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J. Nat. Prod., **1993**, 56 (4), 441-455• DOI: 10.1021/np50094a001 • Publication Date (Web): 01 July 2004

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Journal of Natural Products
Vol. 56, No. 4, pp. 441-455, April 1993

THE ROLE OF RECEPTOR BINDING IN DRUG DISCOVERY[1]

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ABSTRACT.—Radioligand receptor binding has been used extensively to identify and characterize a host of receptors and enzymes targeting virtually every therapeutic area. Many drug discovery programs have been based on the utilization of radioligand receptor binding technology to identify lead compounds which interact with receptors likely to be important in neuronal, immunological, gastrointestinal, and cardiovascular function/dysfunction.

There are several obvious advantages to using in vitro receptor binding as a first level screen when compared to in vivo pharmacometric screens. Scientifically, the structure activity data generated in binding assays is a direct reflection of the ligand/receptor interaction minus the complications which result from secondary events, bioavailability, and pharmacodynamic issues. Technically, the binding studies require only a small amount of test compound (≤1 mg), while whole animal studies routinely need gram quantities. Similarly, only a small amount of tissue is required, compared with the cost of purchase and maintenance of live animals for in vivo screening. Supply and labor costs are drastically reduced due to the limited volume and test tube based technology of receptor binding. For these reasons receptor binding assays have been utilized with considerable success to discover site specific lead compounds in virtually every therapeutic area.

Over the past two decades the radioligand binding assay has been used as a means to study both neurotransmitter and hormonal systems. Today the pharmaceutical industry routinely uses radioligand binding assays to aid in the identification of novel agents which block or mimic the interaction of endogenous chemical messengers with cellular receptors. The use of radioisotope-labeled transmitters or drugs (radioligands) resulted in the development of this simple and sensitive methodology which provides information detailing the molecular interactions between drugs and receptors. Based on its reliable, cost-effective, and rapid nature, receptor binding has replaced many of the whole animal/in vivo assays traditionally used as high volume screening tools in drug discovery. More recently the use of radioligand binding technology has expanded to include the determination of the comprehensive binding selectivity or "profile" of investigational new drug candidates. This article provides an overview of the integration of drug discovery and radioligand binding. Discussion will focus on the basic pharmacology of radioligand binding, the choice of chemical libraries, screening strategies, and data interpretation.

RADIOLIGAND BINDING ASSAY DEVELOPMENT.—A properly developed radioligand binding assay accurately determines the specific binding of a radioligand to a targeted receptor through the delineation of its total and nonspecific binding components. Total binding is defined as the amount of radioligand that remains following the rapid separation of unbound radioligand from radioligand bound to the receptor. The nonspecific binding component is defined as the amount of radioligand that remains following separation of a reaction mixture consisting of the receptor, the radioligand, and excess unlabeled ligand. Under this condition, the only radioligand that remains represents that which is bound to components other than the receptor. The specific

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radioligand bound is determined by simply subtracting the nonspecific from total radioactivity bound (Figure 1). The standard means of reporting specific binding is as a percentage of total binding. The greater this percentage, the more reproducible and reliable that assay will be as a drug discovery tool. Assays having a specific binding component of >80% are suited to high volume screening, while assays that run between 50 to 60% are more difficult to use due to an increase in experimental variation (Table 1).

Many radioligands have the potential to interact nonselectively with other membrane proteins in a receptor preparation or may bind to the glass fiber filters used to separate/terminate the binding reaction. Characteristics of such nonspecific binding, the reaching of the binding equilibrium very rapidly, and the inability to saturate with increasing concentrations of radioligand are important factors to be considered when developing a radioligand binding assay. Since minimizing the non-specific binding component increases the specific binding of an assay, numerous techniques have been employed to lower nonspecific binding, for example: (a) limiting the amount of receptor preparation per reaction volume, since nonspecific binding often increases as the concentration of receptor preparation increases, and (b) pretreatment of the glass fiber filters with either bovine serum albumin or polyethylenimine, which decreases nonspecific binding to the filter.

The kinetic properties that describe the interaction between transmitters and/or drugs and receptor are similar to those used for enzyme studies; binding must be competitive, saturable, specific, and reversible. Mathematical models used to define enzyme kinetics can then be used to analyze data generated by a binding assay. These parameters, i.e., affinity and rate constants and receptor densities, must be clearly established to ensure that the binding data accurately describes the interaction between drug and receptor (1,2).

The equilibrium condition for a particular ligand/receptor interaction is one of the first parameters that should be established. In practical terms equilibrium is defined as the time it takes "specific" binding to reach maximum. It is imperative that all experiments, saturation, inhibition determinations, and screening assays be performed under steady state conditions to validate and standardize results. Equilibrium, or steady-state conditions, is affected by numerous factors that comprise the milieu in which the binding reaction occurs: these include temperature, time, and buffer (ionic strength and pH). The manipulation of these components will allow for the optimization of incubation conditions, ensuring that the assay is run at equilibrium. Knowledge of the equilibrium conditions and the rate constants for a particular binding assay will also serve as an internal check of the reliability of that assay; i.e., higher affinity drugs should dissociate at a slower rate than those of lower affinities.

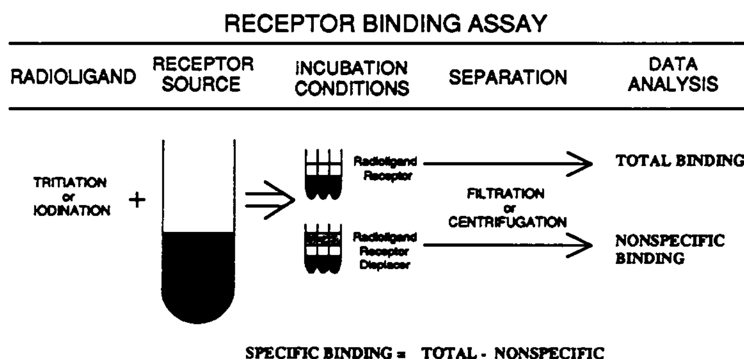


FIGURE 1. Schematic representation of a radioligand binding assay.

