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High-volume cellular screening for anticancer agents with combinatorial chemical libraries: A new methodology

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Summary

A single-step cancer cell cytotoxic assay system for anticancer drug discovery has been developed which facilitates rapid screening of large combinatorial chemical libraries synthesized using the 'one-bead–one-compound' (OBOC) methodology. Each OBOC library bead incorporates two orthogonally cleavable linkers that release the bead-bound compound at a different pH. The assay utilizes high concentrations of tumor cells mixed directly with OBOC beads and plated in soft agarose containing tissue culture medium. One of the orthogonal linkers is cleaved at neutral pH in tissue culture releasing an aliquot of compound to diffuse at a relatively high local concentration into the soft agarose immediately surrounding the bead. Active compounds are identified visually from a clear ring of tumor cell lysis which forms within 48 h around just the rare bead releasing a cytotoxic compound. The bead releasing a cytotoxin is then plucked from the agar and the remaining compound still linked to the bead can be released for structural analysis, followed by compound resynthesis and confirmatory testing. This assay system has been successfully applied to identification of lead cytotoxic compounds from model peptidic and non-peptidic combinatorial chemical libraries. Use of this methodology may facilitate anticancer drug discovery.

Introduction

Development of anticancer drugs has proven to be a difficult task with slow but definite progress having occurred over the past 40 years. Part of the difficulty in anticancer drug development is presumably due to the complexities of cancer. Such complexities include the high spontaneous mutation rate and resultant plasticity of the genome leading to increased expression of growth-promoting oncogenes and inactivation or deletion of tumor-suppressor genes and our still fragmentary understanding of critical steps in cancer cell signalling pathways and drug-resistance mechanisms. Accordingly, most anticancer agents currently available are DNA-damaging antiproliferative agents, and it has been difficult to define essential molecular abnormalities in cancer which can serve as specific targets for rational drug design. An additional difficulty has been limitations in the screening systems available against which to test either rationally designed or empirically identified compounds (e.g. natural products and synthetic intermediates of unknown activity). During the 1960s and 1970s, the U.S. National Cancer Institute (NCI) utilized in vivo murine tumors for anticancer screening. These models were generally limited to one tumor type (e.g. P-388 or L-1210 leukemia) and could screen no more than 10 000 compounds per year [1]. In

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the 1980s and 1990s, the NCI has increasingly conducted the initial screening in vitro with numerous different kinds of human tumor cell lines, with subsequent confirmatory screening of positives in vivo in a relevant human tumor xenograft model. This strategy has as its goal the screening of about 20 000 novel compounds per year in the primary in vitro screen [2–3]. Inasmuch as the finding of a drug lead has been estimated to require testing 50 000 compounds or more, this empirical system is also quite limited.

It has been our goal to develop a new technology which would facilitate cost-effective in vitro testing of hundreds of thousands to millions of compounds each year against a variety of human tumor cell lines in vitro in order to identify additional leads for anticancer drug development. We hypothesized that an increased screening throughput of this magnitude would increase the probability that novel tumor-type selective compounds could be discovered in a more timely fashion, after which traditional approaches could be used to identify novel mechanisms of anticancer action. In order to achieve this goal, we focused on the use of combinatorial chemistry with which large numbers of compounds could be screened. The combinatorial chemical approach that we have exploited uses solid-phase chemistry in which individual resin beads within a library of combinatorial chemical beads each display a single unique compound ('one-bead–one-compound' or 'OBOC'), while the entire synthesized library contains many thousands or millions of distinct compounds capable of exploring a vast array of molecular spaces and molecular recognition patterns [4–9]. Some of the compound on each bead is attached with a cleavable linker so that it can be tested in solution for anticancer activity, while an additional aliquot of the compound remains on the bead for subsequent structural identification of 'hits' from the cytotoxicity assay system. Our first approach used a two-stage assay in 96-well microassay plates [10–12]. While this assay system proved useful for exploring known molecular targets, logistic difficulties compromised its capacity for in vitro testing of tumor cell lines in culture (as discussed in this paper). Therefore, we devised an alternative strategy intended to circumvent the major limitations of the two-step multiwell microassay for anticancer activity. Our solution was to develop a single-step screening culture system in which both the OBOC beads and a high concentration of tumor cells were mixed in molten agarose and allowed to gel into a semi-solid medium wherein the action of active cytotoxic compounds released by the beads could be readily identified via their induction of a zone of cell death around the active bead(s) [9]. This assay system appears to overcome major limitations of prior antitumor screening systems and in studies to be described herein has yielded several lead compounds of unique structure with selective activity against specific hematologic malignancy or solid tumor cell lines in vitro. An alternative in situ releasable approach of OBOC bead libraries on polyethylene films above a soft agar layer has been adapted by Jayawickreme et al. to identify peptide ligands for G-protein-coupled receptors on intact cells [13].

