 J. Comb. Chem. **2010,** *12,* 332–345

Fully Synthetic Phage-Like System for Screening Mixtures of Small Molecules in Live Cells

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Received October 4, 2009

A synthetic "phage-like" system was designed for screening mixtures of small molecules in live cells. The core of the system consists of 2 μm diameter cross-linked monodispersed microspheres bearing a panel of fluorescent tags and peptides or small molecules either directly synthesized or covalently conjugated to the microspheres. The microsphere mixtures were screened for affinity to cell line PC-3 (prostate cancer model) by incubation with live cells, and as was with phage-display peptide methods, unbound microspheres were removed by repeated washings followed by total lysis of cells and analysis of the bound microspheres by flow-cytometry. Similar to phage-display peptide screening, this method can be applied even in the absence of prior information about the cellular targets of the candidate ligands, which makes the system especially interesting for selection of molecules with high affinity for desired cells, tissues, or tumors. The advantage of the proposed system is the possibility of screening synthetic non-natural peptides or small molecules that cannot be expressed and screened using phage display libraries. A library composed of small molecules synthesized by the Ugi reaction was screened, and a small molecule, Rak-2, which strongly binds to PC-3 cells was found. Rak-2 was then individually synthesized and validated in a complementary whole cell-based binding assay, as well as by live cell microscopy. This new system demonstrates that a mixture of molecules bound to subcellular sized microspheres can be screened on plated cells. Together with other methods using subcellular sized particles for cellular multiplexing, this method represents an important milestone toward high throughput screening of mixtures of small molecules in live cells and in vivo with potential applications in the fields of drug delivery and diagnostic imaging.

Introduction

High-throughput screening (HTS) of small molecules is mostly based on the panning of candidate molecules (organic small molecules, peptides) for binding to isolated target proteins (like enzymes or antibodies), usually anchored to a solid matrix that allows automation of the process, thus providing high throughput. HTS brought about an exponential growth in the number of detected biologically relevant small molecules (hits). On the other hand, current HTS systems do not assay pharmacokinetic (PK) parameters such as bioavailability, stability and selectivity, which are essential for transforming the identified in vitro hits into in vivo leads. Therefore, it is of great importance to develop new screening techniques in live cells and in vivo aimed at facing and solving problems related to pharmacokinetic barriers.

Panning of carrier-supported mixtures has been shown to be robust in two different and extensive approaches applied during the last twenty years: (a) Millions of molecules in "mix and split" combinatorial libraries (one bead one compound) were panned for binding to a variety of proteins,[1−5] antibodies in immunoassays,[6,7] or for catalysis of a variety of reactions in vitro.[8,9] (b) Phage display peptide libraries of millions of peptides, were panned in live cell[10] and in vivo assays[11] for binding of peptides to specific tissues or cell types without a particular known target. A common feature shared by these two techniques is that the molecules (mostly peptides) are linked to a carrier (microspheres of 100−500 μm diameter or phages of about 1 μm size). In both cases, binding of cells to the carrier is mediated by the specific molecule bound to its surface.

Between these two approaches we were especially attracted by the phage display random peptide libraries screening method, which is uniquely available for *live cells* and in vivo HTS in mammalians.[11] This method can provide some of the PK parameters such as bioavailability: In an animal tumor model, molecules targeting to the tumor area were isolated from the tumor after IV administration of a phage display peptide library. Many targeting peptides have been identified by this method, and some of them have been used for drug targeting[11] and diagnostics.[12] However, this approach is limited to short natural peptides that bind to specific cells or organs. Recent studies[13] proposed the use of subcellular-sized particles for applications in live cells such as cargo delivery (streptavidine,[14] si-RNA[15]) and sensing[16,17] (fluorescent dyes,[18] β-galactosydase substrate[19]). These systems

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10.1021/cc900156z © 2010 American Chemical Society
Published on Web 03/26/2010

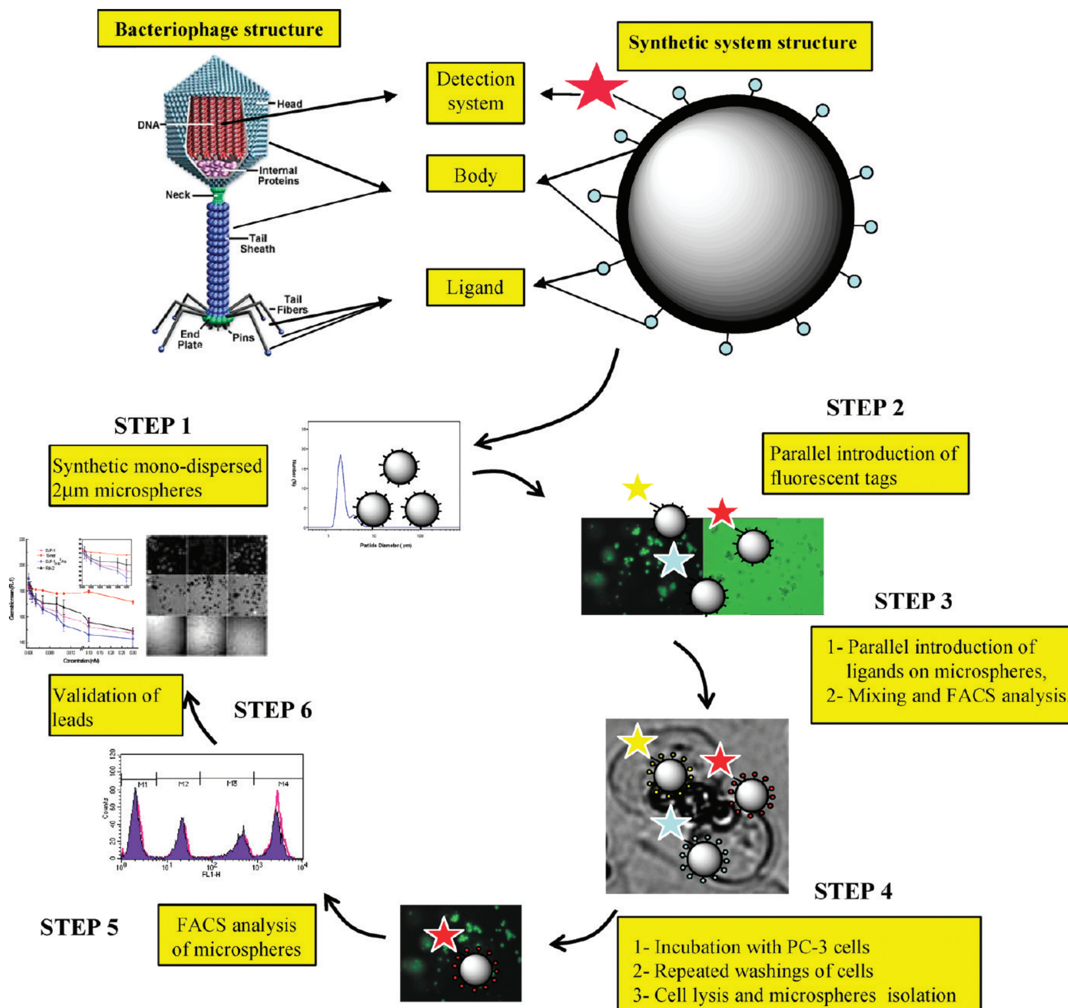

Figure 1. Design of the synthetic phage-like system and steps of the screening technique.

are based on incubation of the modified microspheres with cells of interest for prolonged times (3−24 h). The microspheres are internalized and then the cargo can interact with a known intracellular target, while either linked to the microsphere or be released by reduction of disulfide groups for further interactions.[20] The cells are then analyzed by flow-cytometry or directly observed by microscopy. All together, these studies provide strong, direct evidence that screening methods for evaluating interactions (binding and/or specific internalization) between unknown cellular targets and ligands tethered on particles in live cell models can be developed as a synthetic alternative to phage display peptide screening.

Here, we present a fully synthetic "phage-like" system based on carrier supported mixtures, aimed at identifying not only peptides but also small organic molecules with high affinity for specific cell types in a live cell assay. We called the system phage-like because the main elements found in the phage technique were recreated by synthetic elements. Moreover, the cellular targets of the synthetic system here described are unknown, exactly as in the phage display peptide screening approach. Thus, a mixture of different ligands anchored to subcellular sized microspheres is added to a cell line. After a simple incubation and washing procedure, similar to that employed in phage display peptide screening, bound small molecules are detected by flow cytometry based on the differential fluorescence intensities of the different microspheres. At this point we are presenting a proof of concept, using a mixture of only four different small molecules. Nevertheless, this approach can be expanded to a larger number of different molecules by combining a variety of fluorescent colors.

Together with recently published approaches that used microscopy for identifying cellular multiplexing of colored nanoparticles in live cells,[18] our results contribute another step toward developing and transforming live cell panning into a high content screening system (HCS), where a mixture of ligands bound to appropriately tagged particles is added to cells and the interacting ligands are identified through specific tags. A live cell screening method for panning mixtures of molecules bound to particles is a significant step toward in vivo combinatorial screening in animal models, which will need the development of additional histological methods to trace the presence of particle-bound ligands in tissues of interest.