TABLE 1. Radioligand Binding Assays Available in PROFILE®.[a]

Receptor/Selectivity	Reference Radioligand	Compound	K_i (nM)	Percent Specific Binding
Adenosine				
Adenosine 1	[^{3}H]CPX	2-Chloroadenosine	18.40	90
Adenosine 2	[^{3}H]NECA + CPA[b]	MECA	202.70	85
Adrenergic				
Alpha 1	[^{3}H]Prazosin	Phentolamine	13.00	95
Alpha 2	[^{3}H]RX 781094	Phentolamine	1.70	90
Beta	[^{125}I]Dihydroalprenolol	Metoprolol	45.00	70
Amino Acids				
Excitatory				
NMDA	[^{3}H]CGS 19755	NMDA	4200.00	70
Quisqualate	[^{3}H]AMPA	AMPA	11.80	90
Kainate	[^{3}H]Kainic Acid	Kainic Acid	35.00	90
Glycine	[^{3}H]Glycine	D-Alanine	895.00	90
PCP	[^{3}H]TCP	PCP	62.30	90
Sigma	[^{3}H]DTG	Haloperidol	11.50	90
Inhibitory				
Benzodiazepine	[^{3}H]Flunitrazepam	Clonazepam	2.00	90
$GABA_A$	[^{3}H]GABA	Muscimol	2.60	90
$GABA_B$	[^{3}H]GABA + Isoguvacine[b]	GABA	176.00	75
Glycine	[^{3}H]Strychnine	Strychnine Nitrate	52.50	80
Biogenic Amines				
Dopamine 1	[^{3}H]SCH 23390	Butaclamol	37.30	90
Dopamine 2	[^{3}H]Sulpiride	Spiperone	0.10	90
Histamine 1	[^{3}H]Pyrilamine	Triprolidine	1.60	80
Serotonin 1	[^{3}H]5-HT	5-HT	4.60	60
Serotonin 2	[^{3}H]Ketanserin	5-HT	531.00	65
Channel Proteins				
Calcium, T&L	[^{3}H]Nitrendipine	Nifedipine	1.60	90
Calcium, N	[^{125}I]Omega-Conotoxin	Omega-Conotoxin	0.10	90
Chloride	[^{3}H]TBOB	TBPS	112.40	70
Potassium, Low Cond.	[^{125}I]Apamin	Apamin	0.05	90
Cholinergics				
Muscarinic 1	[^{3}H]Pirenzepine	Atropine	0.30	90
Muscarinic 2	[^{3}H]AF-DX 384	Methoctramine	0.60	98
Nicotinic	[^{3}H]NMCI	Nicotine	0.90	75
Opiate				
Mu	[^{3}H]DAGO	Naloxone	1.60	90
Delta	[^{3}H]DPDPE	Naloxone	42.60	80
Kappa	[^{3}H]U-69593	Cyclazocine	0.20	90
Prostanoids				
Leukotriene B_4	[^{3}H]LTB_4	LTB_4	2.90	70
Leukotriene D_4	[^{3}H]LTD_4	LTD_4	0.60	90
Thromboxane A_2	[^{3}H]SQ 29548	U 46619	5.50	75
Reuptake Sites				
Norepinephrine	[^{3}H]DMI	DMI	580.00	87
Serotonin	[^{3}H]Citalopram	Imipramine	20.30	85
Dopamine, Cocaine Site	[^{3}H]WIN	Nomifensine	32.50	75
Second Messenger Systems				
Adenylate Cyclase				
Forskolin	[^{3}H]Forskolin	Forskolin	29.40	85
Protein Kinase C				
Phorbol Ester	[^{3}H]PDBU	PDBU	16.50	90

[a]Abbreviations: 5HT, 5-Hydroxytryptamine; AMPA, Amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CPA, cyclopentyladenosine; CPP, 3,2-carboxypiperazion-4-yl-propyl-phosphonic acid; CPX, Cyclopentyl-1,3-dipropylxanthine; DADLE, 2-D-Alanine-5-D-leucine enkephalin; DAGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; DMI, Desmethylimipramine; GABA, γ aminobutyric acid; LTB_4, Leukotriene B_4; LTD_4, Leukotriene D_4; MECA, 5′-*N*-Methylcarboxamidoadenosine; NECA, 5′-*N*-Ethylcarboxamidoadenosine; NMCI, *N*-Methylcarbamyl choline iodide; PCP, Phencyclidine; PDBU, Phorbol-12,13-dibutyrate; QNB, Quinuclidinyl benzilate; TBOB, *t*-butylbicyclo orthobenzoate; TBPS, *t*-butylbicylophosphorothionate; and TCP, *N*-(1-[2thienyl]cyclohexyl) 3,4-piperidine.

[b]Competing drug added for selectivity.

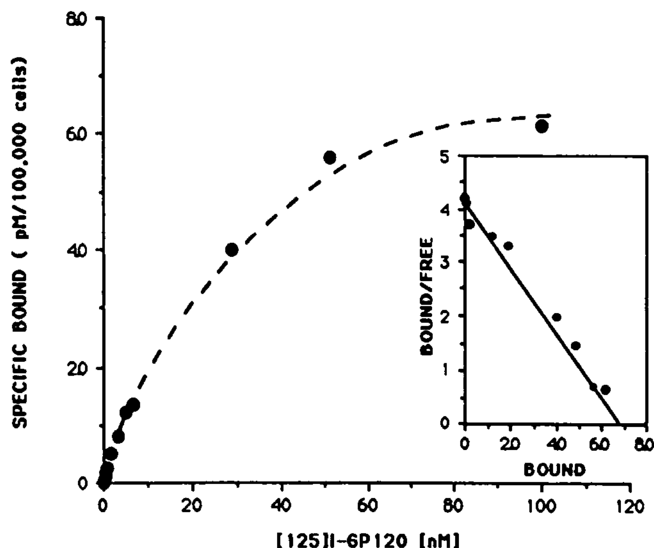


FIGURE 2. Saturation plot: gp 120 specifically bound to the CD_4 receptor is plotted as a function of free radioligand concentration. Scatchard analysis (inset). Linear analysis of saturation data allows for the easy determination of both K_D ($K_D = -1$/slope) and the B_{max} (intercept of the x-axis).

