Drug Identification through in vivo Screening of Chemical Libraries

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Specific cell-surface molecules can direct leukocytes and certain tumor cells to particular organs.[1–3] Recent work by our group has shown that peptides, selected by using the in vivo phage-screening approach, are also capable of mediating selective in vivo localization of phage to individual organs as well as tumors.[4–6] To develop our targeting technology beyond peptide-based systems, we investigated the feasibility of screening a chemical library to identify small molecules other than peptides that possess a preferential affinity for particular organs or tissues. As a proof of principle, we screened

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two different chemical libraries in vivo and identified three compounds that preferentially accumulated in individual organs: a pharmacologically active benzodiazepine localized to the brain, another compound specifically homed in on the liver, and the third on the kidneys. These results show that it is possible to use in vivo chemical library screening to identify compounds that distribute themselves to specific sites in the body. Such knowledge can focus drug discovery on compounds with promising pharmacokinetic and tissue specificity profiles.

For in vivo screening of chemical libraries, we injected mixtures of small molecules into the circulation of mice, harvested selected organs in organic solvent to precipitate proteins, and detected the presence of compounds from the library in the soluble phase by mass spectrometry. We initially tested a library of ten compounds. Mass-spectrometric analysis of extracts from organs harvested 10 min after the intravenous injection of the library showed that one compound preferentially accumulated in the brain (Figure 1 A). Breaking the code for the compounds revealed this 301 Da compound to be a benzodiazepine known as Oxazepam.[7] A biologically inactive 265 Da benzodiazepine also present in the library was not detected in the brain (Figure 1 A). Thus, it appeared possible to obtain organ-targeting small molecules by screening chemical libraries in vivo. We also learned from these early studies that it was easier to detect library compounds and differentiate them from endogenous tissue molecules in organic extracts analyzed by electrospray mass spectrometry when the library molecules had molar masses greater than 300 Da.

To test a larger library, we assembled a mixture of 75 compounds with molar masses between 300 and 600 Da and screened for compounds that home in on the brain, liver, lungs, or kidneys. Mass spectrometry performed on organ extracts from library-injected mice identified ten molecules as candidate organ-homing compounds. These ten compounds were tested individually for their ability to specifically target individual organs. Compounds 5862461 and 6074428 were found to accumulate in the kidneys (Figure 1 B and C). The other tissues tested negative for these two compounds. Compound 5343617 was found primarily in the liver and, to a lesser extent, the lungs and kidneys (Figure 1 D). The spectral patterns of compounds 5862461 and 5343617 were particularly distinct because these compounds contain bromine, which exists as two equally abundant natural isotopes,[8] and causes a characteristic two-mass-unit split in the spectral peak (Figure 1 B, inset). One compound accumulated in the lungs, kidneys, and liver, but not the brain; and another localized to the brain, kidneys, and liver, but not the lungs (data not shown). These compounds are likely to bind to receptors that are expressed in more than one tissue, but the varying tissue selectivity of these compounds clearly indicates tissue-specific homing. Extracts from the organs of control-injected mice confirmed that no molecules matched the spectral pattern of the homing compounds. Two other candidate organ-homing compounds localized to all four tested organs. These compounds might bind to molecules present in all tissues, but it is also possible that their concentration in blood remaining in tissues is high enough to allow detection. As these compounds did not show any tissue-specific homing, we did not study them further. For three compounds, the specific organ homing could not be confirmed in individual testing. The remaining 68 compounds were not detected in any tissue, apparently because they did not sufficiently accumulate in any of the test tissues to bring the concentration above the detection limit.

We next quantified the organ accumulation of the three compounds with the most promising organ-homing properties. We used the mass spectrometer to compare the relative