Materials and Methods

Combinatorial bead library synthesis

OBOC combinatorial chemical libraries were prepared using 130 μm or 220 μm diameter TentaGel Resin beads (polystyrene beads engrafted with polyethylene glycol). Chemical synthesis of peptidic or non-peptidic libraries was conducted using a split-synthesis protocol [4,5,14,15] for solid-phase synthesis, so that each chemical building block was added to a separate aliquot of the beads in a reaction vial and the coupling reaction performed. After completion of coupling, all bead aliquots were then mixed together in a common vessel for randomization, after which they were re-aliquoted into separate reaction vials for the next split-synthesis reaction step. This process was repeated until the desired compounds were synthesized. Peptidic libraries were synthesized using 80–100 natural and unnatural Fmoc-protected amino acid building blocks. Non-peptidic libraries were synthesized from various organic precursors onto compact scaffolds [7]. Using the split-synthesis protocol, each 130 μm bead contains approximately about 100 pmole ($>10^{13}$ molecules) of a single unique compound. A portion of the peptidic or non-peptidic compound on each bead was connected via cleavable linkers using a 'reverse diketopiperazine' (DKP) or ester bond [6,11,16,17]; some of the compound on each bead could be released into solution under specific conditions. For the DKP linkage, cleavage occurred at neutral pH, such as that of tissue culture medium. After a positive bead was identified and isolated, the active compound could be recovered for structure determination by placing individual beads in 0.1 M NaOH to facilitate the cleavage of the ester bond. For these exploratory studies, three libraries with distinctive characteristics were synthesized. Library 1 was a tetrameric peptide library that included both natural and unnatural amino acids. Library 2 was a trimeric peptide library constructed from a very large array of unnatural amino acids. Library 3 was a non-peptidic library in which each compound consisted of 3 organic subunits.

Tumor cell lines and media

A variety of adherent human solid tumor cell lines (A375 melanoma, A549 lung, SW480 colon, MCF-7 breast, and PC3 and LNCAP prostate cancer), as well as non-adherent lines from human hematologic malignancies (HL-60 leukemia and 8226 myeloma) and the murine P-388 leukemia, were obtained from the American Type Culture Collection (ATTC, Rockville, MD, U.S.A.) grown

in complete tissue culture medium with 5% fetal bovine serum (FBS) and maintained in a humidified 37° incubator gassed with 7% CO_2 in air. For the solid tumor lines, M15 medium was used, and for the hematologic tumor lines, RPMI-1640 was utilized. The cells were split on the day before the assay and sufficient cells were harvested on the day of assay to set up the required number of 35 mm petri dishes to test many thousands of compounds from combinatorial libraries comprised of OBOC beads.