Saturation analysis is an important step in the development of an assay and, as suggested by its name, will determine if the specific binding component of an assay is saturable, reflecting the finite number of receptors available in any tissue or cell preparation. By plotting the specific binding/mg protein against an incremental increase in the radioligand, it is possible to generate a saturation curve (non-linear analysis) which can easily be transformed using a Scatchard plot for linear analysis of binding data (Figure 2). Two of the important pieces of information derived are the B_{max} and the K_D values, which are approximations of the receptor density in the preparation used and the dissociation constant of the receptor for the ligand, respectively (3,4).

The completion of radioligand binding assay validation work requires that pharmacological specificity be detailed to ensure that the assay optimally targets the receptor of interest. Dose-response studies are used to determine this specificity. This information is generated by holding the receptor concentration, radioligand concentration (typically one tenth the K_D as determined by saturation analysis), and all other experimental conditions constant, while varying the concentration of the inhibiting or unlabeled drug (Figure 3). These curves will accurately determine the concentration of drug or test compound needed to inhibit 50% of the specific binding in the assay (IC_{50}). IC_{50} values tend to vary slightly between assay runs and laboratories, with the most common differences being the radioligand concentration used and the apparent K_D of the receptor. To correct for these differences, the IC_{50} values are transformed to inhibition constants (K_i) by the Cheng-Prussof equation, which takes into account these variations (5).

Many radioligands interact to some degree with receptor subtypes not primarily targeted or with seemingly unrelated receptors, making it necessary to establish a rank order of potency for various reference agents known to act at those receptors. This determines the selectivity of a radioligand for the receptor being targeted for screening (Tables 2 and 3). The development of very selective radioligands, however, does not lessen

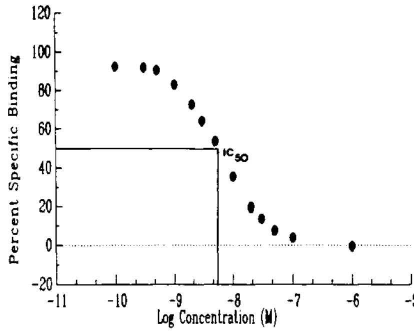


FIGURE 3. Log concentration-response curve (inhibition or displacement curve, typical of those obtained in radioligand binding studies). The IC_{50} for this curvilinear plot represents the concentration of drug required to inhibit 50% of the amount of radioligand specifically bound.

the necessity for running the various reference compounds previously used to study that receptor during assay development.

The development of a radioligand binding assay that conforms to the laws of mass action is essential in establishing a successful screening program. The actual design of the assay may at times push conditions to the edge of kinetic relevance to accommodate a high volume and/or automated format, but the basic principles cannot under any circumstances be compromised, or the information generated will be technically invalid.

HIGH VOLUME SCREENING.—When developed properly the radioligand binding assay is a sensitive, reliable, and reproducible in vitro technique capable of generating quantitative data which can be analyzed rapidly. Used as a screening tool, the radioligand binding assay can provide information on thousands of test compounds in a short period of time while utilizing limited amounts of radioligand, drug, and animal tissue. This is in direct contrast to the use of whole animal/in vivo approaches traditionally employed by drug companies. The majority of pharmaceutical companies now have in place systems for in vitro high volume screening, and each approach is somewhat dif-

TABLE 2. Radioligand Assay Selectivity in K_i Values for Serotonin Receptor Subtypes.[a]

Reference Compound	$5HT_1$	$5HT_2$
Serotonin	4.6	500.0
5-Methoxytrypatamine	45.8	>1,000.0
Methysergide	14.8	6.8
D-LSD	>7,500.0	1.5
Ketanserin	>7,500.0	0.4

[a]The number of serotonin receptor subtypes makes it imperative to demonstrate a particular assay's selectivity. Several major distinctions between $5HT_1$ and $5HT_2$ are the relative potencies of serotonin and ketanserin.

TABLE 3. Radioligand Assay Selectivity in K_i Values for Calcium Channel Types.[a]

Reference Compounds	Type T & L	Type N
Nitrendipine	1.0	>10,000.000
Saxitoxin	13.8	>10,000.000
Verapamil	500.0	>10,000.000
Apamin	>10,000.0	>10,000.000
TBPS	>10,000.0	>10,000.000
Neomycin Sulfate	ND	1,500.000
Omega-conotoxin	>10,000.0	0.1

[a]The demonstration of calcium channel types' selectivity reveals that agents which are potent at type T&L are not potent at type N and vice versa, suggesting the binding domain of each channel type is pharmacologically distinct.

ferent (6,7). Screening may target the molecular mechanism of action, using radioligand binding or enzyme assays, or more biologically complex mechanisms, such as cellular cytotoxicity or proliferation. This section attempts to address issues universal to high volume screening programs, including: library to be screened, disbursement of that library, and format of the screening program.

Chemical library to be screened.—One of the greatest impediments to establishing a successful high volume screening program is access to and the logistical management of compounds to be evaluated. Individual drug discovery efforts are not usually afforded access to a wide selection of chemical libraries for screening. The structural diversity within a chemical library is important to the success of any high volume screening effort, and actively acquiring new libraries should increase the chances for success. If the resources are available, screening all compounds in one or more libraries is advantageous, though more resource intensive. Such a serendipitous approach to drug discovery has been used successfully by many pharmaceutical companies. Scientifically this approach eliminates the intellectual input required to select groups of compounds to be screened based on compound structure. Given the limited information compiled on the structural conformation of the ligand binding domains for the majority of identified receptors, a random format may be an advantage over the more rational selection of libraries pre-selected on the basis of molecular modeling of receptors. Libraries which include structurally diverse entities, a natural product library being a good example, are ideally suited for large scale random screening in which thousands of samples can be tested in a period of several days in a cost-effective manner.