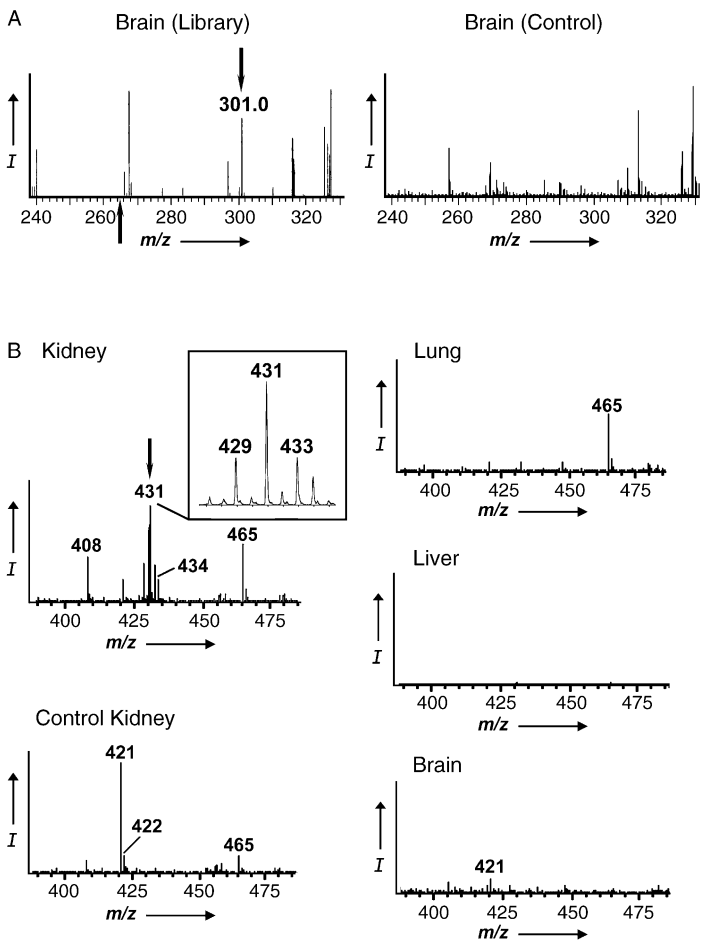


Figure 1. *In vivo targeting of small molecules to particular organs. A) Detection of the benzodiazepine, Oxazepam, in the brain 10 min after intravenous injection with a ten-compound library. "Control" mice were injected with vehicle alone. The downward pointing arrow denotes the spectral peak for Oxazepam. The arrow below the axis denotes the* m/z *of the biologically inactive benzodiazepine in the library. No peak is seen at this position. B–D) Mice were intravenously injected with individual compounds from the 75-member library, and tissues were analyzed 10 min later by mass spectrometry. B) Detection of compound 5862461 in the kidneys after intravenous injection and circulation for 10 min. "Control" denotes mice injected just with DMSO. C) Compound 6074428 targets primarily the kidneys. D) Compound 5343617 targets the liver and lungs. Compound peak heights are shown as relative signal intensity (I). A "+" denotes compound-injected mice and "−" denotes DMSO-injected mice. The downward pointing arrows mark the spectral peaks for the organ-homing compounds.*

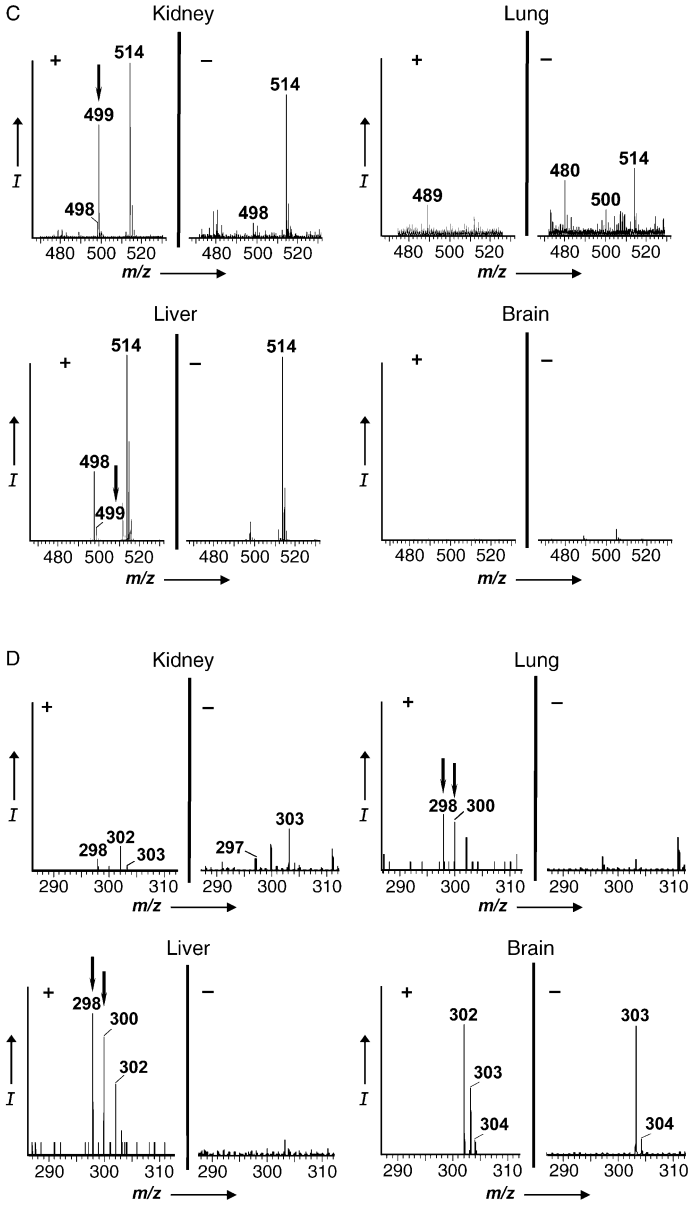
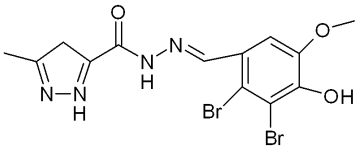
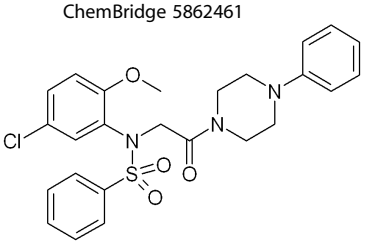
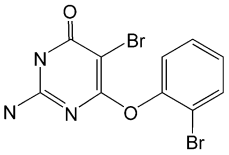


