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ONE BEAD, ONE CHEMICAL COMPOUND: USE OF THE SELECTIDE PROCESS FOR ANTICANCER DRUG DISCOVERY

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Technology for chemical synthesis and testing of libraries of millions of chemical entities has been oped for rapid molecular and cellular screening for drug leads. Each individual compound in the y is on a separate resin bead. Screening for binding activity can be conducted directly on the s. Biological activity is assessed in solution phase assay by cleaving a portion of the compound each bead. The molecular structure of the compound of interest is obtained by automated peptide encing from the bead of origin. We have applied this technology to anticancer drug discovery as as to other pharmaceutical targets. For anticancer drug development, current molecular targets de B-cell lymphoma, the EGF receptor, and the HER2-neu receptor. Solution phase screening with cleavable libraries is being used for growth inhibition of human tumor cell lines. Initial in vitro have been identified in each of these areas of anticancer drug discovery.

ancer drug discovery has focused on two major aches: rational drug design and random screening of al and synthetic products. Both approaches have d some success, and there are now over 40 anticancer in clinical use. The majority of current anticancer are natural products or their semi-synthetic deriva- of plant alkaloids or natural products. When ap- ed with random screening, 50 000–100 000 unique unds are evaluated in order to discover a promising ompound. Despite progress to date, there is major need for new anticancer drugs, particularly in the treatment of common solid tumors, as the existing agents have limited efficacy and substantial side effects. Living organisms have used peptides and proteins as key molecules for cell structure, enzymatic activity, hormonal signaling and interactions with the internal and external environment. The diversity of peptidic compounds that can be synthesized from just the 20 coded amino acids is enormous. About 3 200 000 different pentapeptides can be synthesized from just the 20 natural amino acids. A complete library of 10-mers from these same amino acid building blocks would include about 10 240 000 000 000 peptides. We have used the generation of solid phase libraries of random peptides and more recently of other organic compounds as a new approach to drug discovery via molecular screening and biologic signaling assays.

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The 'one-bead, one-peptide' concept and related technology

Several years ago we reported the development and screening of large libraries of chemically synthesized peptides based on an original 'one-bead, one peptide' synthesis and screening concept known as the 'Selectide technology' (1). Several other library technologies have been developed for similar purposes. Geysen et al. (2–3) devised a method

to chemically synthesize small peptide libraries using a multi-pin technique in 96 well plates for epitope mapping. Very large peptide libraries can be generated by inserting a randomized oligonucleotide into the genome of the filamentous phage pIII coat protein (4–6). Such phage libraries can be screened by immune adherence techniques, selecting those phages which express peptides within the pIII coat protein with the desired ligand binding activity. The adherent phages are then subjected to a series of cloning and expansion steps after which the active peptide is identified by sequencing the phage nucleic acid and determining the peptide sequence from the genetic code. Houghten and his colleagues developed a modification of Geysen's chemical process in which larger batches of peptides of known structure are synthesized and cleaved into solution and screening to identify peptides with various functional activities (7). As with Geysen's technique, Houghten's approach is also iterative and starts with very short peptides and converges on a lead peptide as additional batches of peptides are produced of longer chain length which incorporate the sequence found in the initial shorter peptide. The Selectide technology utilizes a 'split synthesis' approach for random generation of compounds produced via solid phase chemical synthesis (8) conducted in such a fashion that each individual compound in the library is represented on a separate solid phase resin bead on which it was synthesized (1). The individual beads within the batch of resin beads used for synthesis were divided up into different reaction vessels, one for each reactant species (e.g., amino acid) to be added to a growing polymer or compound. After each cycle of deprotection and building block addition (e.g., using Boc or Fmoc protected amino acids and sufficient time to allow the reaction step to go to completion), the beads were recovered and pooled from the various vessels, washed and thoroughly mixed and then redistributed into the vessels again for the next addition step. In an initial model experiment using three different amino acids to produce a library of tripeptides, all 27 theoretically generated tripeptides were successfully produced and identified and found to be produced in approximately equimolar quantities. Of importance, each individual bead contained a sufficient quantity of its unique peptide to permit its sequence to be determined via Edman degradation using an automated protein sequencer. This approach proved to be readily adaptable to the generation of libraries of various sized peptides (e.g., pentapeptides to dodecapeptides), using as many as 40 amino acids. However, with greter than 7-mers, complete libraries cannot be synthesized due to size and expense. Therefore, only a percentage of the total structures are synthesized for such libraries.