Synthetic chemical libraries, such as those assembled from pharmaceutical chemistry, may lack extensive structural heterogeneity and be of only limited use in drug discovery. One contributing factor to the low heterogeneity of such a library arises from expansion due to structure activity research performed by medicinal chemists in support of ongoing lead compound development. This is a common practice when attempting to design better second generation drugs with enhanced potency, selectivity, and/or bioavailability. The result is that the number of totally unique structures in a core sub-library may be limited. Therefore a semi-random approach, where only the core portions are evaluated, seems a logical choice but with one shortcoming: a potential lead compound may not be screened as it may possess only a minor structural modification from a previously selected core compound. A minor structural modification, however, could have significant biological consequences.

The pre-selection of a core library may be necessary if the receptor binding assay being utilized as a screening tool is technically difficult and has low throughput. Such conditions can rapidly exhaust a screening budget and the technical staff as well. An example of this is a peptide/protein growth factor binding assay which utilizes viable whole cells as a tissue source, expensive radioligands, and a non-filtration termination step. In the end, the final choice of chemical library should be based not only on the composition of the library but a balance between technical and budgetary constraints as well.

Chemical disbursement and solubilization.—The weighing out of thousands of samples can become a bottleneck in a screening program (8). Several strategies may be employed when disbursing a chemical library to increase the efficiency and accuracy of this rather tedious and time-consuming work. A "scoop" method may be used in which approximately 1–2 mg is placed into each vial and the actual amount is recorded. A bar code number previously assigned to catalogue each compound in the library is matched with the bar code number on a disbursement vial. The mol wt for each compound is retrieved from the data base and calculations are performed to determine the appropriate volume needed to yield a 1 mM stock solution. Adjusting the dilution volume in this way can be a significant advantage over the time it may take using accurate weighing and standard dilution volumes.

One of the biggest problems in drug development is the solubility of compounds. DMSO is one of the most effective solubilizing agents (typically greater than 70% of synthetic chemicals are soluble in neat DMSO). All assays must be developed to withstand varying concentrations of DMSO (0–10% final in assay) and corrections made, using proper controls, to adjust for decreases in specific binding, etc. One approach in a high volume screening program is to solubilize compounds in 100% DMSO followed by the addition of a volume of H_2O/buffer to yield a 1 mM stock solution in 4% DMSO. Any further dilution of the stock is carried out in 4% DMSO solution. Careful note should be taken to detect compounds which precipitate out of solution when diluting from 100% to 4% DMSO and beyond. If this happens, appropriate adjustments in DMSO concentration can be made, or a different vehicle employed. Other vehicles have been examined, such as alcohols, ketones, and acid/base protocols, but none appear to be as effective as DMSO. If compounds are only soluble in an exotic vehicle, a control may be run side by side with the compound to determine any adverse effects the vehicle might have on the receptor assays. Often an assay must be specifically developed for certain vehicles, and one cannot assume that a well-developed assay will tolerate a change in solubilizer or other chemical modification (e.g., the addition of an antioxidant such as HOAc).

Insoluble compounds are classified as unscreenable due to the fact compounds not in solution do not function in a radioligand binding assay. In most compound libraries, only a small percentage of the samples are likely to be insoluble in the chosen vehicle. Partially soluble (i.e., as determined by visual examination) compounds can be screened taking into account that if biologically active, such compounds may give a "hint" of activity, e.g., detectable binding affinity at what may appear to be high concentrations. It is important that the solubility of each compound be noted so data interpretation can be adjusted accordingly. In high volume screening, one strategy is to limit the work associated with creating a sample repository by screening all compounds, ignoring apparent solubility. If the cost of the compound per assay is low, this strategy can be acceptable and may yield unexpected results.

With natural products the question of solubility is an extremely important one and correlates directly with the extraction procedures used to derive the final material to be screened. Questions routinely arise about which extraction procedure to use to

maximize the compound yield. Typically, aqueous and non-aqueous phases are produced; all phases can, and perhaps should, be screened. Effective methods must be used for the removal of trace levels of solvent that would compromise the performance of the assay, or these trace levels must be corrected for. Special issues, such as the presence of tannins, polysaccharides, and other plant compounds that could compromise the assay, must also be understood and procedures adjusted. The question of how to interpret data from samples that are composed of numerous chemical entities will be discussed in detail later.

Chemical disbursement, solubilization, and repository generation capabilities have a direct impact on the level of sample throughput that can be achieved in any high volume screening effort. While it is important for a system to obtain maximum output, it is equally important to design a system which is flexible, adjusting to the needs of a changing screening program, and is accurate with respect to sample tracking and other operations.

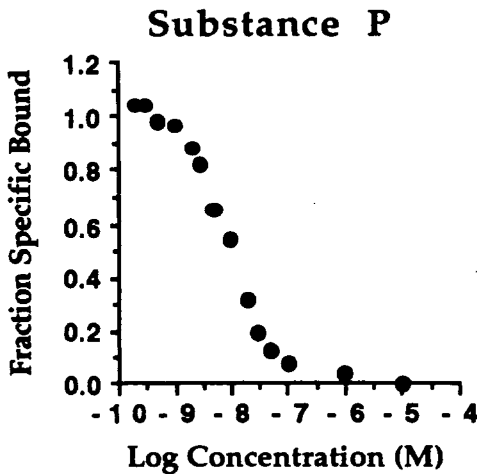
High volume screening formats.—The format of the screening program will have a direct impact on the level of sample throughput in either a high volume or selectivity screen. As an example, a high volume format for a neurokinin 1 assay in which a synthetic chemical library was screened is detailed in Figure 4A (9). The format consisted of samples run at a single concentration (10^{-5} or 10^{-6}) in duplicate tubes and the corresponding quality assurance measures, i.e., positive controls and a standard reference curve (Figure 4B). These measures are designed to provide a complete pharmacological profile of the assay and to assure an accurate assessment of the inhibitory potential of a compound.