Tissue culture assay system

Tumor cells were added to molten 0.5% sea-plaque agarose in complete medium plus 5% FBS to achieve a final concentration of 1.8 million cells/ml in a sterile tissue culture tube and maintained in the molten state after which approximately 1000 library beads were quickly added for each ml of agarose-cell suspension and the combination immediately mixed by swirling and 1.0 ml of the mixture was immediately plated under aseptic conditions into each of a series of 35 mm plastic petri dishes. The dishes were kept at room temperature in a biosafety hood for 10 min for gelation. Thereafter, the petri dishes were all transferred to a 37° humidified incubator for solid tumor cells in M15 medium, and also gassed with 7% CO_2 in air for leukemia, lymphoma or myeloma cells in RPMI media to allow compound diffusion from the OBOC beads and cytotoxicity against tumor cells to occur. After 48 h of incubation, the dishes were removed from the incubator and 150 µl of a 5 mg/ml solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each dish. Two to four hours after MTT addition, the plates were visually inspected to detect clear zones around active OBOC beads. Using a marking pen, the clear zones were marked on the bottom of each dish after which they were further evaluated under a low power dissecting microscope fitted with an eye-piece micrometer with which the ring diameter could be determined in arbitrary units. Active beads causing the largest zones of lysis were individually recovered under microscopic visualization using a micro-pipette for subsequent structure determination.

Structure determination

Determination of combinatorial compound structures on active peptide beads were achieved using the Edman degradation method with an automated protein sequencer [4]. For non-peptidic compounds and those containing certain unnatural amino acids difficult to identify via Edman degradation, structure determination was achieved using LC-MS methodology [7]. As this paper relates primarily to the exploration of a new anticancer cytotoxicity assay for drug discovery using combinatorial libraries, the specific structure of putative 'hits' in the assay system have been coded.

Results

The in situ soft agar screening assay technology proved to be applicable to the various cell lines tested. Many thousands of compounds from the three distinct model combinatorial libraries described in the methods section were tested during the initial evaluation of this new drug discovery technology for identification of cellular cytotoxicity. While 500–1000 combinatorial library beads were evaluated for cytotoxicity in each of the 35 mm plastic petri dishes, most dishes lacked zones of clearing around individual library beads. However, for each of the three libraries tested, a few dishes contained unambigous clear zones of a circular shape surrounding one or more combinatorial beads.

From Library 1, about 15 beads of many thouands tested were found to be active against P-388 leukemia in the initial screen, but no active compounds were noted with the solid tumor cell lines tested. All 15 active beads were recovered from the agarose plates. One of these beads, which had one of the largest clear ring diameters of cell lysis surrounding it was selected for structure identification (Fig. 1). Subsequent to its identification by Edman degradation, this compound was resynthesized in releasable format on 130 µm diameter resin beads for confirmatory testing in soft agar assay. In these second-stage tests, activity against P-388 leukemia was confirmed (Fig. 2), but the compound was found to be inactive against all six solid tumor cell lines tested. This compound (designated 'L-1'), was then resynthesized and purified for solution-phase testing and determination of the IC_{50}. In the initial solution-phase testing, IC_{50}'s against P-388 leukemia ranged from 1–10 µg/ml.

From Library 2, which was constructed as a series of sublibraries to facilitate structure identification, one sublibrary containing a unique unnatural amino acid (usually at a terminal position on the tripeptide) was found to be active against MCF-7 breast cancer and several other solid tumor lines, including lung cancer and melanoma, but inactive against P-388 leukemia or other hematologic malignancies tested. Ring diameters of active compounds in this library were relatively small (Fig. 3), suggesting that the compound is of relatively low potency.

From Library 3, comprised of various small organic compounds, one sublibrary contained beads which caused very large zones of lysis in the plates containing 8226 human myeloma cells. The large lysis zones suggest that this compound is very potent against its target tumor cell line. This same sublibrary from Library 3 did not exhibit antitumor activity against the solid tumor cell lines tested in the primary screen. Subsequent to compound identification from several active beads from the sublibrary, second stage tests confirmed that the initial compound had reproducible activity against 8266 myeloma. Additional testing is now underway of the lead compound in this series.

