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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 two large libraries and discovered three lead compounds that showed relative specificity against breast cancer cell lines. Work is underway in our laboratories to confirm their activities. Compound A4 was discovered during the first year of this grant. Preliminary preclinical studies indicate that A4 has in vivo anti-breast cancer activity (human xenograft SCID mouse model), it is non-myelosuppressive and is well tolerated by the mouse. During the coming year, we'll continue to develop A4 and at the same time characterize the three new leads that were discovered this last year.

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Andrew Lam 9/29/98

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: TL022, total 12000 compounds

Library 2: TL026, total 3100 compounds

Both libraries 1 and 2 are trimeric libraries, and compounds were released from beads in a 96-well plate format for subsequent cytotoxic assays.

RESULTS:

Library Screening

During the second year of this grant, the above two libraries were screened against a series of cell lines for anticancer activity. These cell lines were MCF7, A375, A549, PC3, SW480, and 8226S. After screening over 12000 compounds, we succeeded in identifying three compounds with anti-breast cancer activity with IC_{50} in the low micromolar range (< 5μM). Work is currently underway to confirm their anti-breast cancer activity.

Pharmacologic Evaluation of Lead Compound

In the first year of this grant, we identified a compound, A4, with anti-breast cancer activity (IC_{50} in the low micromolar range). We also discovered that compound A4 blocks DNA synthesis but it is not an intercalator. It does not inhibit topoisomerase II activity. During the second year of this grant, we continue to evaluate the toxicity and anti-tumor activity of compound A4 *in vivo*. MCF-7 (human breast cancer) xenograft in SCID mouse model was used. The anti-tumor activity is shown in Figure 1. In this experiment, each mouse received three i.p. doses of A4 three days apart at daily doses of up to 1 mg/kg. The drug is generally well tolerated by the mice and there is a definite dose response anti-tumor activity for this compound.

We have also studied the toxicity of compound A4 on male DBA/2 mice. In this experiment, the mice were treated at doses of 5 and 10 mg/kg on days 1, 5, 9 via the i.p. route. Control mice were treated with diluent alone. Each group consisted of four animals and their blood was pooled for analysis. Blood was drawn via cardiac puncture 24 hours following the day 9 injection and sent to a commercial laboratory for CBC and chemistry panel. The result of this study is shown in Table 1. It is clear that the drug is very well tolerated and there is no evidence of myelosuppression. Furthermore, the chemistry panel remained unchanged even at the 10 mg/kg dosing. The only exception is that the creatinine kinase (CK) activity is double that of the control. The significance of this elevation of CK is not clear. The mice appear to be able to tolerate the drug without any difficulty.

CONCLUSIONS:

We have demonstrated that compound A4, identified during the first year of this grant, has *in vivo* anti-breast cancer activity, and is very well tolerated by the animal. We have also screened additional small molecular libraries and discovered three additional compounds against MCF-7 lines.

Future experiments include: (1) to send compound A4 to NCI for further profiling the anti-tumor activities in their large panel of tumor lines; (2) to perform anti-tumor as well as pharmacokinetic studies of A4 in the murine xenograft model; (3) to confirm the anti-tumor activity and specificity of the three new compounds identified in year 2; and (4) to screen additional libraries for anti-breast cancer activities.