Figure 1. *(Continued)*

amounts of targeting compound in extracts of different organs. Compound 6074428 was at least 30-fold more concentrated in the kidneys than in the liver, lungs, and brain (Table 1). At least 2.4 times more compound 5862461 localized to the kidneys than to the liver, lungs, and brain. Compound 5343617 accumulated very strongly in the liver; about 55-fold higher levels were detected in the liver than in the kidneys, which contained a trace amount of the compound. This compound was also present at moderate levels in the lungs, but was not detectable in the brain. As each of these three compounds accumulated in different tissues, their organ-selective homing is clearly specific and not due to the presence of blood or nonspecific trapping in the target organs.

We then measured two parameters that influence the sensitivity of in vivo chemical library screening. First, we used the

Table 1. *Homing specificity of compounds and their accumulation in target organs. The structure, target organ, and homing activity of the three organ-homing compounds are shown. The quantity of homing compound in the target organs 10 min after an intravenous injection of individual compounds was determined as described in the Experimental Section. The accumulation of targeting compound was expressed as normalized signal intensity level relative to the detection limit.*

Organ-targeting compound	Target Organ(s)	Signal Intensity[a] (fold higher than detection limit)
 ChemBridge 5862461	kidney	2.4-fold (±0.6)
 ChemBridge 6074428	kidney	32-fold (±5.4)
 ChemBridge 5343617	liver lung kidney	67-fold (±2.1) 8.2-fold (±0.6) 1.2-fold (±0.3)

[a] Accumulation data is represented as mean percentage (±standard deviation) for two experiments per variable.

mass spectrometer to analyze the spectral intensity of nine different compounds added to organ extracts, and found that the smallest amount of an individual compound that could be detected in a tissue extract was between 34 and 215 pmol. For the second parameter, we determined the smallest amount of homing compound that could be injected and still detected in our in vivo screening system. For this analysis, the signal intensity of compound 6074428 in kidney extracts from mice injected with various amounts (2 to 125 nmol) of the compound was determined by mass spectrometry. The spectral peak at *m/z* 499 from compound 6074428 was detectable in kidney extracts from mice injected with as little as 7.8 nmol of targeting compound (Figure 2). In the initial library screen with 75 compounds, about 33 nmol of each molecule was present in the injected library mix. Therefore, it is likely that 300 compounds could be tested in a single screening round for organ-targeting compounds. Given the ease and simplicity of this screening technique, a library of 10000 compounds could be screened in vivo in a few weeks with a relatively small-scale effort.