Once activity is identified, several approaches that utilize radioligand binding can be used to accelerate the early phases of the drug development process. Ideally, initial activity should be verified in a repeat assay followed by IC_{50}/K_i determinations ($N \geq 3$) to identify the potency of the compound. Most compounds identified in a high volume screen tend to be of μM potency and require a significant chemical effort to enhance the potency so as to be of pharmacological significance. The radioligand binding assay then serves as a method of monitoring the relationship of compound structure to potency as the lead compound is modified by chemists. If nanomolar potency can be attained, functional screening in in vitro or in vivo assays will determine if that compound mimics (agonist) or blocks (antagonist) the action of the endogenous ligand or ligands at that receptor. Several important considerations generally make functional screening a later phase activity rather than the initial discovery screen. First, the vast majority of compounds identified by screening interact at a receptor site but lack the ability to turn that receptor on, analogous to identifying a key which will fit into a lock but not turn it. Second, a micromolar lead compound is difficult to evaluate in a functional assay, which may lack the sensitivity of the corresponding receptor binding assay. This results in using millimolar concentrations to detect functional activity, and increases the probability of non-specific interactions and complicating data interpretation. Third, functional assays are not as controllable, from a mechanistic, specific target viewpoint, as a properly developed radioligand binding assay.

Baseline binding inhibition values for random high volume screening are in the range of $0 \pm 10\%$ when considering the vast majority of compounds that are screened will be inactive at the targeted receptor (Figure 4C). A common problem in the screening of natural products is a significant shift from this 10% baseline. Two approaches may be used to correct for this problem. The simplest is to adjust the base line mathematically to 0%. The adjusted positive control values and the knowledge of the targeted inhibitory potential of the controls serve as reference points to determine whether this adjustment is acceptable. The more difficult approach, but a scientifically

A

LEGEND	DPM	AVG	%INHIB	RACK POSITION
TOTAL	6002.0			1
	6235.1			
	6122.4	6119.9		
NON-SPECIFIC	345.7			
	388.2			
	430.5	388.1	93.7%	6
NPC-1001	6776.3		-11.5%	7
NPC-1001	6432.5	-8.5%	-5.5%	8
POS CONTRL	381.2		100.1%	25
POS CONTRL	421.3	99.4%	99.4%	26
NPC-1014	3014.9		51.6%	33
NPC-1014	3074.1	50.6%	49.5%	34



C BASELINE DATA

RACK	BASELINE VALUES
1	-2.2%
2	-1.7%
3	4.0%
4	-1.8%
5	-4.4%
6	-0.7%
7	-2.9%
8	-3.6%
ASSAY	-2.2%

FIGURE 4. A: High volume screening data for the neurokinin 1 receptor (substance P). (1) The specific binding determination run for each 48 tube rack. (2) The known positive control is adjusted by the technician performing the assay to inhibit binding between 80 and 100%. (3) The hidden positive control is treated in an identical manner to the compounds being screened and is adjusted to inhibit binding between 50 and 75%.
B: The standard reference displacement curve run with every eight screening racks. The K_i for this curve must fall within 2- to 10-fold depending on the particular assay being run.
C: The baseline data for each rack is monitored and must fall within ± 10%.

more valid one, is to redefine the incubation conditions of the assay to correct for the shift. The baseline shift shown in Figure 5 for a gp 120/CD4 assay is a significant problem because the improper assessment of inhibitory activity of an extract could lead to fruitless efforts at isolating what is in actuality a non-bioactive entity. After simple adjustment of the baseline, known positive controls fell within the appropriate range, and

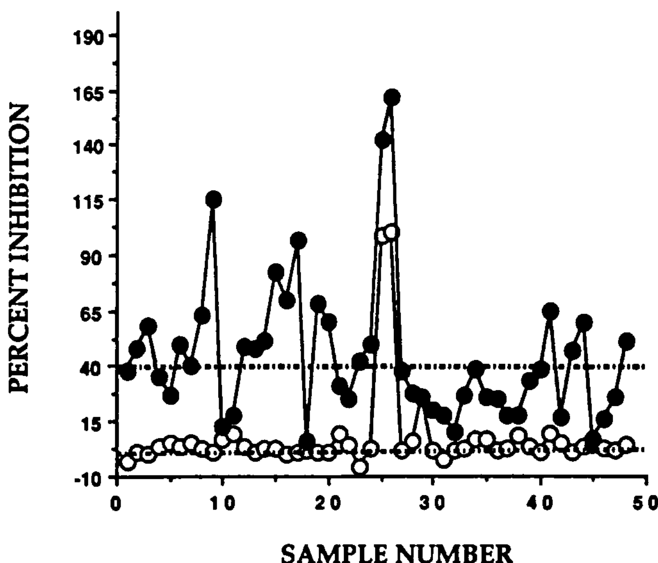


FIGURE 5. The baseline variation for the ^{125}I-gp120/CD4 radioligand binding assay. Range of variation for one rack when screening plant extracts (●). Range of variation for identical compounds when rescreened using a new methodology designed to better deal with plant extracts in this particular assay (○).

several extracts were determined to have inhibitory activity. This activity was determined to be false after no detectable pattern of significant inhibition was reported in subsequent screening in which alterations in the receptor preparation were able to eliminate the shift. In general, radioligand binding assays can be extremely variable (especially those that use peptide radioligands) if not properly developed for natural product screening programs.

SELECTIVITY SCREENING AS DEVELOPMENT TOOL.—Selectivity screening is a recent development by the pharmaceutical industry to help deal with an increased need for mechanistic data, stronger competition, and increasing research and development costs. In addition, the effects of faster product development and shorter product life cycles on revenues and profitability have prompted biopharmaceutical companies to seek ways to optimize development time and to reduce the risk of drug failures in the clinic. One tool in this effort is receptor selectivity screening, which helps predict possible side effects associated with lead compounds early in their development cycle. Such information can help guide ongoing synthetic chemistry efforts and allow direct comparison of that compound to other products in the development pipeline or on the market and thus speed the development of drugs and lower the risk of failures.