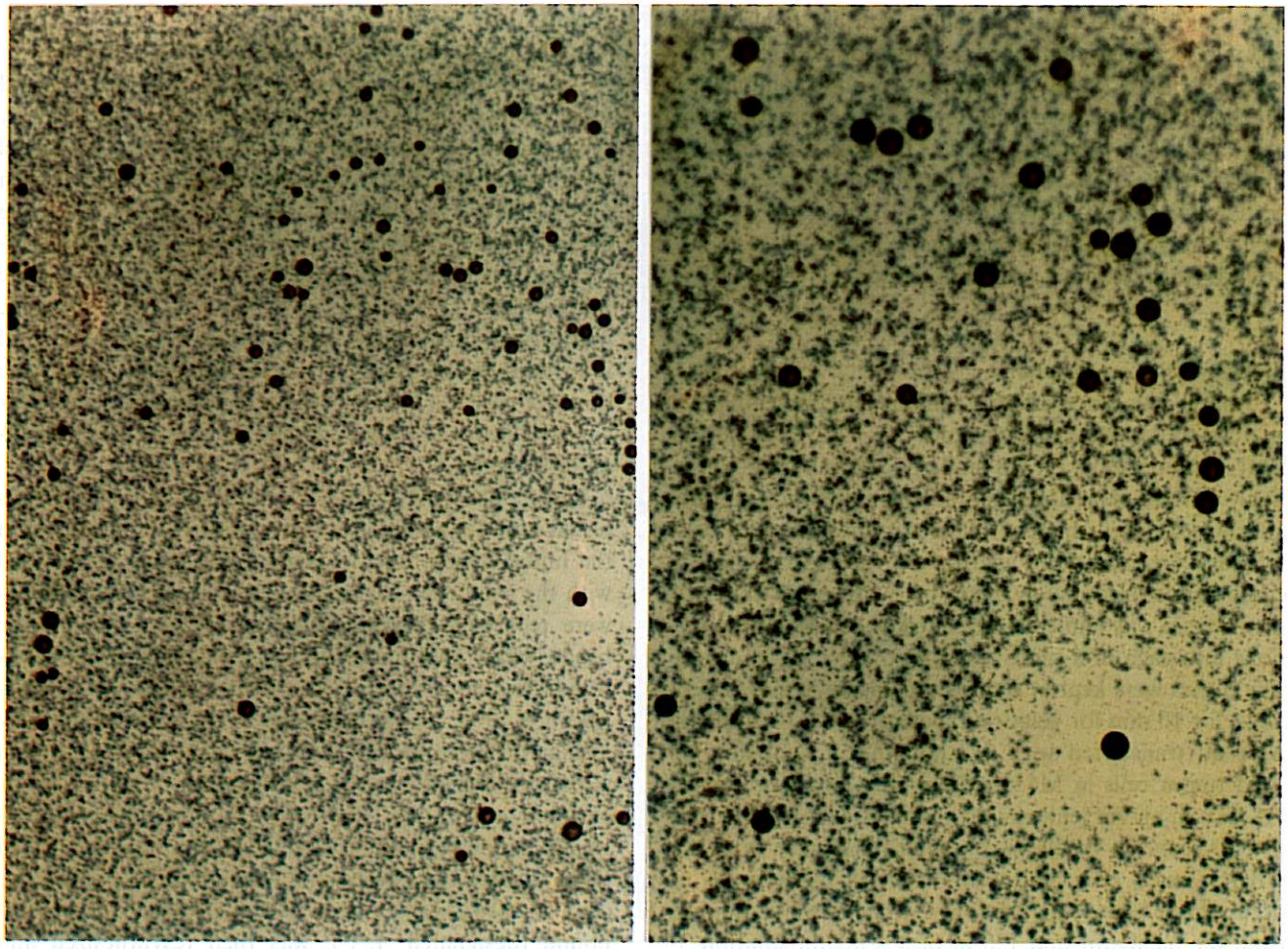

Fig. 1. In situ releasable soft agar assay for screening anti-cancer agents against P388 lymphoid leukemia. There is a clear zone of inhibition surrounding one positive bead. Left: low power; right: higher power. See text for experimental details.