Using natural or unnatural amino acids and varying peptide length, and by introducing conformational constraints to induce cyclic or alpha-helical structures, a wide variety of library formats was developed. In as much as each individual peptide in the library was represented separately on an individual bead, binding assay fluorescent or enzyme marker-coupled molecular a were used to identify unique peptides that bound to antibody, enzyme or receptor. The technique prov quite effective for high volume testing as an in library containing millions of compounds could be sized within a few days. Screening of a library of ten peptides using one of the marker-bead binding ass be accomplished within a single day.

Using several model macromolecular targets, established that structurally unique high affinity from within a series of pentapeptide libraries co approximately three million different peptide st could readily be identified (1). For epitope analy cific staining of the few beads within a library tha specifically to a tagged monoclonal antibody pro rapid means for identifying 'mimotopes' as well immunizing epitope within pentapeptide librarie affinity peptide ligands for an anti-β-endorphin clonal antibody (clone E-7) and for the biotin-bind of streptavidin were the demonstrations used to do the specificity of this screening process and establis it was based on molecular-recognition (1, 5). development of this technology for drug disco quired application of suitable chemistries, materi assay techniques.

We have also extended the Selectide technology testing of free peptides in solution by incorporati cleavable linkers, with orthogonal cleavage charac (diketopiperazine and ester bonds), as well as non-cleavable linker onto each bead. The same oli synthesized on each of these linkers on an individu producing a construct where one-third of the oligo be released and tested, subsequently the second third released and tested, and the final third remains on t enabling its identity to be determined (9). This a provided a means to test massive libraries of comprised of either natural or unnatural amino ac conventional microplate pharmacological assays of signaling, etc. A two-stage assay was developed f volume screening in solution phase microassays. In phase, 500–1 000 beads were placed in each well of microplate and and the first linker cleaved at neutra form a diketopiperazine and release the free pepti peptide solution from each well of each 'master' pl then transferred by filtration into a corresponding t for solution phase testing. After identifying the well(s)' in the first stage assay, the beads from th sponding master well(s) were then recovered an tributed by pipetting into new master plates with ab bead per well (range 0–6) and the second stage lin ester bond) was cleaved using either base or ammonia to release free peptide. Following tran filtration and assay, the active well(s) were then id and the corresponding beads from the second stage

ere recovered and subjected to peptide sequencing. nstrate the specificity of this release-technology for specific ligands, we utilized two molecular targets: anti-beta endorphin monoclonal antibody as used g the specificity of the bead binding assay, and the platelet IIb/IIIa receptor for fibrinogen (10). Using nology, we were in fact able to rapidly identify ligands for both of these molecular targets. The technology is now being used to evaluate growth n effects or other signaling assays that can directly functional as well as structural interactions.