Unlike the logistics of high volume screening with thousands of compounds going through one assay, selectivity screening examines small numbers of compounds, most already known to be biologically active, through a large number of different assays. It is often difficult for individual pharmaceutical companies to validate and run more than a few radioligand assays at one time, even in a central core facility, or, worse, to coordinate the collection of assay data from a number of laboratories. As an alternative, contract research companies offer services such as the NovaScreen ® Profile® system, to provide researchers a tool to determine the binding selectivity of their lead compounds in dozens of different assays. NovaScreen®, for example, offers formats for screening com-

pounds in up to 55 receptor binding assays in a rapid (thirty day turnaround), reliable, and cost effective manner.

Selectivity screening format.—In one standard selectivity format a compound is examined at three concentrations, 10^{-5}, 10^{-7}, and 10^{-9} M, in duplicate tubes. This approach allows for the discovery of activity in pharmacological (nM) and non-pharmacological (μM) ranges. Compound activity of greater than 50% at any of the three concentrations is verified using a freshly solubilized disbursement of the compound in a newly performed assay. The ability of selectivity determinations to detect inhibitory activity of pharmacological significance is a major improvement from approaches that target only potentially non-pharmacological activity (such as the micomolar range typically used for high volume screening). At an early stage of drug development, studies are normally designed to examine therapeutically relevant dosages (nM ranges). Lead compound screening at only one concentration (instead of three or more) offers no advantage in terms of increasing sample throughput, as pharmaceutical companies may generate fewer than 20 solid lead candidates a year to undergo this type of screening. These numbers are below the maximum throughput loads of an automated or nonautomated system.

A selectivity report for nifedipine, Procardia® (Pfizer), reveals the simplest interpretation of a screening profile (Figure 6) (10). In this study nifedipine was shown to interact selectively and potently with T and L calcium channels as expected, and no other activity was detected. A more complex selectivity profile, but equally satisfying,

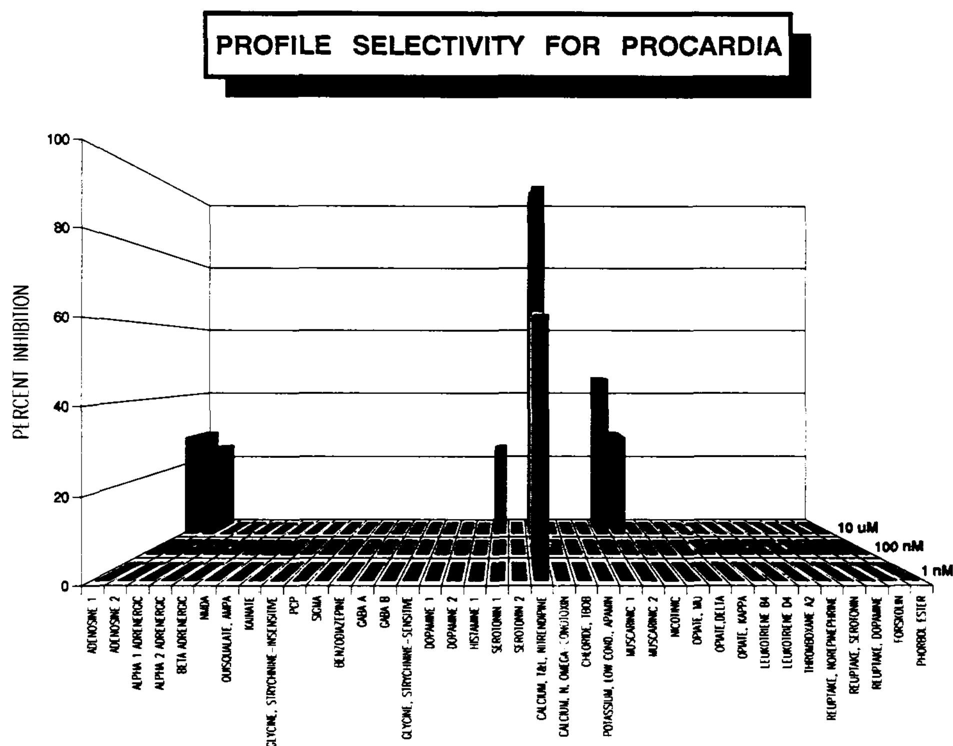


FIGURE 6. Selectivity report for Procardia® (nifedipine). Nifedipine potently inhibited binding to the T&L calcium channel (>50% inhibition at 1 nM). Nifedipine also inhibited binding to adenosine 1 and 2, serotonin 1, low conductance potassium channel, and nicotinic binding sites (<50% inhibition at 10 μM). This profile confirms the high degree of selectivity of nifedipine for the T&L calcium channel binding site.

revealed that fluoxetine, Prozac® (Eli Lilly), is a potent and selective serotonin uptake inhibitor (11). Fluoxetine was shown to inhibit binding at a number of related and non-related receptors, although at concentrations not deemed to be pharmacologically or physiologically significant (μM range) (Figure 7) (12).