Results and Discussion

Design of the Screening System. The system is composed of monodispersed cross-linked 2 μm microspheres (Figure 1, step 1) bearing a panel of intensity-level fluorescent labels (Figure 1, step 2) on which libraries of ligand candidates are either directly synthesized or linked after synthesis

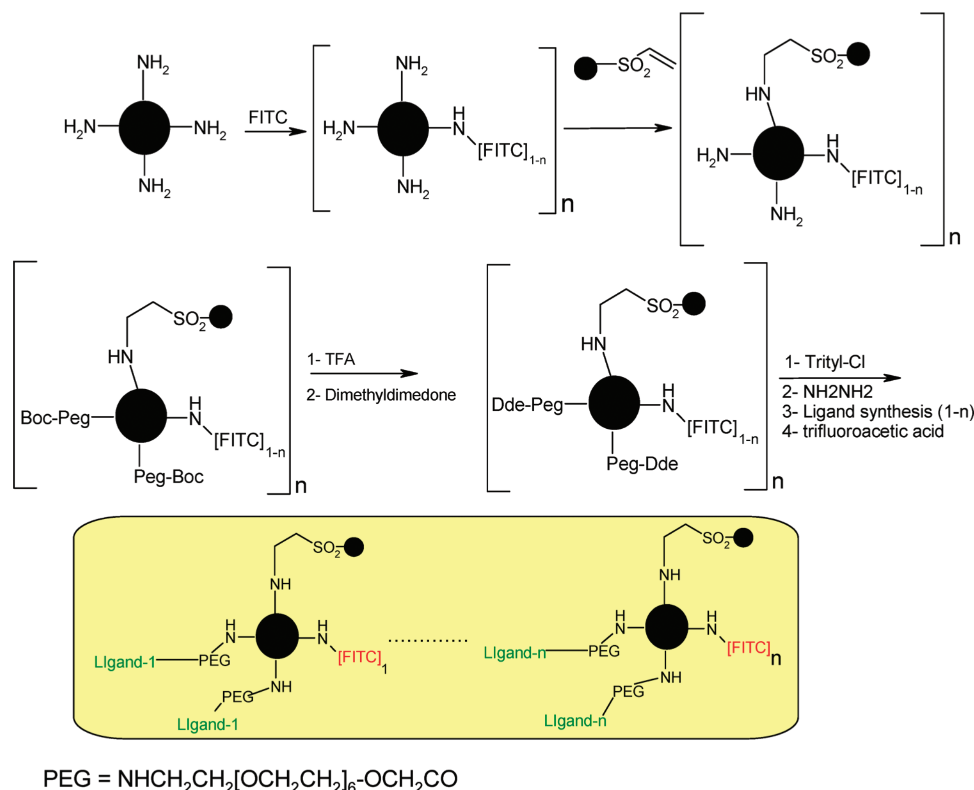

Figure 2. General strategy for microsphere functionalization.

(Figure 1, step 3). Each fluorescence intensity represents a specific ligand. The subcellular size of the microspheres allows their incubation with cells grown on microplates, and consequently, their interactions with cells can be observed after repeated washings and cells lysis (Figure 1, step 4). The remaining cell lysates contain those microspheres with significant affinity for the cells and which were either bound to the cell surface or internalized prior to lysis. These microspheres are visualized by flow-cytometry and ranked by comparing the FACS plots to an appropriate control in the mixture (Figure 1, step 5). Finally, validation of molecules with high affinity is carried out either by binding assays and microscopy (Figure 1, step 6).

The model peptide DUP-1, which is a known high-affinity ligand for PC-3 cells[12] from a phage display library (with unknown cellular target), was synthesized on microspheres and used as a standard positive control.

The synthetic system also possesses a magnetic arm that can be eventually used for tracing and for isolating microspheres from tissues in future applications.

Choice of a Polymeric Carrier. Most of bead-based assays developed during the last years were demonstrated for beads in the range of 6−500 μm.[21−23] This range is excluded from assays where beads are incubated with plated cells and from in vivo administration. On the other hand, it has been shown that subcellular particles can be incubated with cells and internalized after 3−24 h. Some of these systems were used for intracellular multiplexing and sensing.[13] The system proposed here aims at discovering molecules with significant affinity for a specific cell line, mediated by a tethered molecule on the microsphere, rather than by a global internalization process obtained after long hours of incubation with cells.

In a previous work, peptides were synthesized on 6 μm organosilica microspheres that must be subjected to stabilization procedures for maintaining their morphology, as well as their encoding, during exposure to the solvents used for solid-phase synthesis. In contrast with the present application, those microspheres were used only in immunoassays for recognition of selected antibodies.[22] Thus, cross-linked polystyrene microspheres with a narrow size distribution (2.2 $\pm$ 0.2 μm) were prepared and characterized according to a previously reported procedure.[24] Although this size is larger than the bacteriophages used in phage display screening, it is well suited for incubation with plated cells and for FACS analysis of microsphere mixtures properly labeled with a panel of fluorescence labels (See Figure a in Supporting Information for physical properties of microspheres).

Functionalization of Microspheres. The diversity of the employed labels will determine the throughput of the biological screening. We chose to label the microspheres with differing levels of fluorescein isothiocyanate (FITC) as previously used for multiplex analysis.[25] The general strategy for functionalization of microspheres is described in Figure 2. After transformation of chloromethylene-microspheres into amino-microspheres by known procedures,[26] batches of amino-microspheres were reacted with different concentrations of FITC solutions for an identical time to produce labeled populations of defined fluorescence intensity. Mixtures of microspheres labeled with differing FITC intensity levels can be easily analyzed using flow-cytometry. Figure 3a shows flow cytometry of microspheres bearing 6 defined fluorescence intensity levels of FITC.

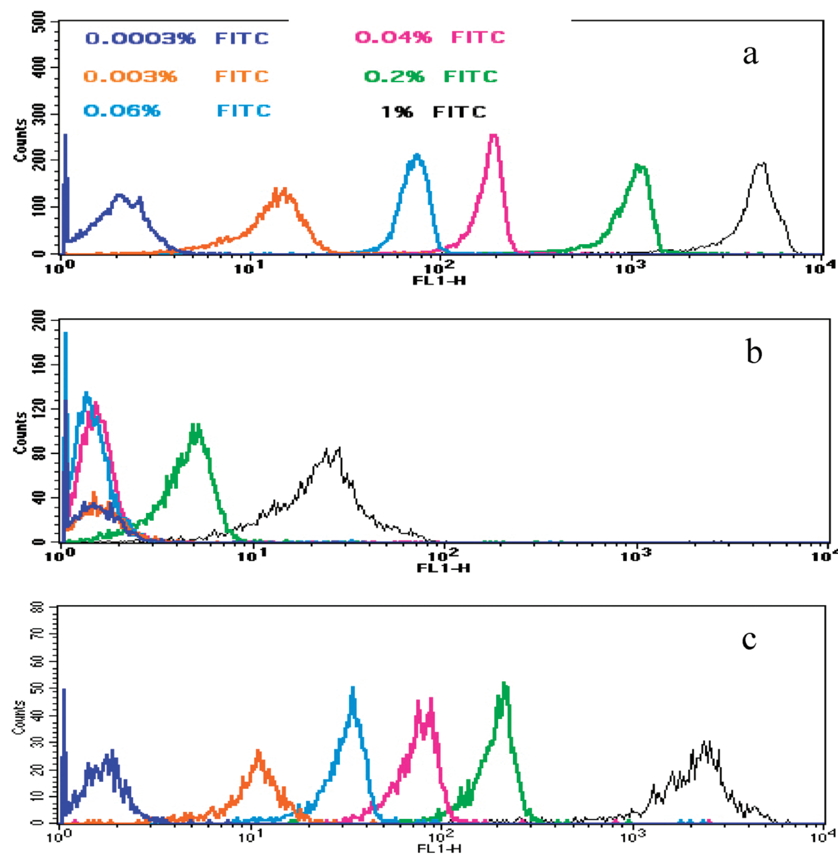

Figure 3. FACS histograms of microspheres: (a) after fluorescence labeling with 6 levels of FITC, (b) after tritylation of microspheres in a, and (c) after trityl cleavage from the microspheres from b.

This procedure leaves more than 99 mol % of the free amino groups available for the subsequent steps of the synthesis (for detailed procedures, see Experimental Section, Method 1).

To introduce magnetic susceptibility, vinyl-sulfonyl magnetite nanospheres were prepared as previously reported[27−29] and linked by Michael addition to a small fraction of the free amines of the microspheres. This functionality permits easy isolation of the microspheres from cells or tissues in future applications. The microspheres were then reacted with Boc-$(PEG)_6$-COOH, which generates a hydrophilic layer between the hydrophobic polystyrene in the core and the ligands on the microspheres. This layer prevents hydrophobic interactions between ligands and the polystyrene allowing free interactions between the tethered ligands and targets in the cells.

The Boc groups were cleaved and the free amines were reacted with Dde protecting group[30] followed by exhaustive tritylation of the free carboxylate and phenolate groups of the FITC labels using trityl chloride. This prevented amidation of the carboxylate group and consequently the irreversible loss of fluorescence during the synthesis of ligands. This orthogonal strategy allowed recovery of the free amino groups for ligand synthesis after Dde removal using a hydrazine solution. (Experimental Section, Method 1).

Finally, after synthesis of the ligands, trityl groups were removed using trifluoracetic acid. The feasibility of this strategy was demonstrated by flow-cytometric analysis of labeled microspheres as shown in Figure 3: before tritylation (a), after tritylation (b), and after trityl removal using trifluoroacetic acid (c). The observed slight shift of the fluorescence levels in the FACS plots after removal of trityl groups by TFA (compare panel c to panel a in Figure 3) can be explained by the well-known effect of the pH on the fluorescence intensity of FITC,[31] Thus, equilibration of the microspheres suspension with an appropriate buffer prior to analysis improves the signal's reproducibility so that the peaks appear at the same intensity levels of the starting mixtures (see Experimental Section, Method 7).