studies established that we could identify peptides bound to the active sites of antibodies and of cell receptors. More recently, the technology has been ully applied by the Selectide Corporation to identify enzyme inhibitors. We also sought to advance the technology beyond peptides to the more general of organic compounds, as peptides have only ally been used as drugs. This is because most do not retain biological activity after oral admin- nor do they gain access to the intracellular ment. The cyclic peptide antibiotic cyclosporine is ption as it is orally active and also internalized in its lls via the cyclophyllin receptor. The technology for ing libraries of non-peptides (including both poly- uctures based on subunits linked as amides, ureas as derivatives of biologically interesting molecular s such as steroids, sugars, etc.) has proven feasible. e now been built with Selectide process by using a of precursor organic molecules that are available in d form for chemical coupling. Screening of non- libraries can be conducted with either the bead- or release assay screening technologies as described after which the active bead(s) can be recovered. A problem that had to be addressed with the non-pep- raries was that of structure determination from the r quantities present on a single bead. Some struc- n be determined with mass spectroscopy that is not feasible. Therefore, an encoding technique was ed using a peptide to code for each non-sequence- ucture (10). This was accomplished by incorporating no acid to code for each randomized component of sequenceable compound. Beads in the non-peptide therefore contain two separate structures: a non-se- able oligomer or other organic compound, and a sequenceable peptide (the amino acids which serve code for the structure of the non-sequenceable nd) (11). An analogous technique has recently been d (12).

Current applications of the Selectide process to anticancer drug discovery

re currently using the Selectide technology in an to discover new anticancer drugs. One of us, (KSL), first developed a new concept for using random peptide libraries in order to discover mimotopes which might be used for 'antigen-directed immunotherapy" (ADI) for B-cell non-Hodgkin's lymphoma (13). This approach was designed to be patient specific and to identify peptides binding to the cell-surface monoclonal immunoglobulin idiotype ('id') which is unique to each individual patient's B-cell lymphoma (14). The overall concept of ADI includes recovery of cell surface id, identification of a peptide from within the library which binds specifically to the id (and not other immunoglobulin components), followed by resynthesis of and radioiodination of the patient specific peptide for use in radioimmunotherapy (13). Use of a peptide to deliver tumor-specific radiotherapy would have the advantage of using a small molecule with more favorable tissue distribution and clearance than a monoclonal antibody for tumor imaging, dosimetry and treatment. This concept is currently being tested in preclinical models by Dr. Lam at the Arizona Cancer Center. In addition to the logistic obstacles to be overcome in the development of individualized ADI, there is the potential biological obstacle of in vivo evolution of tumor clones expressing variants of the idiotype or no immunoglobulin whatsoever (15).

In 1992 we substantially expanded efforts at drug discovery using the Selectide technology with a collaborative program involving laboratories at The University of Arizona, New York University, The Max Plank Institute, The Selectide Corporation and the National Cancer Institute (NCI). This program is intended to discover new anticancer drugs of more general use than described above for B-cell lymphomas. This initiative is supported in part with 'National Cancer Drug Discovery Group', (NCDDG), funding from the NCI. In this research, we use both bead binding technology (for specific recombinant receptors) and the release assay technology for identifying antiproliferative peptides. While this is still a new program, we have already obtained preliminary evidence that the Selectide technology can be used for lead discovery for anticancer agents.

Molecular recognition of cancer-related receptors

As one objective in our NCDDG program, we are attempting to identify relatively small peptide ligands which bind to the external domains of tyrosine kinase receptors which are overexpressed in some carcinomas and thought to be involved in the growth signaling process (16). The specific receptors we are initially studying are the epidermal growth factor receptor (EGFR) and the HER2-Neu oncogene expression product (HER2R), HER2R has structural homology to EGFR, although its specific ligand remains to be confirmed. The recombinant external domains have been expressed in forms that are of particular use for molecular screening efforts. We postulate that a binding peptide that occupies the growth factor binding

site on either of these receptors may inhibit cancer growth. For both EGFR and HER2R, the bead binding technology was employed after coupling the extracellular domain of the relevant receptor to either an alkaline phosphatase or a fluorescent tag. With EGFR, the natural ligand EGF can be used in competitive binding studies. This approach increases the specificity of the screening assays and provides experimental evidence that the discovered peptides bind at the ligand binding site. While a variety of libraries have been screened, our initial leads were obtained from a library of cyclic 7-mers. Our lead peptides bind to EGFR and can block the binding of EGF. We are not yet certain of the affinity of these peptides for EGFR and they have yet to be tested in functional assays. Additional screening as well as lead optimization based on the initial peptide leads is now underway.