We encountered some limitations with in vivo chemical library screening that will be addressed in future studies. The volume of library injected into the mice (25 μL) was limited by

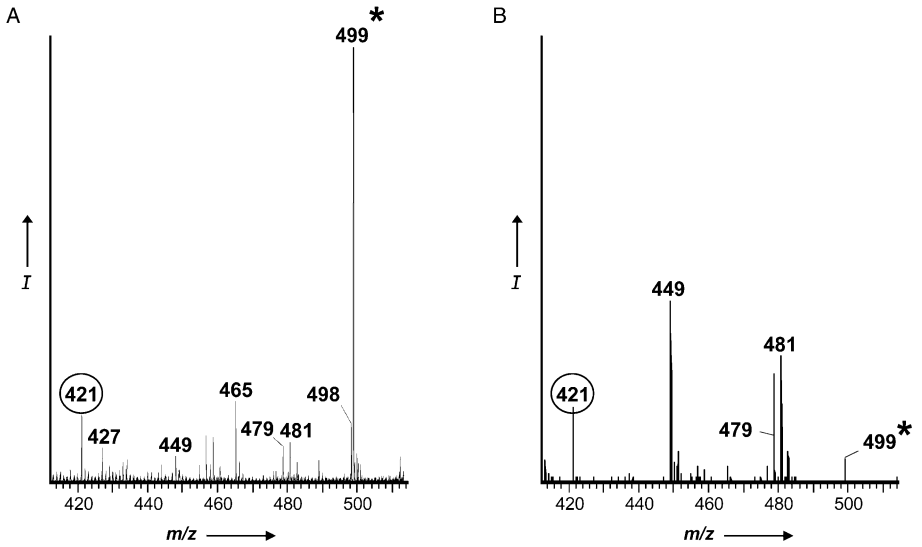


Figure 2. The lower detection limit of in vivo chemical library screening. Mass-spectrometric analysis of kidney extracts from mice injected with either A) 125 nmol or B) 7.8 nmol of compound 6074428. The peak intensities were normalized to the height of an endogenous tissue molecule at m/z 421 that was consistently detected in kidney extracts (circled). The asterisk denotes the spectral peak for the kidney-homing compound, 6074428. The spectral peak intensities of other endogenous tissue molecules (e.g. the molecules at m/z 449 and 481) varied between experiments; as a result, they were not used to normalize the spectral peak intensities of compound 6074428. Compound peak heights are shown as relative signal intensity (I).

the toxicity of the solvent, dimethyl sulfoxide (DMSO). With a less toxic solvent, it should be possible to inject up to 200 μL of library and screen potentially as many as 3600 compounds in one round. Emulsifying agents like Cremophor® EL, Emulphor®, polysorbate 80, Solutol® HS15, or solvents containing *N*-methylpyrrolidone could be used as an alternative to DMSO when solubilizing the chemical library before in vivo screening. In addition, only 1% of the organ extract could be analyzed by mass spectrometry due to the presence of various endogenous tissue compounds in the acetone extracts. A more selective extraction and prepurification method could increase the sensitivity of the compound detection by mass spectrometry.

The biological basis for the targeting activity of some of the compounds identified in the screen has yet to be determined. However, it seems likely that binding to benzodiazepine receptors mediated the brain-homing activity of the pharmacologically active benzodiazepine, as the related inactive compound did not accumulate in the brain. The kidney-homing compound, 6074428, contains a benzenesulfonamide group that is known to have diuretic properties; perhaps this group mediates the kidney-homing activity of this compound.

This work provides the first demonstration that it is possible to conduct large-scale screening of chemical libraries in vivo. Such screening can identify targeted small molecules for use in a variety of applications and has some advantages over previous methods. In vivo phage screening primarily targets the vascular endothelium. Low-molecular-weight chemical compounds can target the vasculature, but are also likely to gain access to parenchymal cells in tissues. That parenchymal cells can be targets is suggested by our recovery of a benzodiazepine as a brain-homing molecule, as most receptors for these compounds are on the neurons. As an additional advantage, this screening approach does not require encoded or tagged library compounds. This is an improvement over other approaches that require separate chemistries for coupling different small molecules to synthetic or genetically engineered tags such as bacteriophage.[9] In addition, the absence of compound tags eliminates the possibility of interference by the tag with the in vivo homing activity.

The localization of selective molecules to specific "addresses" on the endothelium suggests that each tissue puts a specialized signature on its vasculature.[10] Organ-specific vascular molecules are attractive targets for the delivery of therapeutics to particular sites. By conjugating targeting moieties to drugs, diseases such as cancer can be treated with increased efficacy and fewer side effects;[11,12] phage-derived homing peptides and peptidomimetics have been used in this manner to target malignant tumors.[11–15] Organ-homing compounds isolated from chemical libraries are likely to be useful for similar purposes.