Synthesis of Peptides on Microspheres. To further validate the synthetic phage-like system, direct synthesis of peptides on the microspheres and the conjugation of a nonpeptide Ugi-library[32] were tested.

To allow a direct structural chemical analysis of the obtained peptides, we began the synthesis by reacting a mixture of Fmoc-Gly/Fmoc-Rink linker[33] with the microspheres. This approach permits partial cleavage of the obtained ligands after completion of the synthesis and a direct high resolution mass spectrometric and HPLC analyses of the peptides. Feasibility was demonstrated by the synthesis of two peptides: 15-mer WGLRALESRWDRYYF **1**, a peptide with high affinity for ubiquitin[34] and used in the assay as negative control, and DUP-1 FRPNRAQDYNTN **2**, a peptide with high affinity for an unknown antigen in PC-3 cells (found by phage display peptide screening method[12]), used here as positive standard for the establishment of the screening technique.

The peptides were synthesized using conventional fmoc strategy protocols (see Experimental Section)[35,36] in parallel

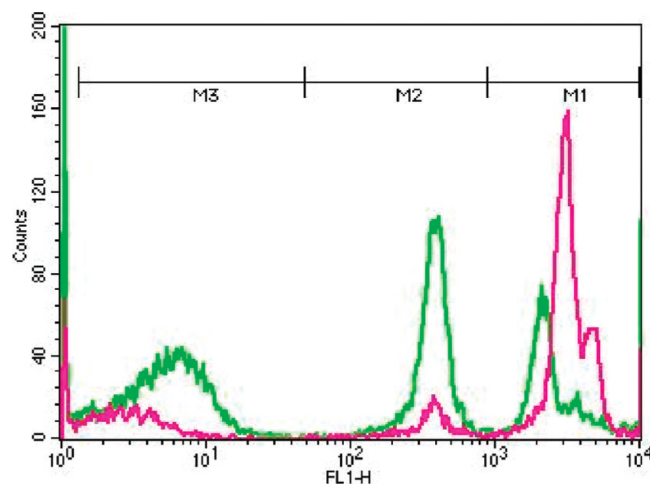

Figure 4. Flow cytometry analysis of a mixture of microspheres: KLBM, DUP1 peptide (M1); MBM1, 15-mer (M2); MBM2, naked microspheres (M3). Overlaid histograms of initial mixture (green line) and of the extract from cells after incubation, washing, and lysis (pink line). (Values for each population are given in Table 1 below).

wells of a 96 well/0.45 μm filter microplate (Pall) placed into a Millipore manifold connected to a vacuum pump (see Figure b in Supporting Information). Microplates were placed onto an orbital shaker during the coupling reactions of the amino acids. After partial cleavage with TFA, the crude soluble peptides **1** and **2** were analyzed by HPLC and HR-MS (see Experimental Section, Method 2, and Supporting Information for HRMS/HPLC).

The results indicate that peptides can be reliably synthesized on monodispersed 2 μm microspheres using classical FMOC chemistry. To our knowledge, these are the smallest particles ever used for a Merrifield synthesis.[35] Slightly bigger organosilica particles of 6 μm have been used in Merrifield synthesis; however, the organosilica microspheres were sensitive to acidic conditions and were used for immunoassays on isolated proteins. We chose to not use microspheres of 6 μm as they are too large to be used for binding to plated cells. Two micrometer microspheres can be assayed with the ligand of interest tethered on their surface for binding to cells, using the method described below.

Establishment of a Flow-Cytometry-Based Biological Assay for Binding of Microsphere Mixtures. The assay is based on the comparison of binding of a molecule bound to a micropshere, MBM*n* (*n* is the number of a specific ligand, member of a library composed of n members), and a known ligand bound to a microsphere, KLBM, defined as the positive calibration control. Each microsphere population bearing a different MBM*n* is labeled with a defined fluorescence level in a way that individual and different molecules can be unambiguously followed and quantified by flow cytometry.

The relative amount of a specific MBM*n* in a mixture is defined relative to the KLBM standard. To calculate this, the total number of events representing the MBM*n* is divided by the total number of events representing KLBM (each event represents one microsphere, and the overall number of events for MBM*n* or KLBM is represented by the corresponding peak in the histogram, see Figure 4). The number of events is automatically calculated by the software of the flow-cytometer which can also calculate the % of each population (each ligand) out of the total number of microspheres in the mixture. The data are automatically obtained in the form of a table containing the percentage of each member in the mixture (events of each population out of the overall events of the mixture). These percentages are placed in eq 1 to obtain a rank for each compound (see explanation below).

If the initial ratios ($MBMn_{initial}/KLBM_{initial}$) are calculated by performing a FACS analysis of the starting mixture (prior to incubation with cells), we can rank the relative binding extent of each MBMn to cells by comparing the ratios of the bound microspheres obtained after incubation with cells and washings to the starting ratio. This relationship is easily expressed by eq 1, where KLBM and MBMn represent number of events detected for each type of microspheres (or their percentage in the mixture which is the same).

$$(KLBM_{initial} \times MBMn_{bound})/(KLBM_{bound} \times MBMn_{initial}) = X \quad (1)$$

In this way, the relative binding of different molecules of a library can be ranked. *X* larger than 1 shows higher binding of MBM*n* compared to the KLBM positive standard, and ratios lower than 1 indicate lower binding of MBM*n* compared to the KLBM positive standard. Overall, one can estimate if a given molecule (MBM*n*) displays significant binding to a tested cell line as compared to the binding of a molecule (KLBM) known to have strong binding to the cell line. This process is especially interesting for finding molecules with strong affinity for specific cells without early knowledge of the cellular targets involved in the affinity. This is exactly the way phage display peptide libraries are evaluated.

To estimate the nonspecific binding in the assay and the signal-to-noise ratio, KLBM mixed with a panel of ligands that should not bind to the specific cells used in the assay (negative controls) must be tested (see Figure 4).

In our assay, PC-3 cells, a model cell line for prostate cancer, were seeded on six-well plates and grown to 75% confluence. A mixture of microspheres containing the following ligands was added to the cells: (1) KLBM, DUP-1 peptide (positive standard, highest fluorescence label), (2) MBM1, 15-mer peptide (negative control, medium fluorescence label), and (3) MBM2, naked microspheres (background control, no fluorescence label). The initial microsphere mixture was analyzed by flow-cytometry prior to incubation to obtain the initial ratios of the components (Figure 4, green line). The mixture was then applied to the cells (as detailed in the Experimental Section, Method 7). After incubation and repeated washings to eliminate unbound microspheres, cells were lysed, and the total well content, containing only the cell-bound microspheres, was suspended in an appropriate buffer and analyzed by flow-cytometry (Figure 4, pink line).

The "initial" and "bound" values of KLBM, MBM1, and MBM2 were introduced into eq 1, and a ranking of binding was established as compared to the calibration positive control KLBM (see Table 1). This experiment was repeated

Table 1. Population Values for FACS Analysis in Figure 4 for Mixtures Composed of DUP-1, 15-mer Peptide, and Naked Microspheres[a]

name	sequence	FITC-label	marker	% initial	% bound	rank eq 1
DUP-1 KLBM	FRPNRAQDYNTN	1%	M1	25.9	76.5	1
15-mer MBM1	WGLRALESRWDRYYF	0.04%	M2	34.64	7.03	0.062
− MBM2	naked microspheres	no	M3	36.3	14.21	0.152

[a] The table gives the ranking of each population according to eq 1.

Table 2. Summary of Ugi Organic Molecules Sets According to their FITC Labeling Levels

	FITC levels				
set number	0.003%	0.04%	0.06%	0.2%	1%
1	MBM1		MBM2	DUP-1 (KLBM)	MBM3
2	MBM4		MBM5	DUP-1 (KLBM)	MBM6
3		MBM7	MBM8	DUP-1 (KLBM)	MBM9
4		MBM10		DUP-1 (KLBM)	MBM3

three times. A representative FACS histogram disclosing the results obtained for the mixtures (M1 = KLBM = DUP-1, M2 = MBM1 = 15-mer, M3 = MBM2 = naked microspheres) is shown in Figure 4. The green line in the histogram represents the initial mixture and the pink line the "bound" microspheres after incubation with PC-3 cells, washing and lysis (for more details, see Experimental Section). While for the positive control (DUP1, M1) the pink line is higher than the green one, that is, there is a clear binding of this peptide to the cells, the pink line for the 15-mer (M2) and the naked microspheres (M1) are lower than the green line, showing very low binding.

