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PRINCIPAL INVESTIGATOR: Sydney Salmon, M.D.

CONTRACTING ORGANIZATION: The University of Arizona
Tucson, Arizona 85719

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Sydney Salmon, M.D.

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Tucson, Arizona 85719

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The goal of this project is to discover new drugs that have specificity and significant anticancer activity against breast cancer. The approach uses several novel aspects that permit high volume screening for drug discovery. To maximize the number of unique chemical compounds available for testing, we have employed the new technology of combinatorial chemistry to produce large libraries containing thousands of compounds using the "one-bead, one compound" technology in which a portion of each compound can be released in vitro in a semisolid agarose culture of breast cancer and other tumor types as controls (1-3). During the past year we have screened three large libraries and discovered one lead compound that showed specificity against two breast cancer cell lines as compared to the control tumor types. Analogs of this lead compound have subsequently been tested and one discrete compound was found to be active in vitro at the micromolar level. This compound is currently undergoing preliminary evaluation of mechanism of action and will also be advanced to in vivo study in mice. During the coming year we will study additional large libraries in search of other lead compounds.

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Jerry Silverman 9/15/97

PI - Signature Date

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INTRODUCTION

Discovery of new anticancer drugs has been an arduous task. Both rational and empirical approaches to drug discovery have been used with modest success having been achieved by both approaches, although most currently active drugs have been discovered after detecting "leads" from screening of natural products or synthetic compounds in vitro or in vivo. Such studies have been classically limited by both the limited number of compounds available for screening for discovery of initial "hits" and by the low throughput available for in vitro screening assays. We have attacked both of these limitations by applying the new technology of combinatorial chemistry to produce large libraries of unique structures, using a mix and split technology for combinatorial synthesis in which each unique compound is synthesized on a separate resin bead, and from which, a portion of the compound can be released for in vitro screening in soft agarose culture. This combinatorial synthesis and screening approach that was developed in our laboratories has been applied subsequently elsewhere as well as in academia and industry. The technology has been licensed by The University of Arizona to Hoechst-Marion-Roussel, Inc., (HMR) which has collaborated with us by providing libraries of compounds for in vitro testing in our novel high volume screening assay system. Our goal is to identify at least one new lead compound each year from the thousands of compounds that undergo initial screening.that can then be further studied for mechanism of action, pharmacology and further preclinical study including initial murine model studies.

BODY

Experimental Methods:

Chemical Synthesis and Screening.

Combinatorial chemical libraries are synthesized using several different new technologies. The synthetic approaches often use discrete or proprietary steps by HMR, and therefore the approaches will be discussed in general, with description of the types of libraries synthesized and tested, but specific reactions used and compounds synthesized will be kept discrete and identified by project code rather than chemical structure.

The first of these technologies, the "one bead, one compound" process (OBOC), is based on the approach reported by the co-PI's on this research grant (4). This technology, first applied to synthesis of peptides, uses a "mix and split" approach wherein peptide or non-peptidic compound synthesis is carried out on partially cleavable linkers on resin beads. A series of different subunits intended to couple to the linker are exposed to different aliquots of beads in different vessels. After the first chemical binding step goes to completion, the beads from the various vessels are all mixed together to randomize them, and they are then re-aliquoted into the different vessels for the second chemical synthesis step to be conducted. In a typical peptidic synthesis, 20 or more different natural or unnatural amino acids are utilized to synthesize relatively small peptides (e.g. tripeptides). More than 100 different natural and unnatural amino acids are now commercially available as building blocks for the OBOC technology. OBOC beads can release up to 100 picomoles of peptide for in vitro testing. Prior studies in our laboratory have demonstrated that libraries comprised of just natural L-amino acids are inactive with respect to anticancer activity. Therefore, unnatural amino acids are now regularly utilized in OBOC. After a series of mix and split steps, many thousands of different compounds have been synthesized, with each individual resin bead carrying only a single unique compound (OBOC). These compounds are then tested for anticancer activity by mixing the beads with breast cancer or control cells in a soft agarose culture system and after several days of incubation, the plates are stained using the MTT technique (1). Beads releasing an "active compound" against breast cancer form a clear "halo" around the active bead. The active bead is then physically removed from the agarose plate and subjected to chemical analysis to determine the structure of the active compound. The agar plate method is extremely sensitive and can identify active compounds at the nanomolar level released from beads. On the other hand, less potent compounds can be identified up to the high nanomolar or low micromolar range. Compound identification is determined by HMR using either a protein sequencer or by LC-MS technology. Thereafter, the active compound is resynthesized on partial release beads and retested against both breast cancer cell lines and various control cell types using the same format of soft agarose culture technology. Confirmed positives with specificity to breast cancer but not to control cell types are then resynthesized in solution or on beads (in which case the compound is cleaved from the beads as part of the preparation phase) and the active compound is then purified and tested as pure compounds using solution phase assays against cancer cells in 96 well microwell plates with staining by either the SRB or

