 cells/well (HL-60 cell line) in 100 μ L of the appropriate cell culture media. Adherent A549, PC3, HCT116 and MCF7 cells were allowed to attach and grow for 24 h before treatment with serially diluted compound solutions, and incubated further for 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 using CellTiter-Glo® (Promega). Compound concentrations that gave 50% reduction in cell growth when compared with vehicle control cells were calculated from sigmoidal plots of cell viability versus the logarithm of drug concentrations.

Chromatin Condensation

HL-60 cells were harvested by centrifugation (1500 rpm, 5 min) and resuspended in complete growth medium. Cells were counted using a hemacytometer. For each sample, approximately 1×10^6 cells were transferred into a non-treated 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 (1500 rpm, 5 min), washed with 1 mL of PBS. The cells were then resuspended in 1 mL of PBS and treated with 1 μ L of 5 mg/mL Hoechst 33342 solution. 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 1 mL of PBS and resuspended in 0.5 mL of 10 μ M solution dihydroethidium (DHE) in PBS. The cells were protected from light and incubated at 37 °C for 15 min, followed by analysis on a BD FACSCanto flow cytometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Synthesis of 936-member small-molecule library. B) Structure of triazatricyclamide (1) and growth inhibitory constants (IC₅₀) in five cancer cell lines. C) Structure-activity relationship of triazatricyclamides measured by the ability to inhibit growth of A549 cells; compound 1 was assigned a relative activity of 100%, which corresponded to IC₅₀=160 nM. D) Chromatin condensation, which was visualized by staining HL-60 cells with Hoechst 33342. E) Time-dependence of caspase-3/7 activation in HL-60 cells treated with 1 and staurosporine (STS). Error bars, standard deviation (SD); n=3. F) 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. G) Release of ROS in HL-60 cells in response to **1** (20 μ M) for 3 h (shown in black) compared to DMSO control (shown in gray) measured by monitoring dihydroethidium (DHE) fluorescence. H) Effect of z-VAD-fmk on PS exposure and membrane integrity in HL-60 cells treated with **1**. I) DNA fragmentation in MCF7-Fas cells overexpressing Bcl-xL upon treatment with **1**, LzFasL and DMSO for 24 h. Error bars, SD; n=3.