This is clearly shown when the total number of events in the peaks are placed in the ranking equation: MBM1 = 0.062 and MBM2 = 0.152 (see Table 1). The relatively high ratio value obtained for MBM2 (M3 peak in Figure 4) is the result of the low autofluorescence of cell debris with intensities close to those of the naked beads (as determined in a separate experiment where cells were just lysed and extracted exactly as the cell-microsphere mixtures of the relevant assay, not shown). On the other hand, the very low ratio value obtained for MBM1 (M2 peak in Figure 4) represents the nonspecific binding of 15-mer peptide microspheres to the cells. Although these 15-mer peptide microspheres are well represented in the starting mixture, after incubation and repeated washings they almost disappear, thus the final ratio is very close to zero. Thus, when comparing two fluorescently labeled beads (DUP-1 = M1 and 15-mer = M2 in table 2) the signal-to-noise ratio is consistently high for using this protocol in screening of coded-ligands tethered on microspheres as mixtures. We also note that to overcome noise generated from cell debris, it is necessary to use only FITC labeled microspheres for any ligand, in this way the noise from cell debris which falls at a very low fluorescence level, is moved out from the histogram. The ideal incubation time of microspheres with cells was 40 min. For this time range no significant unspecific internalization was observed as the negative controls remained very low. These results are in agreement with previous studies which have shown that significant nonspecific internalization of subcellular sized particles only occur over incubation times of 3−24 h.[13]

These results show that the assay can be used for testing any new ligands tethered to fluorescently labeled microspheres either separately or in mixtures. The use of the positive calibration standard DUP-1 bound to microspheres bearing a defined fluorescence label allows a comparative ranking of different tested ligands.

Synthesis and Screening of a Library of Ugi's Small Molecules. The goal of the present screening is to identify molecules with high binding affinity for prostate cancer cell line PC-3 as compared to a known binding molecule DUP-1. Although the throughput of the presented technique is at this stage low, it is of interest as it permits the screening of non-natural peptides or small molecules which cannot be screened in a phage display library approach. Therefore, we synthesized a library of novel small molecules using the well-known Ugi reaction.[32] The library was generated in solution, permitting us to easily characterize the molecules and attach them to the labeled microspheres. Briefly, the four component Ugi reaction was performed using a panel of 10 aldehydes, cyclohexyl-isocyanide, heptyl amine and Boc-GlyOH which served as acid component (Figure 5).

A specially developed microwave energy assisted reaction was carried out that reduced the time of the reaction to several minutes (see Experimental Section, Method 3). The products were deprotected with trifluoroacetic acid to generate a free amino group suitable for conjugation to the microspheres. Amino-microspheres were reacted with diglycolic anhydride to generate carboxylic acid microspheres that were reacted with the deprotected Ugi compounds using classical peptide coupling. Completion of this reaction was monitored by malachite green assay,[37] which confirmed the presence of the molecules on the microspheres (see Figure 5). Like in peptide synthesis, these compounds were linked to the microspheres through a Rink linker or Gly (9:1). This enables cleavage of a part of the molecules and allows a secondary characterization by HPLC and HRMS to confirm their linkage to the microspheres (see in Supporting Information the full characterization of synthesized molecules and table a for analyses of their cleavage products).

The screening of the Ugi library was conducted according to Method 7 in the Experimental Section as follows: we generated 5 FITC levels on microspheres (see Table 2). The 0.2% level (M2 population in FACS) was used exclusively for the control DUP-1-microspheres (KLBM), and the rest were used for the MBM*n*'s. Experiments were repeated three times.

Figure 6 shows the histograms obtained for four groups of the Ugi's library, and Table 3 summarizes the percentage obtained for each population and the ranks obtained by applicaton of eq 1. The screening shows that compound Rak-2 (MBM-2, figure 6, set 1, M3 peak) displays strong affinity for PC-3 cells as compared to the DUP-1 standard peptide, although the two compounds are totally different. Rak-3 (MBM-3, Figure 6, set 1, M1 peak) shows binding levels slightly lower than those of DUP-1.

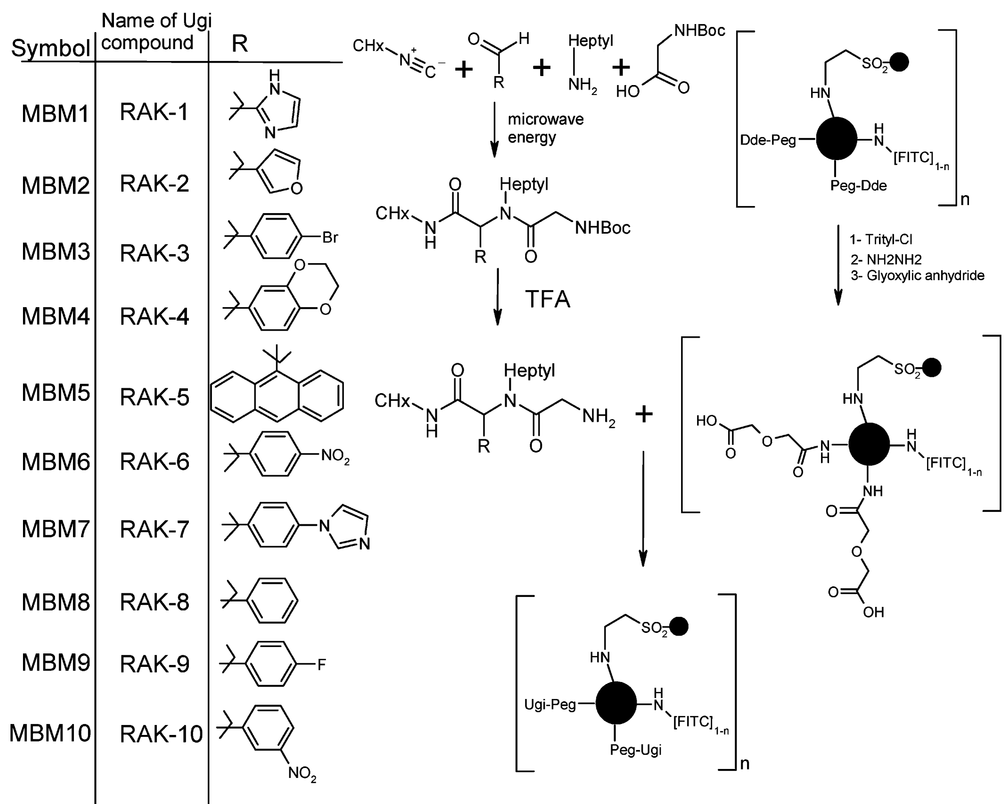

Figure 5. Synthesis Ugi library and attachment of products to microspheres.

Validation Studies. Validating the affinity of small molecules for an unknown target in vitro is of course impossible. However, it is possible to establish a semiquantitative comparative binding analysis of small molecules in a live cell assay to evaluate the approximate extent of binding to specific cell types. Zitzman et al.[12] have already validated the binding of DUP-1 to PC-3 cells using a radioactive derivative of DUP-1 in the presence of increasing concentrations of unlabeled DUP-1. An IC_{50} of 1 μM was thus obtained for DUP-1. Additionally, in the same work the binding of fluorescently labeled DUP-1 was observed qualitatively using confocal microscopy. To further demonstrate the reliability of the proposed screening technique, we performed a competitive binding assay on cells in suspension based on this work, using fluorescently labeled probes and FACS analysis.[38] In this assay, unlabeled molecules to be tested at different concentrations and fluorescently labeled DUP-1 at a given concentration are added to the cells and their fluorescence intensity, representing the amount of the known molecule bound, is tested by flow cytometry. It should be pointed out that with this method, full competition between the molecules and DUP-1 is not expected: the ranking obtained for each molecule as compared to DUP-1 is a way for evaluating the extent of binding and not necessarily a proof of full competition with DUP-1. Each molecule can bind to multiple cellular targets, some of them might be common, and if this is the case, competition binding can be qualitatively evaluated.

RAK-2, which was identified by the screening of the Ugi's library as being the molecule with the highest binding ranking to PC-3 cells (Table 3), was synthesized separately and characterized in this assay (see Supporting Information for synthesis and physical properties). Rak-2 was synthesized in a pegylated form to improve solubilization (see Experimental Section). The PEG was identical to that used as linker between the small molecules and the microspheres in the Ugi's library.

First, it was necessary to evaluate the extent of the non specific binding of labeled DUP-1 to PC-3 cells. This procedure is necessary for selecting an appropriate concentration of the labeled DUP-1 to be used in the validation assays and reduce the noise produced by the non selective binding.[38] Therefore, a saturation experiment was carried out as follows (see Supporting Information). Cells were preincubated with a high concentration of non labeled DUP-1 (1 mM) followed by the incubation with increasing concentrations of labeled DUP-1. Binding was determined using the geometric means obtained from the FACS histograms at the different concentrations of the ligands.[38] The plot in figure c in the Supporting Information shows the non specific binding of labeled DUP-1 at a variety of concentrations. Linear increments of fluorescence are observed up to 10 μM labeled DUP-1. Within this range we are certain that inner filter effects (bleaching) which might be caused by very high concentration of the fluorescent probe remains insignificant. Zitzman[12] has already established an IC_{50} of 1 μM for DUP-1 against a radiolabeled derivative. At this value the noise from non specific binding (84 arbitrary units of geometric mean) was in the linear zone. Thus, we chose to use 1 μM of labeled DUP-1 in the following binding assay.