MTT staining techniques. Active compounds are then advanced for analog synthesis or identification (e.g. often using variations in the OBOC synthesis using slightly different building blocks at one or more positions in the compound) and the analogs retested for potency. Potent compounds are then advanced to mechanistic and pharmacology studies. The OBOC technique provides the ability to synthesize extremely large libraries of many thousands of compounds, but is not compatible with all chemical synthesis methods. It is ideal for synthesis of peptidic compounds, but is not always amenable to the synthesis of non-peptidic compounds due to the harsh and varying conditions used in organic synthesis.

A second chemical synthesis approach to synthesis of combinatorial libraries is that of robotic synthesis (RS) using 96 well plates as a template for robotic synthesis. Robotic synthesis has the advantage of permitting a much larger variety of synthesis technologies to be used that are not compatible with the OBOC technology due to incompatibilities of reactants, linkers, chemical bonds, or beads. As with OBOC, with RS a variety of different chemical compounds are used as "building blocks" for the synthesis of a combinatorial library. However, with RS a computer driven robot carries out the different chemical synthesis steps in a set order in the individual microwells in a series of 96 well plates. In this instance, as all steps and reactants are known, the compound in each microwell is known based on the series of building blocks used and the reaction employed for synthesis in each microwell. Compounds synthesized using RS are normally synthesized in solution and provided for cytotoxicity testing as coded aliquots in the microwell plates and aliquoted into test plates for solution phase testing against breast cancer or control cell lines in simultaneous controlled experiments using SRB or MTT staining to identify active compounds which inhibit breast cancer growth but not control cell types. Libraries synthesized with the RS technique are available with higher concentrations of compounds for testing than is the case with OBOC libraries, so that leads at the micromolar level can be identified. Active compounds can be retested immediately against additional breast cancer or control cell types because additional compound is still available in the synthesis wells for dose response against breast cancer and control cells for confirmatory studies. Using the RS approach, chemical identification of active compounds is confirmed to be that which the synthesis was intended to produce. Once the actual active compound is identified or confirmed, additional quantities of the active compound are synthesized for pharmacology studies. Additionally, structural analogs can often be identified from chemical catalogs or synthesized on a one-by one basis to further explore structure-activity relationships. Potent compounds are then advanced to mechanistic and pharmacology studies.

For both OBOC and RS libraries, putative active compounds are subjected to confirmatory dose-response testing in solution in order to determine IC50 concentrations against various cell lines.

Tumor cell lines:
During the past year, a total of nine different breast cancer cell lines have been used in either initial or confirmatory tests looking for breast cancer specificity for anticancer activity. In addition, 15 other cancer cell lines have been used as controls for specificity. These include 4 leukemia, 2 colon, 2 lymphoma, 2 myeloma, and 2 prostate cancer cell lines as well as 1 lung, 1 melanoma and 1 renal carcinoma cell line. In first stage testing, 1 or 2 breast cancer cell lines are tested along with 1 leukemia and 1 solid tumor cell line against the various compounds. Specific cell lines used were either obtained from the American Type Culture Collection (ATCC) or from the Arizona Cancer Center's (UACC) own cell line series from our tissue culture shared service. UACC lines were developed over the past 15 years and maintained as early passage cell lines. Quality control of cell line purity and non-contamination is determined periodically by ACC's cytogenetic shared service. Cell lines are maintained by our tissue culture service as in both cell passage and viably cryopreserved cell stocks. The latter is important for line replacement if and when contamination or change in line genotype or phenotype is identified.