Discussion

This paper describes a new technology for the rapid screening and structure identification of agents from combinatorial chemical libraries which exhibit cytotoxicity against human or murine tumor cell lines in vitro. The preliminary results obtained using less than 100 000 compounds from three model combinatorial libraries in this paper support the hypothesis that this assay system provides a means to identify specific compounds within combinatorial OBOC libraries that exhibit selective antitumor activity against specific cancer cell lines. As these studies were developmental, no attempt was made to comprehensively study all compounds within any one of the libraries utilized, but rather to determine whether the strategy and logistics of assay performance, structure identification and confirmation of activity provided sufficient throughput to support high-volume screening for identification of initial lead compounds for further development into potential anticancer drugs. The soft agarose system using a single-step release of compounds into the semi-solid medium for identification of cytotoxic compounds proved to be far more precise and rapid than our previously described solution-phase two-step screening assay [11,12] and eliminated the problem of false-positive wells resulting from spontaneous growth failure of tumor cells within individual wells containing 500–1000 compounds in a 96-well assay plate. Each such false positive in the 96-well format resulted in the requirement for secondary plate assays of an additional 50–100 additional 96-well plates before a decision could be reached as to whether an antitumor compound was present or not. Moreover, false negative may well have also been encountered in that system due to the dilution of individual compounds that necessarily occurred in microwells. In the microwell system, the volume of tissue culture medium and the number of replicates required to test multiple tumor cell lines of differing histology resulted in diluting compounds to the range of 0.1 mM or less, which effectively limited the screen to the detection only of very highly potent compounds. Therefore, more typical initial hits in the micromolar range could not be detected.

Unlike the solution-phase system, the soft agarose system appears to have markedly reduced false-positive

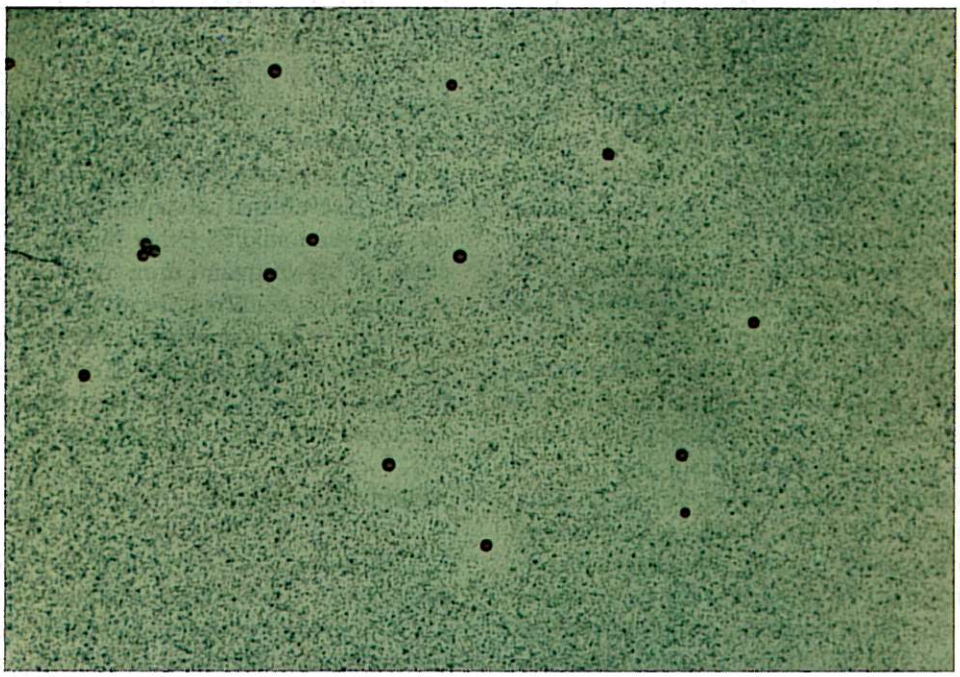

Fig. 2. Confirmatory test on the anti-P388 leukemia agent discovered from the initial lead.