PC-3 cells were preincubated with increasing concentrations of ligands: non labeled DUP-1 (positive control), peptide 15-mer (negative control), or Rak-2. one micromolar (FITC-^{13}Lys)DUP-$1_{(1-12)}$ peptide was then added, and cells

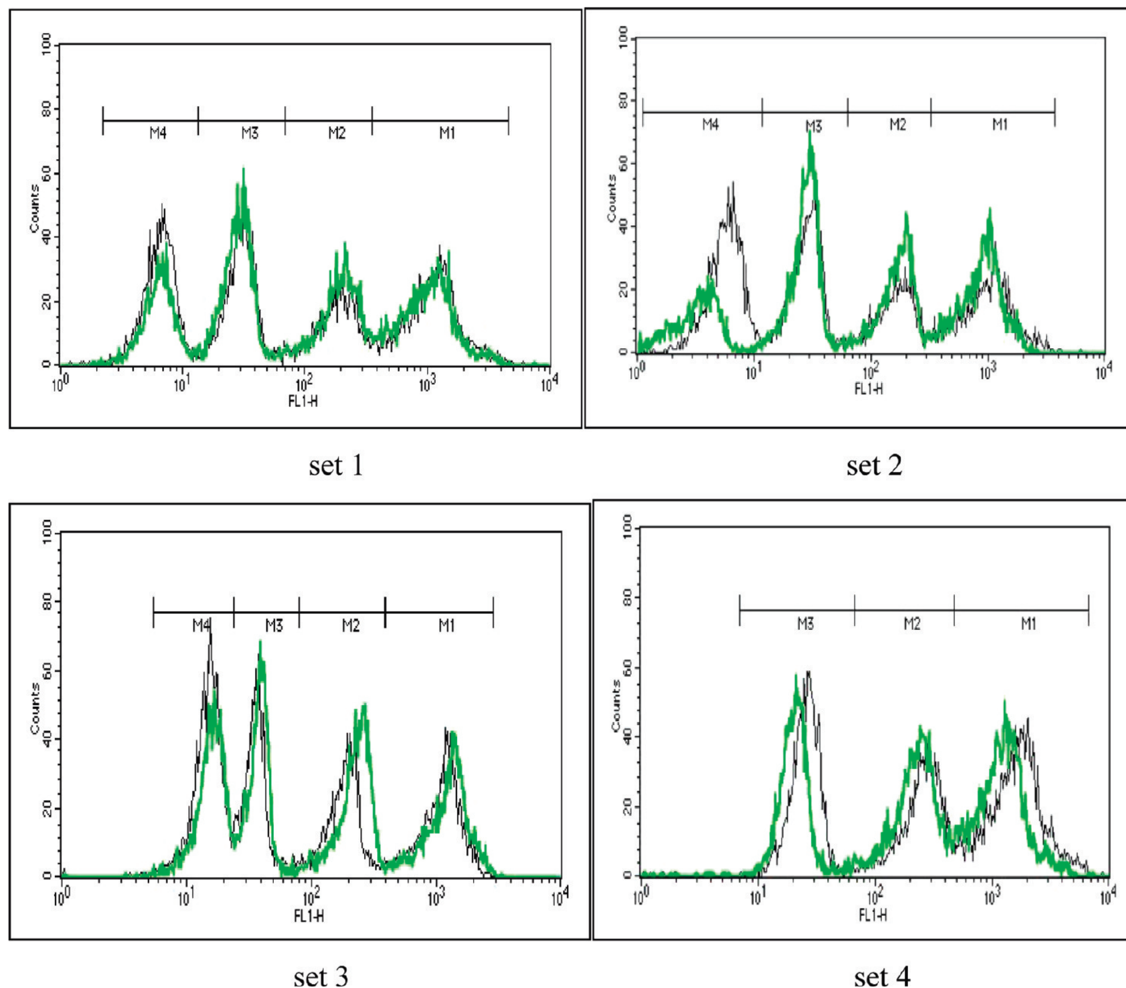

Figure 6. M2 = DUP-1 in all the experiments. Black histograms: Initial composition. Green histograms: End composition. Experiments were repeated 3 times.

Table 3. Population Values for FACS Analysis in Figure 6 for four Sets of Mixtures Composed of DUP-1 and Ugi Compounds[a]

set number	member of library	name of compound	FACSmarker	initial %	end %	rank eq 1
1	MBM1	Rak-1	M4	27.79	19.11	0.60
1	MBM 2	Rak-2	M3	26.18	30.10	1.01
1	KLBM	DUP-1	M2	18.30	20.65	1
1	MBM 3	Rak-3	M1	27.64	30.01	0.96
2	MBM 4	Rak-4	M4	31.69	15.24	0.35
2	MBM 5	Rak-5	M3	27.94	32.81	0.85
2	KLBM	DUP-1	M2	17.14	23.38	1
2	M BM 6	Rak-6	M1	23.18	27.17	0.85
3	MBM 7	Rak-7	M4	33.23	27.05	0.64
3	MBM 8	Rak-8	M3	23.09	23.81	0.82
3	KLBM	DUP-1	M2	20.44	25.85	1
3	MBM 9	Rak-9	M1	23.35	23.35	0.78
4	MBM 10	Rak-10	M3	33.49	29.82	0.70
4	KLBM	DUP-1	M2	25.89	32.71	1

[a] The table gives the final ranking for each population according to eq 1.

were tested by flow-cytometry. Binding was determined using the geometric means obtained from the FACS histograms at the different concentrations of the ligands as previously described.[38] The competitive binding inhibition obtained for each of the tested ligands is shown in Figure 7. (see Experimental Section, Methods 4 and 5 for probe synthesis and Method 8 for procedure).

As expected, DUP-1 (□) significantly inhibited binding of (FITC-^{13}Lys)DUP-1$_{(1-12)}$. The new small organic Ugi molecule Rak-2 (▲) inhibited FITC-^{13}Lys)DUP-1$_{(1-12)}$ peptide binding to an extent close to that found for the DUP-1$_{(1-12)}$ peptide. This indicates that this molecule binds to the same target/s molecule in the cell at least at a significant extent. On the other hand, we did not observe significant reduction of the fluorescence of labeled DUP-1 in the presence of the same range of concentrations of the nonrelated 15-mer peptide WGLRALESRDWRYYF (●), meaning that this peptide does not compete with (FITC-^{13}Lys)DUP-1$_{(1-12)}$ for binding to cells (negative control).

None of the ligands completely blocked the binding of the fluorescently labeled DUP-1 molecule. We suggest that part of the residual fluorescence results from early penetration

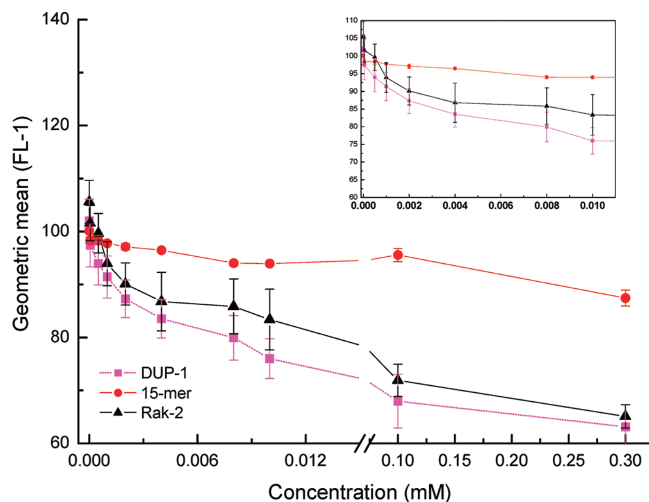

Figure 7. Competitive binding inhibition of fluorescent (FITC-^{13}Lys)DUP-$1_{(1-12)}$ from PC-3 cells by unlabeled DUP-1 (□), small molecule Rak-2 (▲), and 15-mer peptide (WGLRALESRW-DRYYF) (●) as measured by flow-cytometry. Non specific binding of DUP-1 at 1 mM shown in Figure c of Supporting Information was subtracted from the obtained values. Mean values ± S.E.M., $n = 3-5$ (see Experimental Section).

of part of the non labeled ligands into the cells, which diminishes their effective concentration in the medium. This suggestion is supported, qualitatively, by confocal microscopy studies, which demonstrate that DUP-1 and Rak-2 are internalized by PC-3 cells.

Thus, microscopy studies were carried out to directly observe the binding and incorporation of Rak-2 to PC-3 cell as compared to DUP-1.

Intracellular Fate of Rak-2. Live cell microscopy was used to provide an independent method of demonstrating affinity for PC-3 cells, and as a preliminary way for understanding the mechanism of interaction, PC-3 cells were incubated either (FITC-^{13}Lys)DUP-$1_{(1-12)}$ and FITC-Rak-2 (see Experimental Section, Methods 4 and 6 for synthesis), following which confocal microscopy was used to determine whether uptake of the fluorescently labeled peptides occurred. The compounds were incubated at the same concentrations in living cells, followed by two washings and addition of sulforhodamine B (SRB) (to a concentration of 1 μM) which labels the medium surrounding the cells, but is excluded from live cells. Further details of the microscopy can be found in the Experimental Section. Confocal microscopy was done on an Olympus FV-1000/IX-81 confocal inverted microscope, using a 40×/NA = 0.9 air objective. The excitation wavelengths were 488 nm for the FITC and either 561 or 594 nm for the SRB. The FITC was detected using a 505−545 nm emission filter, while either a 585−615 nm barrier filter or a 610 nm low pass filter was used to detect SRB. Regardless of the excitation wavelength and emission bands used for SRB, the SRB channel contained no signal from FITC. FITC and SRB were imaged sequentially, to avoid crosstalk between the two fluorescence channels. A nonconfocal DIC transmitted light image was also acquired. The DUP-1 and control images shown in Figure 8 were acquired under the same acquisition conditions. The Rak-2 images were acquired under similar conditions. However, since they were acquired on a different day, a separate set of control images were acquired under identical conditions as the Rak-2 images, with similar negative results (data not shown).