Cancer cell lines:

Name	Code	Description
MCF-7	ATCC HTBWW	breast, adenocarcinoma, human
SKBR3	ATCC HTB30	breast adenocarcinoma, human
BT474	ATCC HBT20	breast adenocarcinoma, human
CEL	UACC 2087	breast adenocarcinoma, human
NOR	UACC 812	breast adenocarcinoma, human
DUD	UACC 3199	breast infiltrating ductal, human
BUTV	UACC 893	breast adenocarcinoma, human
SYNC	UACC 3133	breast adenocarcinoma, human
PREB	UACC 3132	breast adenocarcinoma, human
A549	ATCC CCL 185	lung carcinoma, human
SW480	ATCC CCL228	colon adenocarcinoma, human
HT29	ATCC HTB38	colon adenocarcinoma, human
LNCAP	ATCC CRL 1740	prostate adenocarcinoma, human
PC3	ATCC CRL1435	prostate adenocarcinoma, human
A375	ATCC CRL1619	malignant melanoma, human
HL60	ATCC CCL240	promyelocytic leukemia, human
K562	ATCC CCL 243	chronic myeloid leukemia, human
AML193	ATCC CRL 9589	acute monocytic leukemia, human
ARH77	ATCC CRL 1621	plasma cell leukemia, human
DAUDI	ATCC CCL213	Burkitt lymphoma, human
OCILY8		B cell lymphoma, human
8266	ATCC CCL155	multiple myeloma, human
U266	ATCC TIB196	multiple myeloma, human
786-O	ATCC CRL 1932	renal adenocarcinoma, human

All cell lines could be grown in complete tissue culture medium with 10% fetal bovine serum. Cultures for OBOC screening were normally carried out for 48-72 hours at which time MTT staining would be performed. Cultures in 96 well plates for SRB or MTT staining were normally cultivated for 96 hours.

Combinatorial Libraries:

Library 1: Unique Tripeptide Library: A diiodotyrosyl tripeptide library prepared with almost 100 natural and unnatural amino acids. The library was synthesized in two subsets containing over 18,000 unique compounds synthesized on OBOC beads containing cleavable linkers.

Library 2: A Nucleo/Glyco/Sterol/Lysyl-Diamid chimeric library. This library was synthesized using the RS approach in which the structure R1-K(R2)-OH was synthesized with each of 19 organic acids. This library contained 361 unique compounds synthesized in a series of 13 sublibraries.

We are currently awaiting a new library format from HMR in order to initiate testing for year 2 discovery efforts.

RESULTS:

A total of about 18,500 unique combinatorial compounds were screened in vitro in this project over the past year in an effort to discover new agents with unique activity against breast cancer.

While a few potential leads were obtained from Library 1, the leads could not be confirmed on retesting after resynthesis for solution phase testing. It is possible that some residual intermediary within the OBOC beads could have accounted for the activity which was observed. This activity was lost on compound purification and could not be identified.

Library 2 testing utilized a total of 230 96 well plates and screened the three sublibraries in Libary 2 as well as 166 unique compounds. One lead compound was identified within sublibary 16 and was confirmed on retesting, including after resynthesis. This compound was active with an IC50 in the micromolar range. Its structure is heterocyclic and it had clear specificity against two breast cancer cell lines as compared to a series of control cell lines.