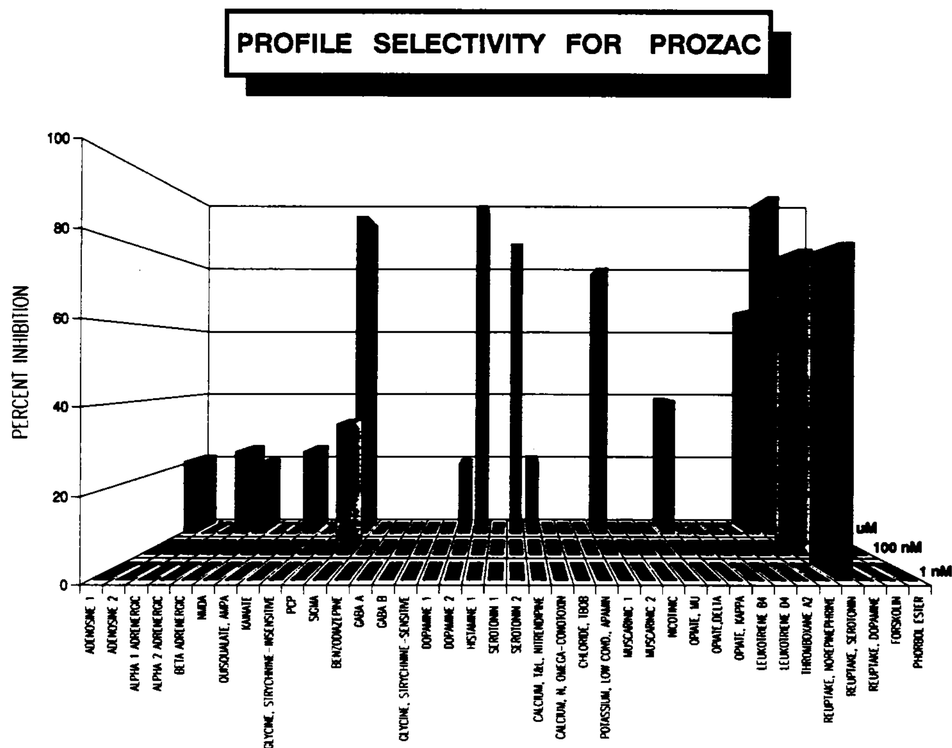


FIGURE 7. Selectivity report for Prozac® (fluoxetine). Fluoxetine potently inhibited binding to serotonin reuptake site (>50% inhibition at 1 nM). Fluoxetine also inhibited binding at the sigma, histamine, serotonin 2, low conductance potassium channel, and norepinephrine reuptake binding sites (>50% inhibition at 100 nM). None of this ancillary activity appeared to be at pharmacologically relevant concentrations.

This type of selectivity screening will not provide all the answers that researchers may need, and in such instances the screening format or assay list may need to be customized or expanded. For example, use of three different concentrations does not supply enough information to generate an accurate IC_{50} or K_i value; thus a receptor selectivity format of this kind should not be used for this particular purpose. It will, however, provide the type of broad information on multiple receptors required to establish the appropriate range of concentrations needed for more accurate IC_{50} determinations. Selectivity reports for triazolam, Halcion® (Upjohn) (Figure 8), and flurazepam, Dalmane® (Hoffman-La Roche) (Figure 9) (13,14), benzodiazepine derivatives used in the treatment of insomnia, provide an example of where the assay list may need to be expanded. By examination of the profile of each, one might envision that flurazepam and not triazolam administration would be implicated in several central nervous system side effects. This report suggests that the interaction of triazolam with any of the receptors studied is unlikely to be the potential mechanism for these side effects. However, more

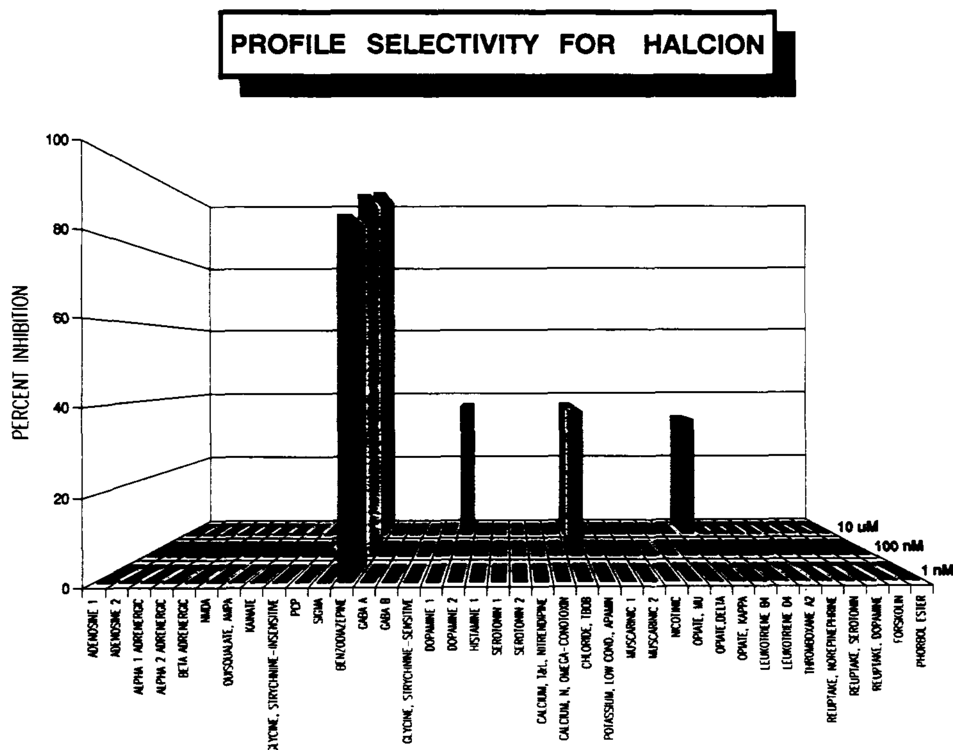


FIGURE 8. Selectivity report for Halcion® (triazolam). Triazolam potently inhibited binding at the benzodiazepine receptor in the nanomolar range. Triazolam also inhibited binding at dopamine 2, kappa opiate, and chloride channel (TBOB) binding sites in the micromolar range. The inhibitory action at the chloride channel may be explained by the close physical association with the benzodiazepine site; both are part of the $GABA_A$ supramolecular complex.

recent evaluations of triazolam and flurazepam hve confirmed the ability of selectivity screening to indicate that flurazepam and not triazolam is more prone to contraindications (15).