All image processing was done in ImageJ.[39] The SRB images were thresholded using the Otsu automatic thresholding option within ImageJ. The resulting binary image was used to find the edges of the cells. The edges which were found are highlighted on the FTIC and SRB images. The dark areas are definitely within the cells, as these are areas from which SRB is excluded.

We verified that these areas are separated from the edge of the cell by checking the adjacent z-planes (data not shown). The images were processed as follows. The 12 bit images were divided by 16 and then converted to 8-bit images without scaling, so as to avoid various autoscale functions which distorted the relative strength of the images. A rolling ball filter of radius 100 was used to remove slowly varying background. A global linear contrast stretch was applied to all of the images from the FITC channel. This last step was only done to make the images easily visible. An identical contrast stretch was applied the control images. The upper limit of the stretch was high enough that the signals did not reach saturation. A confocal plane which definitely passed within the cell was chosen. The edges of the cell at this level were found from the corresponding SRB image, and these edges were overlaid onto the FITC channel images to show the cell locations.

The DIC images are shown to indicate normal cell morphology, and to confirm cell location. The edges do not correspond exactly to the DIC images of the cells, because the DIC images are not confocal.

Figure 8 shows a typical result. The top row is the FITC channel, the middle row contains the SRB images, in which the cell interiors are dark, and the bottom row are the corresponding DIC images. The DIC images are shown in Figure 8 to indicate normal cell morphology, and to confirm cell location. The edges found from the SRB images do not correspond exactly to the DIC images of the cells, because the DIC images are not confocal. (see Experimental Section, Method 9).

The important point of Figure 8, is that under similar acquisition conditions, there is easily visible incorporation of FITC-Rak-2 and (FITC-^{13}Lys)DUP-$1_{(1-12)}$, while the control cells show some auto fluorescence, but much lower signals than the cells which were incubated with the labeled peptides.

These results correlate and complement the results obtained by displacement of (FITC-^{13}Lys)DUP-$1_{(1-12)}$ by non-fluorescent Rak-2 shown in Figure 7. There, we have already shown that non fluorescent Rak-2 displaces the (FITC-^{13}Lys)DUP-$1_{(1-12)}$peptide, while a nonfluorescent nonrelated peptide 15-mer does not compete with the DUP-1. Our microscopy results also correlate with and complement those obtained for (FITC-^{13}Lys)DUP-$1_{(1-12)}$ in a previous work.[12]

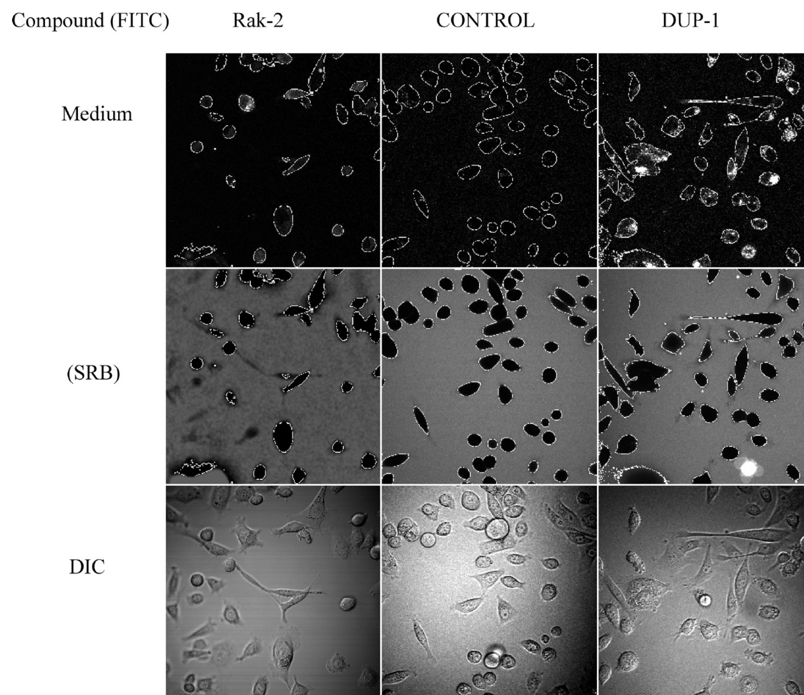

Figure 8. Confocal images of PC-3 cell which were incubated with FITC-Rak-2, (FITC-^{13}Lys)DUP-1$_{(1-12)}$ or nothing (controls), as indicated. The cell culture medium contained about 1 μM SRB. Top row: FITC channel, which images the labeled peptides. Middle row: SRB images, which shows the location of the interior of the cells. Bottom row: nonconfocal DIC images. The cell boundaries, as found from the SRB images by edge detection, are indicated in white on the FITC and SRB images (top two rows).

Overall we have demonstrated that the molecule Rak-2 identified in the screening has a high affinity for PC-3 cells as compared to control DUP-1.

Conclusions

We have established a live-cell screening technique for testing mixtures of ligands. The technique is based on the design of a synthetic phage-like system composed of a monodispersed 2 μm microsphere surrounded by a PEG-spacer, fluorescent labels and ligands directly synthesized or conjugated on their surface. The robustness of the synthesis was demonstrated by HPLC and MS analysis of partially cleaved peptides. To our knowledge, the 2 μm particles used here are the smallest particles ever used in Merrifield synthesis. The interest of these microspheres resides in their subcellular size, which allows their incubation with adherent cells to screen for binding of the ligands tethered to their surface to those cells. An important feature of the use of the microspheres is that multiple ligands can be simultaneously screened in mixtures, by the use of different combinations of fluorescent labels. This will be of great advantage in future HTS using small particles. The testing of a small molecules Ugi-library, together with the subsequent identification of the compound Rak-2 with high affinity for PC-3 cells, as well as the further confirmation of the results using a binding assay on cells in suspension and direct observation of the labeled molecule by confocal microscopy, give us confidence that the concept of synthetic phage-like system can be further developed on the basis of polymeric monodispersed small particles, including direct synthesis on them.

The advantage of the synthetic phage-like system is that nonpeptide molecules can also be synthesized and screened as mixtures, in contrast to the existing phage display strategy, which screens and identifies only peptides. Although there is still a long way to go before this system can be adapted to in vivo high throughput screening, a first step has been taken using relevant cultured cells, and validated by an independent binding assay and microscopy. The small molecules identified in this work are now under further studies aimed at elucidating the mechanism of their interaction with both cancer and somatic cells.

Experimental Section

General. FACS was conducted using FACS Calibur flow cytometer (Becton-Dickinson) equipped with a 488-nm argon laser; results and data acquisition were analyzed using software CellQuest and a Power Macintosh G4. Ugi reactions were conducted using a professional microwave "Initiator" from Biotage. HPLC was performed on Waters Gradient System equipped with a 717-Plus autosampler, a Waters 600 intelligent pump, and a Waters 996-photodiode array detector; the system was piloted with Millenium software from Waters. Selected wavelengths for chromatograms were 220 and 254 nm. Mobile phases were (A) H_2O (0.1% TFA) and (B) MeCN (0.08% TFA). Separation condition for analytical analysis was as follows: Column Chromolith Performance (from Merck) RP-18e 100−4.6 nm, gradient H_20/MeCN, *gradient a* [A/B] 1 min [100/0], 1−8 min [0/100], 8−11 min [0/100], 11.1 min [100/0]; flow = 6 mL/min.

Preparative HPLC was performed on a Gilson's HPLC System with a Gilson 321 pump, and a Gilson 155 UV/vis HPLC detector with option two wavelengths, manual injector and fraction collector Gilson FC 204; the system was piloted with Unipoint LC system software from Gilson. Mobile phases were (A) H_2O (0.1% TFA) and (B) MeCN (0.08%

TFA). Separation condition for preparative analysis was as follows: separation was conducted using Column (250 × 22 mm) Vydac C18 reversed phase: *gradient b* gradient H_20/MeCN, [A/B] 3 min [100/0], 3−40 min [0/100], 40−45 min [0/100], flow = 30 mL/min. After purification, the purity of compounds was determined using gradient a and was more then 95%. MS analyses was conducted using ESI (electron spray ionization) mass spectrometry on a Q-TOF low-resolution micromass spectrometer (Micromass-Waters, Corp.), HR-MS was conducted using AUTOSPEC-FISSONS VG (Micromass) high-resolution mass spectrometer under DCI (desorption chemical ionization) conditions (CH_4) or high-resolution MS-MALDI-TOF spectra with an Autoflex TOF/TOF instrument (Bruker, Germany). 2, 5-Dihydroxybenzoic acid (DBH) was used as a matrix.

^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker DPX-300 and advanced DMX-600 spectrometers. Chemical shifts are in ppm relative to TMS internal standard or relative to solvent resonance.

Microscopy was conducted using a confocal inverted microscope Olympus FV-1000/IX-81, with 40×/NA = 0.9 air objective. All image processing was done in ImageJ.[39]

All solvents were analytically pure grade and were used without further purification. HPLC water was purchased from Beit Dekel Ltd., Israel. HPLC acetonitrile, *N*,*N*-dicyclohexylcarbodiimide (DCC), and triflouroacetic acid (TFA) were purchased from BioLab Ltd., Jerusalem, Israel. RPMI 1640 with glutamine, 0.25% trypsin and EDTA solution, and penicillin-streptomycin solution were purchased from Biological Industries Ltd., Haemek, Israel. Foetal Boving Serum (FCS) was purchased from Invitrogen Ltd. Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), tri-isopropylsilane, and Rink amide-AM resin were purchased from GL Biochem (Shanghai/China) Ltd. Boc-21-amino-hexaoxaheneic osanoic acid (Boc-$(PEG)_6$-COOH) (code no. BA19206) was purchased from NeoMPS, Inc. Anhydride glycolic, ethylenediaminetetraacetic acid (EDTA), and *N*-methyl morpholine (NMM) were purchased from Acros Organic, Israel. 1,2-Ethanedithiol (EDT), thioanisole, phenol flouresecein isothiocyanate (FITC), and malachite Green were purchased from Sigma Aldrich. Sulforhodamine B was purchased from New Biotechnology Ltd., Israel.