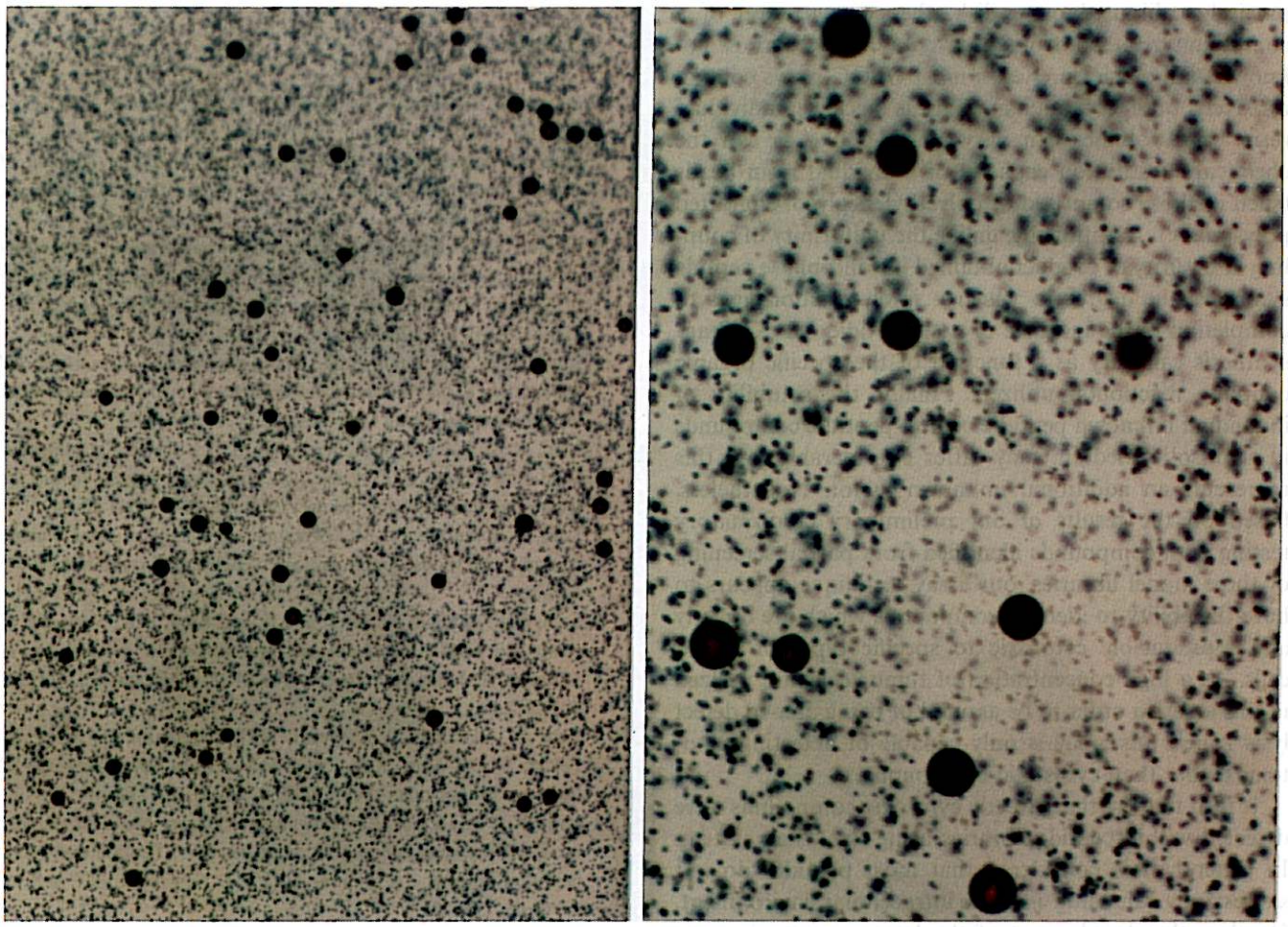

Fig. 3. In situ releasable soft agar assay for screening anticancer agents for a breast cancer cell line (MCF-7). Left panel: lower power; right panel: high power.