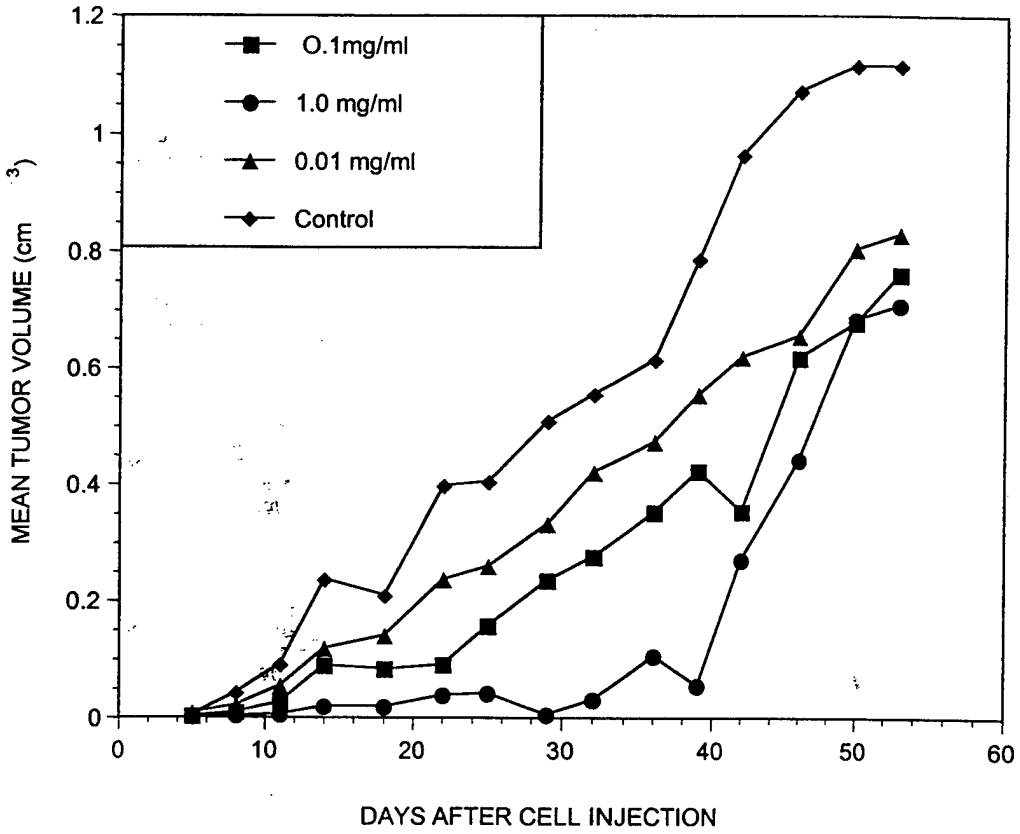

Figure 1. Anti-tumor activity of compound A4 in the MCF-7 breast cancer xenograft SCID mouse model.

Table 1. CBC and chemistries of pooled mouse serum obtained from mice that had been treated with compound A4.

	UNITS	CONTROL	5 mg/kg	10 mg/kg
GLUCOSE	MG/DL	210	197	205
BUN	MG/DL	20	20	22
CREATININE	MG/DL	0.2	0.2	0.2
SODIUM	MEQ/DL	136	135	136
POTASSIUM	MEQ/DL	5.9	5.2	5.3
CHLORIDE	MEQ/DL	96	98	98
NA / K		23	25	25
CALCIUM	MG/DL	7.6	7	7.7
PHOSPHORUS	MG/DL	6.8	7.1	7.2
TOTAL PROTEIN	G/DL	5.3	5.1	5.1
ALBUMIN	G/DL	2.9	2.7	2.6
GLOBULIN	G/DL	2.4	2.4	2.5
TOTAL BILIRUBIN	MG/DL	1	0.8	0.5
ALK PHOSPHATASE	U/L	37	65	75
AST	U/L	80	69	114
ALT	U/L	25	17	20
GGT	U/L	0	0	0
CK	U/L	346	710	749
AMYLASE	U/L	782	791	844
LIPASE	U/L	63	40	40
CHOLESTEROL	MG/DL	144	144	139
OSMOLALITY	MOSM/KG	279		
HEMOGRAM				
WBC	THSN/CU MM	6.4	5.1	3.8
RBC	MILL/CU MM	8	7.95	8.04
HGB	G/DL	13	13	13
HCT	%	36.7	36.3	36.5
MCV	U(3)	45.9	45.7	45.4
MCH	UUG	16.3	16.4	16.2
MCHC	%	35.4	35.8	35.6
PLTS	THSN/CU MM	clumped	clumped	clumped
SEGS	%	28	32	60
BANDS	%	0	0	0
LYMPHS	%	69	66	29
MONOS	%	2	1	10
EOSINOPHILS	%	1	1	1
BASOPHILS	%	0	0	0
ABS SEGS	MM/C3	1.7	1.6	2.2
ABS BANDS	MM(3)	0	0	0
ABS LYMPHOCYTES	MM/C3	4.4	3.3	1.1
ABS MONOCYTES	MM/C3	0.1	0	0.3
ABS EOSINOPHILS	MM/C3	0	0	0
ABS BASOPHILS	MM/C3	0	0	0

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