Cell Culture. The human prostate carcinoma cell line PC-3 (from American Type Culture Collection, Manassas, VA) were generously donated by Prof. Zelig Eshhar from Weizman Institute, Rehovot/Israel. Cells were cultivated at 37 °C in a 5% CO_2 incubator (Water-Jacketed, US Autoflow Automatic CO_2 Incubator manufactured by NuAire, Inc.) in RPMI 1640 with Glutamine supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FCS.

Method 1: Functionalization of Microspheres. Aminomethylpolystyrene. Chlorobenzyl microspheres obtained as previously reported[24] (3 g, 10 mmol) were suspended in 95 mL of dimethylformamide (DMF) and potassium phthalimide (1.85 g, 10 mmol) was added. The mixture was stirred at 50 °C for 18 h. The microspheres were washed with DMF (3 × 100 mL), MeOH (3 × 100 mL), H_2O (3 × 100 mL), and MeOH (3 × 100 mL) and dried in vacuo overnight. IR (KBr): 1716 cm^{-1} (CO), 1772 cm^{-1} (CO). The diameter and size distribution of the microspheres dispersed in aqueous phase were determined using a micrometer particle analyzer. The microspheres showed a single population of 2.1 ± 0.2 μm. (see Figure a in Supporting Information) The microspheres were treated overnight with hydrazine hydrate (12 mL) in refluxing EtOH (50 mL) overnight at 100 °C. The suspension was cooled to room temperature, washed with EtOH (3 × 100 mL), 1 M NaOH (3 × 100 mL), H_2O (3 × 100 mL), EtOH (3 × 100 mL), and dried in vacuo to give 1.6 g of the microspheres. After the mixture was dried overnight in vacuum, ninhydrin test indicated for 1.12 mmol NH_2/g microspheres. The IR (KBr) bands at 1716 and 1772 cm^{-1} were absent, and the main peak was at 2924 cm^{-1} (CH_2).

Introduction of Different FITC Levels on Microspheres. A stock solution of FITC (0.67 mM, 2.61 mg in 10 mL DMF) was diluted to six different concentrations in DMF: 672, 134, 40.2, 26.8, 2.01, and 0.201 nM. One milliliter of each of the 6 solutions and TEA (0.14 mmol) were added to six portions of microspheres (0.06 gr, 0.0672 mmol) in a 1.5 mL tube. The mixtures were shaken for 3 h at rt. Microspheres were washed with DMF (×3), iPrOH (×3), and EtOH (×3) (solvent removed by centrifuge). The theoretical maximal labeling of microspheres can be calculated from the total quantity of FITC in the different reactions. Thus, levels of 0.0003%, 0.003%, 0.04%, 0.06%, 0.2%, and 1% mmol were generated. Typical FACS analysis is disclosed in Figure 3a.

Introduction of Magnetic Susceptibility and Pegylation of Microspheres. FITC-labeled microspheres (60 mg, 0.0672 mmol; 1.12 mmol amine/g) were washed and suspended in 1 mL $NaHCO_3$ (0.1 M). Magnetic nanospheres obtained as previously reported (3 mg in 280 μL water) were added to the microspheres suspension. The reaction was stirred for 10 min. The microspheres were washed with $NaHCO_3$ (1 mL × 5) and water (1 mL × 5). The new amine loading of the microspheres was determined by Fmoc-Gly test that gave 0.48 mmol/g. Microspheres (0.06 g, 0.03 mmol) were washed with DMF (1 mL × 3) and used for the next step. Boc-$(PEG)_6$-COOH (136.6 mg, 0.3 mmol), BOP (132.7 mg, 0.3 mmol), and HOBt (46 mg, 0.3 mmol) were dissolved in DMF and added to the microspheres in a 1.5 mL tube. DIEA (60 μL, 0.3 mmol) was added, and the mixture was shaken overnight. Microspheres were washed alternately with DMF and *i*PrOH (1 mL × 3). Boc groups were cleaved as usually using TFA. A solution of Dde-OH (11 mg, 0.06 mmol) in 200 μL of DMF was added to microspheres (60 mg, 0.03 mmol), and the reaction was shaken for 2 h. Microspheres were washed alternately with *i*PrOH and THF/DCM (1 mL × 3).

Protection of FITC Groups by Tritylation. A stock solution of Trt-Cl (540, 100 mg, 3.6 mmol) and DIEA (62 μL, 3.6 mmol) in DCM (3 mL) was added to microspheres and reacted overnight with shaking. The solution was discarded, and a fresh identical solution was added and reacted overnight with shaking. Microspheres were washed alternately with DMF and THF/DCM (1 mL × 3). Hydrazine hydrate (0.8 mL) in DMF (5 mL) was added to the microspheres, and the reaction was shaken for 1 h. The microspheres were washed alternately with *i*PrOH (3 × 1 mL) and DMF (3 × 1 mL). Microspheres

were used for peptide synthesis or conjugation to Ugi molecules through a diglycolic arm.

Introduction of Rink Linker into Microspheres. Fmoc-Rink linker (165 mg, 0.0306 mmol) and Fmoc-Gly (81.8 mg, 0.2754 mmol) were mixed to obtain a 9:1 mol mixture (10 equiv excess overall) in DMF (0.6 mL), BOP (135.3 mg, 0.306 mmol), HOBt (46.8 mg, 0.306 mmol), and NMM (67.2 μL, 0.612 mmol) were added to microspheres (60 mg, 0.03 mmol). The reaction was shaken for 2 h. The microspheres were washed alternately with DMF (3 × 1 mL) and *i*PrOH (3 × 1 mL). Fmoc test gave 0.3 mmol/g microspheres. Finally, a solution of 20% piperidine in DMF was added to the 1.5 mL tube containing microspheres, and the reaction was shaken at rt for 20 min. The microspheres were washed with DMF (3 × 1 mL) and *i*-PrOH (3 × 1 mL) and used directly for peptide synthesis or conjugation to diglycolic acid.

Introduction of Diglycolic Acid. Diglycolic anhydride (35 mg, 0.3 mmol) and DMAP (4 mg, 0.3 mmol) were dissolved in DMF and added to a 1.5 mL tube containing unprotected microspheres (0.06 g, 0.03 mmol). Then, DIEA (60 μL, 0.3 mmol) was added, and the mixture was stirred overnight. The microspheres were washed alternately with DMF (3 × 1 mL) and *i*PrOH (3 × 1 mL). Malachite Green test shows positive result as green colored microspheres. The miscro-spheres were directly used for conjugation to Ugi molecules.

Method 2: General Procedure for Peptide Synthesis on Microspheres. Microspheres with Fmoc-Rink linker in 1 mL of DMF (0.2 g 0.048 mmol) were uniformly dispensed into 10 wells of a 96 wells polypropylene Pall microplate with 0.45 μm filters (see Figure b in Supporting Information). A solution of 20% piperidine in DMF was then added, and the microplate was stirred with an orbital shaker at rt for 20 min. The microspheres were washed alternately with DMF (0.2 mL × 3) and *i*PrOH (0.2 mL × 3) and used directly for the peptide synthesis using Fmoc chemistry. Each well contains the starting unprotected microspheres (0.02 g, 0.0048 mmol), the Fmoc-amino acids corresponding to the appropriate sequence (0.0484 mmol), BOP reagent (0.021 g, 0.0484 mmol), hydroxybenz-triazole (HOBT) (0.007 g, 0.0484 mmol), and *N*-methyl morpholine (10 μL, 0.096 mmol) in DMF. The reaction was stirred at rt for 2 h under orbital stirring, followed by alternate washings with DMF (0.2 mL × 3) and *i*-rOH (0.2 mL × 3). After each synthetic step Fmoc group was deprotected using 20% piperidine in DMF (20 min.). The wells were washed by multipipetor simultaneously with DMF (0.2 mL × 3) and *i*PrOH (0.2 mL × 3). Final peptides were deprotected in 1.5 mL tubes during 2 h at rt using 0.5 mL of a mixture composed of TFA/TIS/H_2O (95%:2.5%:2.5%). Microspheres were centrifuged and washed with THF. The supernatant containing the cleaved peptide was removed to glass vials, evaporated, washed with ether and analyzed using HPLC and HRMS (data available in the Supporting Information). The peptides-microspheres were thoroughly washed with PBS and used in the screening assays.

Physical Properties of Cleaved 15-mer (WGLRALES-RWDRYYF-Amide) 1 Cleaved from Microspheres. HPLC (gradient a): R_t = 4.3 min. HR-MS (MALDI) calcd for $C_{96}H_{134}N_{27}O_{22}$: 2017.0191; obt. 2017.022.

Physical Properties of DUP-1 (FRPNRAQDYNTN-Amide) 2 Cleaved from Microspheres. HPLC (gradient a): R_t = 3.1 min. HR-MS (MALDI) calcd for $C_{63}H_{96}N_{23}O_{20}$: 1294.7196; obt. 1494.717.