Screening for binding ligands for HER2R is more difficult, as the natural ligand for this receptor-like molecule has not been clearly identified, and has not been available for these studies. Therefore, we cannot carry our competitive binding studies as one of the tests of specificity. The peptide ligands which we determine to bind to HER2R may well bind at alternative sites on the receptor than at the ligand binding site. Functional assays will be required to determine their relevance to HER2R signaling. We have identified some 13-mers which bind with specificity to HER2R. Several of these appear to inhibit functional tyrosine kinase activity, but their affinity remains indeterminate. Characterization of affinity of these HER2R binding peptides and effect on cell proliferation is in progress.

Cancer cell line screening

The release assay as described above and elsewhere (9) is being utilized in an antiproliferative screening program using 6 human tumor cell lines (breast, colon, lung, prostate, melanoma, leukemia) with a replicate plating approach. For first stage screening for antitumor activity the released peptide from each well of the 96 well plates (500–1 000 beads per well) is divided and simultaneously tested against 2–3 cell lines in a 96-h cell growth inhibition assay. Based on bead content and peptide release characteristics as well as plating volumes of releasate, the maximal test concentration of each peptide is approximately 1 μM. Drug effect is determined after four days of culture with the sulforhodamine S (SRB) assay with quantitation by spectrophotometry (17). With releasate from 500 beads per well, each 96 well test plate can be used for the simultaneous evaluation of 50 000–100 000 peptides. A single technician can readily plate 20 per day with each cell line, permitting the screening of 1–2 million peptides in each 4-day assay. After a positive well is identified in the first stage assay, the beads from the master plate for that well are recovered and redistributed by pipetting with approximately one bead per well for second
The second stage test is performed with the sp
that yielded the positive screening result in t
assay. With a positive second stage assay, th
the master well is then recovered for sequen
automated protein sequencer. The sequence
resynthesized and retested against all 6 cell li
response fashion. Thus far, we have conduct
screening against 10 different library formats
prepared with the dual cleavable linkers, and
than 30 million individual peptides. At this s
nology development, library format is explo
specific molecular targets have not been ide
the cell line testing. Structural modificatio
date have included libraries of differing p
length (up to 15-mers), cyclic structure, or i
various end-groups added to the amino ter
peptides. Complete screening of a library was
peptide lengths up to 5-mers, but only a fracti
chain length libraries can be screened at this p
3 million individual peptides were tested fr
the libraries tested. To date we have disc
lead antiproliferative peptide with this assa
lipophillic pentapeptide with differential antitu
against the 6 cell lines. The activity profile of
against the various cell lines was quite differe
of an index antitumor agent (mitomycin-C)
positive control. The greatest degree of growt
with the resynthesized peptide was observe
prostate and colon cell lines, and with minim
the leukemic cell line.

In addition to the work on conventional pep
continuing prospective screening with the re
using new or novel library formats including li
conformational constraints, unnatural amino
recently initiated study of releaseable comp
structed of non-amino acid building blocks. S
are constructed to have a non-releaseable pepti
sequence for structure determination.

Discussion

Our studies provide indication that the Sel
nology can be applied to anticancer drug dis
have focused on cell surface receptors on can
our initial molecular targets. However, it is
enzymes associated with invasion and metastas
targets of interest, particularly since this tech
been successfully applied to the Selectide Cor
identify inhibitors of proteases. Lead compou
ered from peptide libraries also may serve as te
subsequent development of peptidomimetics,
instances may themselves serve as therapeutic
have great interest in pursuing non-peptide
organic molecules, as 'hits' from these libraries

optimize than peptides to produce useful drugs. reason to hypothesize that compounds within non-peptide libraries will have a greater probabil- versing cell membranes and acting on intracellular

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