reactions, as cytotoxicity is manifest only around an individual OBOC bead wherein the compound's concentration is relatively high rather than more generally within an assay plate. However, we cannot be certain that a given zone of lysis around a bead is due to the specific compound on the bead versus a reactive intermediary or other contaminant until confirmatory testing including solution-phase testing of the resynthesized and purified compound has been completed. As it is possible to detect relatively small zones of clearing around an OBOC bead, it appears likely that assay sensitivity is far greater in the semi-solid assay system as well, and this should theoretically reduce the frequency of false-negative assay results.

The assay system as described is analogous to disk sensitivity assays with antibiotics wherein any one disk contains a pure compound. In contrast, during drug discovery of antibiotics, disks are spotted with microbial fermentation broths which contain numerous unknown compounds, which must be purified from the extract if activity is observed. With antibiotic disk sensitivity assays, the size of the zone of clearing around a given disk is sometimes predictive of that specific antibiotic's concentration and activity against given bacterial strains. While the concentration of a specific antibiotic can be precisely calibrated on a disk, the concentration of a compound released from a given OBOC bead is not known with precision, even if most OBOC beads contain relatively pure compounds. This is because individual resin bead volumes can vary considerably, as can the chemical substitution capacity of individual resin beads (such as those comprised of polyethyleneglycol-engrafted polystyrene). Additionally, unlike the uniformity of coupling and purity of product obtained in solid-phase peptide synthesis, the degree to which a given reaction goes to completion in the instance of non-peptidic OBOC libary synthesis can vary as a function of the specific organic coupling reaction(s) utilized. Finally, the solubility of individual unknown peptidic or non-peptidic compounds in semi-solid medium may differ considerably and this represents an additional unknown factor. Nonetheless, based on the results of our preliminary confirmatory testing with compounds identified from partial screening of three model libraries, our assay technique appears to have the ability to identify compounds which are directly and reproducibly cytotoxic to specific types of tumor cells. As a high concentration of tumor cells are utilized in this assay in order to be assured of being able to detect lysis zones, this assay is only capable of detecting cytotoxic rather than cytostatic compounds.

It is of interest that directly cytotoxic compounds were detected within peptide libraries using this assay system. In general, it is considered that most peptides do not successfully gain entry into the intracellular environment. On the other hand, most of the known cytotoxic compounds that are used in cancer therapy exert their actions intracellularly on DNA, microtubules, or specific steps in intracellular macromolecular synthesis. As the mechanism of cytotoxicity of agents detected empirically using this screening system will usually be unknown at the time of their detection, each confirmed compound of potential biological or pharmacologic interest that is discovered will need to be evaluated subsequently in a variety of mechanism of action studies. Other hurdles that must be overcome in downstream drug development after initial screening and confirmatory in vitro testing include demonstration of in vivo activity, pharmacokinetics, and lead optimization. Such steps will of course be required if 'hits' detected in this cytotoxic assay are to be successfully developed into effective anticancer drugs.

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References

1 DeVita, V.T., Oliverio, V.T., Muggia, F.M., Wiernik, P.W., Ziegler, J.L., Goldin, A., Rubin, D., Henney, J. and Schepartz, S., *The drug development and clinical trials program of the Division of Cancer Treatment, National Cancer Institute*, Cancer Clin. Trials, 2 (1979) 195–216.

2 Salmon, S.E., *Applications of the human tumor stem cell assay to new drug evaluation and screening*, In Salmon, S.E. (Ed.) Cloning of Human Tumor Stem Cells, Alan R. Liss, New York, NY, U.S.A., 1990, pp. 291–312.