With the known structure of this lead compound, a series of structural analogs was identified by computerized compound search for analogs. A total of seven analogs were available from the Aldrich chemical catalog and were purchased for in vitro testing. These are designated as A1 through A7. Compound A4 proved to be the most potent compound with IC50's against breast cancer in the low micromolar range, whereas the IC50's for other tumor cell lines were 3-4 times greater. Compound A4 has therefore been advanced for phamacologic testing in Dr. Robert Dorr's laboratory. Initial studies suggest activity against hormone dependent breast cancer cell lines. Dr. Dorr's results are summarized below.

Pharmacologic Evaluation of Lead Compound

Compound A4 was obtained in pure form from Aldrich Chemical for cytotoxic mechanism of action studies in tumor cell lines in vitro. Initial cytotoxicity experiments in 4 cell lines showed a range of sensitivity. The most sensitive cell line was OVCAR-3, a human ovarian cancer line which requires estrogen for optimal growth in vitro (Table 1). There was also evidence of partial (3-fold) resistance in a mitomycin C-selected L-1210 murine leukemia cell line which expresses the MDR1 gene product, p-glycoprotein and is approximately 10-fold cross-resistant to natural products such as the anthracyclines and vinca alkaloids. Because of the marked sensitivity of the OVCAR-3 cell line, mechanistic studies were performed in these cells to determine whether compound A4 inhibited the synthesis of DNA, RNA or protein.

Table 1: Antitumor Activity of Compound A4 in Tumor Cell Lines In Vitro

Cell Line Type	Human (H) or Mouse (M)	Inhibitory Concentration (IC) in µg/mL by MTT-Dye Assay Using Continuous Drug Exposure for 6 Days	
		IC_{50}	IC_{90}
L-1210, Leukemia	M	2.5	7.0
L-1210MDR, Leukemia	M	7.5	> 10
OVCAR-3, Ovary	H	0.1	0.3
A-375, Melanoma	H	2.8	8.0
MCF-7, Breast	H	0.15	1.0

OVCAR cells were exposed to radiolabelled thymidine, uridine or valine after a 24 hour exposure to compound A4 to determine the degree of precursor uptake into macromolecular forms of DNA, RNA or protein, respectively. Positive (inhibitory) controls were doxorubicin for DNA synthesis, actinomycin D for RNA synthesis and cycloheximide for protein synthesis. The results showed that compound A4 selectively inhibited DNA synthesis (Figure 1). In addition, the dose-response for DNA inhibition was comparable to that for cytotoxicity (also plotted in Figure 1 from prior experiments). This suggests that the two phenomena are linked.