In vivo screening may also identify small molecules that have pharmacological effects at the target organ. The identification of a neuroactive compound and a potential diuretic as brain- and kidney-homing molecules, respectively, suggests that this may be possible. Thus, in vivo screening has the potential to advance drug discovery; it allows pharmacokinetics and specificity of action to be studied among large numbers of candidate compounds, or even from completely random libraries. Such approaches may accelerate the discovery and development of new drugs.

Experimental Section

A library of ten small molecules with molecular weights between 200 and 300 Da was prepared by a person not involved in the in vivo experimentation and was tested blindly. The ten-compound library was prepared in phosphate buffer (40 mM, pH 7.2) with each molecule at a final concentration of 1 mM. A larger library of small molecules was prepared from 75 organic molecules (purchased from ChemBridge, San Diego, CA) with molecular weights between 300 and 600 Da. The library compounds were randomly selected from a 420 000-member ChemBridge library, with each compound satisfying the following criteria: 1) the partition coefficient, expressed numerically as log *P*, was less than 5 and 2) the molecular weights of the compounds differed from each other by at least 4 Da. There was high structural diversity in the library, given that the only limitation was the compounds selected from the 420 000-

member parent library needed to fit the parameters described above. The 75-compound library was resuspended in DMSO, with each molecule at a final concentration of 1.33 mM.

To identify molecules that localize to particular organs, two-month-old female Balb/c mice were anesthetized with avertin (0.15 μL g^{-1}) administered intraperitoneally. In experiments with the ten-compound library, 200 μL of library solution (200 nmol per compound) was intravenously injected into the tail vein. With the 75-compound library, 25 μL of library solution (33 nmol per compound) was intravenously injected into the tail-vein. After 10 min of circulation, the lungs, liver, kidneys, and brain were removed. We found 5–15 min to be optimal for the screening of intravenously injected phage for homing to individual tissues and tumors,[16] and we wanted to keep the time short enough to prevent metabolism of the injected compounds, which would change their mass-spectrometric signature.

The organs were washed with PBS (5 mL) to remove excess blood and weighed. Each organ was mixed with acetone (5 mL) and then homogenized with a Handishear hand-held homogenizer (Virtis, Gardiner, NY). For certain organ homogenates, a control compound (ChemBridge 5116670, molar mass 340 Da, 0.25–2.5 nmol) was added as a reference to quantify the amount of homing compound in target organs. The organ/acetone homogenates were transferred to 15 mL centrifuge tubes and incubated at −80 °C for 12 h to precipitate the proteins. Following centrifugation for 30 min at 3000 *g* and 4 °C, the supernatants were recovered and dried in a Speed Vac. A set of control organ extracts was also prepared from mice that were injected with pure DMSO (25 μL).

The dried organ extracts were resuspended in methanol (100 μL), spun in a vortex for about 10–20 min, and separated in a centrifuge to turn the debris into pellets. The supernatants were recovered, further diluted 1:20 in methanol, and the diluted sample (20 μL) was analyzed on a Waters Micromass® LCT mass spectrometer (Milford, MA) at The Scripps Research Institute (La Jolla, CA). Samples were injected into the electrospray by using a mobile solvent phase of methanol/water/acetonitrile (90:9:1). By comparing the masses of the individual compounds and the molecules in the organ extracts of mice injected with DMSO to the molecules in the organ extracts from the mice injected with the library, we were able to identify molecules in the library that localized to a particular organ.

The accumulation of compounds in organs was measured as follows:

We first measured the signal intensities of the targeting compounds using the mass spectrometer and compared them to the signal intensity of a standard compound that was added to the organ extracts; this enabled us to normalize the intensity value of compound peaks from experiments performed on different days. We then determined the smallest amount of an individual compound that could be detected in a tissue extract using mass spectrometry by measuring the spectral intensity of nine different compounds added in small amounts to organ extracts. The detection limit was defined as the spectral intensity level halfway between the background noise and the spectral intensity level generated from the smallest detectable amount of compound in organ extracts (averaged from nine different compounds whose spectra were analyzed and displayed with a scanning window of *m*/*z* 290–610). The normalized intensity values for homing compounds were compared to the detection limit to determine the degree of enrichment of compound in target organs relative to background levels. These enrichment values were not comparable from compound to compound, since each compound has a different ionization efficiency and stability on the mass spectrometer.

The Burnham Institute Animal Research Committee approved the animal experimentation in compliance with the relevent US laws.

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