3 Boyd, M.R. and Paull, K.D., *Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen*, Drug Dev. Res., 34 (1995) 91–109.

4 Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V., Kazmierski, W.M. and Knapp, R.J., *A new type of synthetic peptide library for identifying ligand-binding activity*, Nature, 354 (1991) 82–84.

5 Lam, K.S. and Lebl, M., *Selectide technology – Bead binding screening*, Methods: A companion to Methods Enzymol., 6 (1994) 372–380.

6 Lebl, M., Krchňák, V., Salmon, S.E. and Lam, K.S., *Screening of completely random one-bead–one-peptide libraries for activities in solution*, Methods: A companion to Methods Enzymol., 6 (1994) 381–387.

7 Lebl, M., Krchňák, V., Sepetov, N.F., Seligmann, B., Strop, P., Felder, S. and Lam, K.S., *One-bead–one-structure combinatorial libraries*, Biopolymers (Pept. Sci.), 37 (1995) 177–198.

8 Lam, K.S. and Lebl, M., *Combinatorial library based on the one-bead–one-chemical concept*, In Jung, G. (Ed.) Peptide and Non-peptide Libraries – A Handbook, VCH, New York, NY, U.S.A., 1996, pp. 173–201.

9 Lam, K.S. and Salmon, S.E., *Method of screening a peptide library*, United States Patent No. 5 510 240, April 23, 1996.

10 Salmon, S.E., Lam, K.S., Lebl, M., Kandola, A., Khattri, P., Healy, S., Wade, S., Patek, M., Kocis, P., Krchňák, V., Thorpe, D. and Felder, S., *An orthogonal partial cleavage approach for solution-phase identification of biologically synthesized peptide libraries*, In Hodges, R.S. and Smith, J.A. (Eds.) Peptides: Chemistry, Structure and Biology (Proceedings of the 13th American Peptide Symposium), ESCOM, Leiden, The Netherlands, 1994, pp. 1005–1006.

11 Salmon, S.E., Lam, K.S., Lebl, M., Kandola, A., Khattri, P., Wade, S., Patek, M., Kocis, P. and Krchňák, V., *An orthogonal partial cleavage approach for solution-phase identification of biologically active peptides from large chemical-synthesized peptide libraries*, Proc. Natl. Acad. Sci. USA, 90 (1993) 11708–11712.

12 Salmon, S.E., Lam, K.S., Felder, S., Yeoman, H., Schlessinger, J., Ullrich, A., Krchňák, V. and Lebl, M., *One-bead–one-chemical-compound: Use of the Selectide process for anticancer drug discovery*, Acta Oncol., 33 (1994) 127–131.

13 Jayawickreme, C.K., Graminski, G.F., Quillan, J.M. and Lerner, M.R., *Creation and functional screening of a multi-use peptide library*, Proc. Natl. Acad. Sci. USA, 91 (1994) 1614–1618.

14 Furka, A., Sebestyen, F., Asgedom, M. and Dibo, G., *General method for rapid synthesis of multicomponent peptide mixtures*, Int. J. Pept. Protein Res., 37 (1991) 487–493.

15 Houghten, R.A., Pinilla, C., Blondelle, S.E., Appel, J.R., Dooley, C.T. and Cuervo, J.H., *Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery*, Nature, 354 (1991) 84–86.

16 Lebl, M., Patek, M., Kocis, P., Krchňák, V., Hruby, V.J., Salmon, S.E. and Lam, K.S., *Multiple release of equimolar amounts of linker free peptides from the polymeric carrier*, Int. J. Pept. Protein Res., 41 (1993) 201–203.

17 Kocis, P., Krchňák, V. and Lebl, M., *Symmetrical structure allowing the Selectide multiple release of a defined quantity of peptide from a single bead of polymeric support*, Tetrahedron Lett., 34 (1993) 7251–7252.