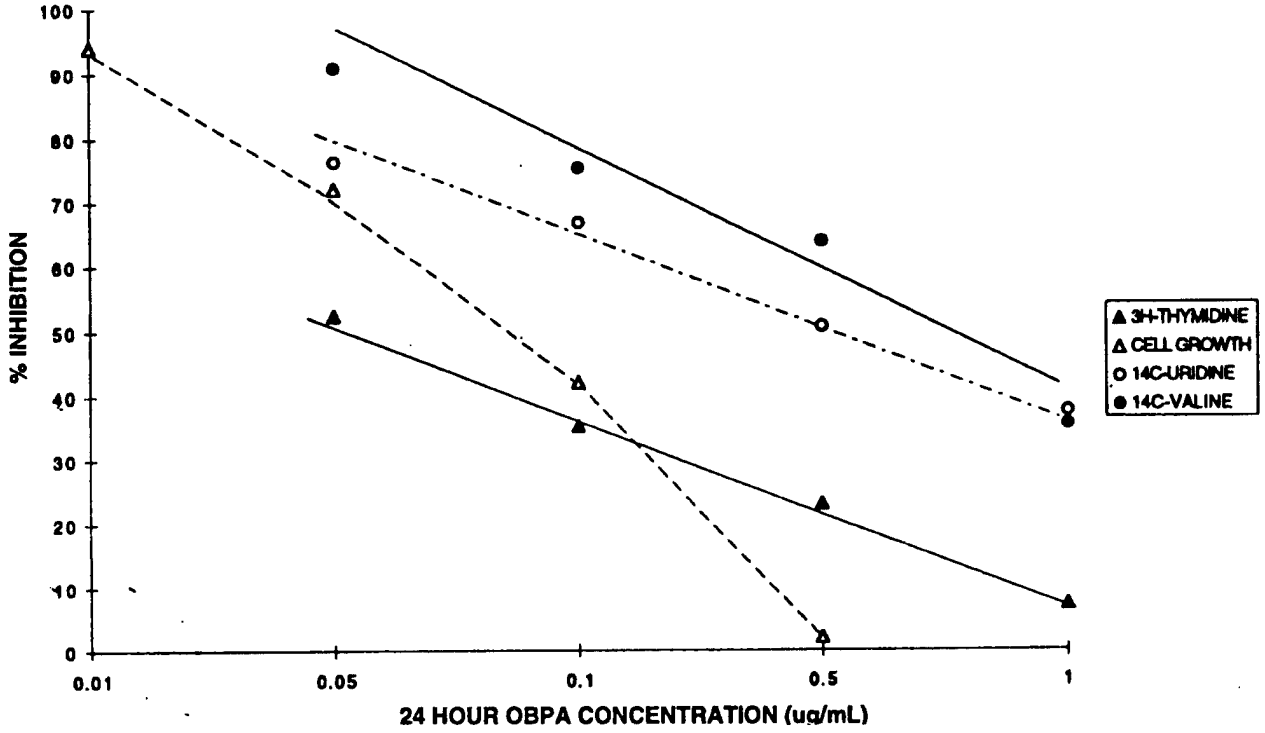

Figure 1

Because compound A4 has a planar heterocyclic structure, experiments were next performed to determine whether the drug intercalated into DNA. These experiments were performed in vitro using a solution of calf thymus DNA heated in a spectrophotometer cuvette to determine the transition profile from double to single-stranded DNA, a process that is significantly retarded if DNA is intercalated. The results showed no evidence of stabilization against thermal denaturation in the presence of up to 10 µg/mL of compound A4 whereas the positive control daunorubicin predictably elevated the transition or melt temperature by the typical 20°C at a concentration of 2.0 µg/mL (Table 2). These results suggest that compound A4 does not intercalate into mammalian DNA to inhibit DNA synthesis and cell growth.

Table 2: Intercalation Studies Using Thermal Denaturation of Calf-Thymus DNA (n = 3)

Agent	Concentration (µg/mL)	50% Transistion Temperature (°C)	Δ_{Tm} (°C)
None	—	68	—
Daunomycin	2.0	88	20
Compound A4	1.0	68	0
Compound A4	10	68	0

Subsequent cytotoxicity experiments showed that compound A4 was also active against human MCF-7 breast cancer cells, confirming the earlier studies performed in Dr. Lam's laboratory. Because cytotoxic activity for A4 was most potently observed in ovarian and breast cancer cells, the possibility that A4 interacted with estrogenic signalling pathways was tested. Using OVCAR-3 cells, A4 cytotoxicity was tested in the presence and absence of a potent estrogenic supplement. Initial studies confirmed that the estrogen antagonist tamoxifen inhibited cell growth with an IC_{50} of 5 μM. If A4 was acting similarly, then concomitant incubation with estrogens should reverse this inhibition. However, the addition of up to 36.7 nM of estradiol did not block A4 cytotoxicity. This suggests that A4 is not acting as an estrogen antagonist.

In summary, mechanistic studies have shown that compound A4 is selectively cytotoxic for hormonally-dependent human tumor cell lines but this does not involve DNA intercalation or antiestrogenic effects. Compound A4 does selectively block DNA synthesis by an unknown mechanism. Future experiments will be directed to in vivo tests of antitumor activity and exploration of other DNA-dependent mechanisms of action such as inhibition of topoisomerase II enzymes.

CONCLUSIONS

We believe we have been successful in our first year's efforts in identifying a lead compound that was confirmed to have specificity against human breast carcinoma cell lines and then identified a more potent structural analog. Literature search does not reveal any prior publications on anticancer activity of our lead compound or its structural analog.

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