Selectivity screening as a discovery tool.—The use of selectivity screening may also be of value when dealing with the discovery of novel therapeutics from natural product sources. Given the ethnomedicinal information about a particular plant, one may choose to screen an extract through a wide variety of assays to determine if a receptor-based mechanism of action can be determined that correlates with the ethnomedicinal properties. We used such an approach to study *Rauwolfia viridis*, a member of the dogbane family. This shrub is indigenous to the coastal thickets of northern South America and many Caribbean Islands and has been used for several centuries by the local inhabitants to relieve pain, fever, and gastrointestinal disorders. By screening the aqueous fraction in over 55 distinct radioligand binding assays an initial profile of receptor has established interactions which may explain several of the tea's reputed biological activities (16). It is generally acknowledged that the level of any distinct chemical entity is relatively low in typical plant extracts. Initially, a tea fraction concentrated tenfold was screened to uncover potential activity. The ability of the tea to completely inhibit the binding at a number of distinct receptors suggests that the tea has several distinct chemical entities with potent biological activities. The rank order of potency was as follows: mu opiate > dopamine > dopamine reuptake (cocaine site) > muscarinic$_2$ > delta

PROFILE SELECTIVITY FOR DALMANE

FIGURE 9. Selectivity report for Dalmane® (flurazepam). Flurazepam potently inhibited binding at the benzodiazepine receptor (>50% inhibition at 1 nM). Flurazepam also inhibited binding at the adenosine 2, NMDA, sigma, dopamine 2, nicotinic, kappa opiate, and low conductance potassium channel binding sites. These results are in accordance with a recent study that suggested that flurazepam and not triazolam administration could result in a greater percentage of contraindications (15), although none of the ancillary activity appeared to be at pharmacologically relevant concentrations.

opiate and muscarinic$_1$. The ability of the fraction to inhibit select opiate receptor subtypes may explain its reported ability to relieve gastrointestinal problems and serve as an analgesic. The interaction of the tea with the opioid/dopaminergic systems could explain the broad spectrum of central nervous system activities described for the plant in Indian folklore. In this regard, the substances isolated from the tea may be useful in the treatment of depression, anxiety, psychosis, and drug addiction.

SUMMARY.—The objective of pharmaceutical drug discovery might best be phrased as the identification of new potential therapeutic agents in a rapid, reliable, and cost-efficient manner. To this end, the simplicity of the radioligand binding assay makes it an outstanding resource to identify and monitor the early stages of the development of a drug. Successful high volume and selectivity screening requires the integration of pharmacology, automation, and data management, as well as the ability to manage and handle a chemical library. It must be emphasized that a successful screening program will be defined by the identification of actual lead chemical entities which can be placed in the research and development pipeline. Scientific integrity must not be sacrificed for the sole purpose of increasing the rate at which data can be generated from a particular screening effort.

ACKNOWLEDGMENTS

This work was in part supported by the following grants to Dr. Paul M. Sweetnam: NCDDG

(NIAID) Grant, U01A127221 and NIMH RFP# NIMH-OD-92-003. The authors also thank Naomi Randolph for typing this manuscript.

LITERATURE CITED

1. J.P. Bennett Jr., in: "Neurotransmitter Receptor Binding." Ed. by H.I. Yamamura, S.J. Enna, and Michael J. Kuhar, Raven Press, New York, 1978, pp. 57–90.
2. J.W. Ferkany, in: "Neuromethods." Ed. by A. Boulton, G. Baker, and J. Wood, Humana Press, Clifton, NJ, 1985, Vol. 3, pp. 117–153.
3. L.P. To, V. Balasubramanian, M.E. Charlton, T.A. Francis, C. Doyle, and P.M. Sweetnam, *J. Immunoassay,* **13**, 61 (1992).
4. D.B. Bylund, "Receptor Binding Techniques," Short Course Syllabus, Society for Neuroscience, Bethesda, MD, 1980, p. 70.
5. Y.C. Cheng and W.H. Prusoff, *Biochem. Pharmacol.*, **22**, 3099 (1973).
6. M. Williams and P.L. Wood, in: "Neuromethods." Ed. by A.A. Boulton, G.B. Baker, and P.D. Hrdina, Humana Press, Clifton, NJ, 1986, Vol. 4, pp. 543–569.
7. W.J. Kinnier, in: "Methods in Neurotransmitter Receptor Analysis." Ed. by H.I. Yamamura, S.J. Enna, and M.J. Kuhar, Raven Press, New York, 1990, pp. 245–258.
8. P.M. Sweetnam, C.W. Bauer Jr., and R. Zeppetello, "Data Management Technologies in Biological Screening: Sample Handling Strategies for High Volume and Selectivity Screening," SRI International, Menlo Park, 1992, p. 31.
9. S.W. Bahouth and J.M. Musacchio, *J. Pharmacol. Exp. Ther.*, **234**, 326 (1985).
10. R.W. Fuller, K.W. Perry, and B.B. Molloy, *J. Pharmacol. Exp. Ther.*, **193**, 796 (1975).
11. American Medical Association, in: "Drug Evaluations." Ed. by K. Lampe, S. McViegh, and B. Rodgers, John Wiley & Sons, New York, 1986, Vol. 6, p. 475.
12. C.W. Bauer, W.J. Kinnier, and P.M. Sweetnam, "Receptor Selectivity of Fluoxetine as Determined by Radioreceptor Binding," *IBRO Abstr.*, **3**, P42.6 (1991).
13. P. Sweetnam, E. Nestler, P. Gallombardo, S. Brown, R. Duman, H. Stefon Bracha, and J. Tallman, *Mol. Brain Res.*, **2**, 223 (1987).
14. J.F. Tallman, J.T. Thomas, and D.W. Gallager, *Science,* **207**, 274 (1980).
15. I. Hindmarch, *Scrip,* **1746**, 18 (1992).
16. L. Caldwell, A. Mezu, T. Ranson, J. Lancaster, K. Barron, L. Zimmerer, W.J. Kinnier, and P.M. Sweetnam, *FASEB Abstr.*, **6**, 354 (1992).