Method 3: Synthesis of Ugi Small Molecules Library and Their Conjugation to Microspheres. Three M solutions of acid, amine and aldehyde components in dry methanol were freshly prepared; 300 μL of aldehyde and 410 μL of acid component were added to a 1.5 tube containing 410 μL of the amine component. The resulting solution was mixed with vortex for 30 s and allowed to stand for 10 min at room temperature. The solution was added to a 0.5−2 mL microwave tube containing the isocyanide component (122 μL). The tube was degassed, sealed, and placed in the microwave. The sample was irradiated for 10 min at 60 °C in the Initiator and then allowed to cool. The solvent was evaporated, and the residual oil was dissolved in 50 mL of dichloromethane; then 50 mL of 1 M $Na_2S_2O_5$ was added, and the resulting mixture was stirred for 1 h. The organic layer was separated and washed with 1 M $Na_2S_2O_5$ (50 mL × 3), 1 M $KHSO_4$ (50 mL × 3), 1 M $NaHCO_3$ (50 mL × 3), brine (50 mL × 3), and dried over magnesium sulfate; the solvent was removed in vacuum. (Physical data including HNMR, CNMR, HPLC. and HRMS for individual products are available in Supporting Information).

Conjugation of Ugi Library to Microspheres. TFA (1 mL) was added to a flask containing Ugi product (0.04 mmol). The solution was stirred for 1 h at rt. The TFA was removed under reduced pressure; 0.5 mL of DMF was added to dissolve the residual oil, and it was transferred to a 1.5 mL tube containing microspheres (20 mg, 0.01 mmol). BOP reagent (0.04 mmol, 20 mg) and TEA (0.08 mmol, 33 μL) were added to microspheres mixture and the suspension was stirred overnight at rt. Microspheres were washed alternately with DMF and *i*PrOH (1 mL × 4). Malachite green test gave negative result.[37] A mixture of TFA/TIS/H_2O (95%: 2.5%: 2.5%) was added to a 1.5 mL tube containing the micro-spheres. After they were stirred for 1 h, the microspheres were centrifuged and were washed with DCM (1mL × 3), DMF (1 mL × 3), *i*PrOH (1 mL × 3), and EtOH (1 mL × 3). The TFA from the cleavage was evaporated, and the remaining oil was washed with ether, dissolved in MeOH and analyzed with analytical HPLC, QTOF MS, and HR-MALDI-TOF. (Analyses are summarized in table a in Supporting Information).

Method 4: Peptide Synthesis for Validation Studies. DUP-1 Peptide (FRPNRAQDYNTN-Amide) 2. The peptide was synthesized by solid-phase Fmoc strategy; the synthesis was performed using 0.25 mmol Rink Amide-AM resin (0.52 mmol/g loading). The resin was introduced in the ABI-431A peptide synthesizer, and all Fmoc-amino acids were coupled using the DCC/HOBt protocol from the company (4-fold excess of the Fmoc-amino acid, 90 min per coupling). The peptide was cleaved from the resin with a mixture of TFA/TIS/H_2O (95%: 2.5%: 2.5%) (5 mL) yielding the crude DUP-1 peptide which was precipitated and washed with cold ether (5 mL × 5). The crude DUP-1 (400 mg) was purified by preparative HPLC (gradient b) and lyophilized to give 155 mg of the peptide product as a white solid. HPLC analysis: R_t = 3.12 min (gradient a). MS analysis: HRMS (MALDI) *m/z* calcd for $C_{63}H_{96}N_{23}O_{20}$ 1494.7196; found 1494.715 (see Supporting Information for analyses).

15-mer Peptide (WGLRALESRWDRYYF) 1. The peptide was synthesized exactly as DUP-1 using the appropriate Fmoc-amino acids. The peptide was cleaved from the resin with a mixture of TFA: EDT: thioanisole: phenol: TIS: H_2O (81.5: 2.5:5: 5:1:5) (5 mL) yielding the crude 15-mer peptide which was precipitated and purified as described above. HPLC analysis: R_t = 4.38 min (gradient a). MS analysis: HRMS (MALDI) *m/z* calcd for $C_{96}H_{134}N_{27}O_{22}$ 2017.0191; found 2017.004 (see Supporting Information for analyses).

FITC-^{13}Lys-DUP-1 for Microscopy Studies. To label DUP-1 peptide for FACS and confocal microscopy experiments, the peptide was synthesized exactly as described above but starting the synthesis with Fmoc-Lys(Mtt)-OH at the C-terminal as in previous studies.[12] The first amino acid was manually loaded using BOP reagent (4-fold excess, 2 h). After peptide synthesis in the peptide synthesizer, the resin (0.1 mmol) was taken out of the synthesizer reaction vessel and the Mtt protecting group on the lysine side chain was removed by gentle mixing in 4 mL of a 1:5:94 mixture of TFA/TIS/DCM for two minutes and was followed by removal of the solution by filtration and a resin wash with DCM. This process was repeated six times. The clear TFA solution became yellow when added to the resin and this yellow color was less intense with each subsequent cycle.[40] Ninhydrin test was positive. Then, the resin was swollen with 3 mL DMF. TEA (160 μL, 1 mmol) and FITC (98 mg, 0.25 mmol) were added and the resin was shaken for 2 h in an orbital shaker. Then, the resin was washed alternatively with DMF/iPrOH. Ninhydrin test was negative. Then, the peptide was cleaved, washed and purified as described above. HPLC analysis: R_t = 3.76 min (gradient a). MS analysis: HRMS (MALDI) *m/z* calcd for $C_{90}H_{119}N_{26}O_{26}S_1$ 2011.8504; found 2011.838 (see Supporting Information for analyses).

Method 5: Synthesis of Pegylated RAK-2 for Validation Studies. Trifluoroacetic acid (TFA) (1 mL) was added to a 5 mL round-bottom flask containing 150 mg (0.32 mmol) of RAK-2. The solution was stirred for 1 h at rt. Then, the TFA was removed under reduced pressure and the compound was dried in vacuum for 2 days. Then, 200 μL acetonitrile (CH_3CN) was added to the flask to dissolve the deprotected RAK-2 and a solution of Boc-$(PEG)_6$-OH (143 mg, 0.32 mmol) in 92 μL CH_3CN and BOP (155 mg, 0.352 mmol) were added. TEA (1.4 mmol, 200 μL) was added to the flask, and the solution was stirred for 3 days. Then, CH_3CN was evaporated, and the product was dried under vacuum to give a yellow oil. The residual oil was dissolved in 18 mL of ethyl acetate, washed with a water (10 mL × 4), 1 M $KHSO_4$ (5 mL × 3), 1 M $NaHCO_3$ (5 mL × 3), saturated NaCl (5 mL × 3), and dried over magnesium sulfate. The solvent was removed under vacuum. The obtained oil was dissolved in 5 mL MeOH and 140 mg Dowex 50 WX2-100 ion-exchange resin (0.7 mmol/g) was added to the solution and stirred for 20 min to give 100 mg of pure product (40% yield). This compound was used for validation studies. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.68 (s, 1H, H-3′), 7.42 (m, 1H, H-2′), 6.40 (bs, 1H, H-4′), 5.66 (s, 1H, H-14), 4.12 (m, 2H, H-12), 3.71 (t, 2H, H-18), 3.65 (m, 20H, H-19 to H-21), 3.53 (t, 2H, H-22), 3.31 (t, J = 5.1 Hz, 2H, H-23), 3.2 (m, 2H, H-5), 2.57 (t, J = 6 Hz, 2H, H-17), 1.86 (m, 2H, H-2), 1.62−1.68 (m, 4H, H-2+H-3), 1.44 (s, 9H, H-26), 1.11−1.32 (m, 16H, H-5 to H-10, H-3+H-2+H-4), 0.85 (t, J = 7.2 Hz, 3H, H-11). ^{13}C NMR (50 MHz, $CDCl_3$): δ 171.78 (C-13), 168.90 (C-16), 167.861 (C-15), 156.03 (C-24), 143.36 (C-3′), 143.08 (C-2′), 118.98 (C-1′), 111.04 (C-4′), 70.54 (C-20), 70.39 (C-21, C-22), 70.20 (C-19), 67.04 (C-18), 55.24 (C-14), 48.58 (C-1), 46.11 (C-5), 41.62 (C-12), 40.50 (C-23), 36.47 (C-17), 28.41 (C-26), 22.47 (C-10), 14.01 (C-11), 32.74, 31.59, 29.35, 28.68, 26.77, 25.43, 24.71 (C-9, C-8, C-7, C-6, C-4, C-3, C-2). HPLC analysis: R_t = 5.76 min (gradient a). MS analysis: HRMS (MALDI) *m/z* calcd for $C_{41}H_{72}N_4O_{12}$ 835.5039; found 835.507 (see Supporting Information for analyses).

Method 6: Synthesis of FITC-PEGYLATED RAK-2 for Microscopy Studies. TFA (1 mL) was added to a 5 mL round-bottom flask containing 24 mg (0.03 mmol) of Boc-$(PEG)_6$-RAK-2 (synthesized according to Method 5). The solution was stirred for 1 h at rt. Then, the TFA was removed under reduced pressure. The oil was dissolved in 1.5 mL of DMF containing FITC (24 mg, 0.06 mmol), and TEA (15 μL, 0.1 mmol) was added to the solution under stirring for 3 h. The solution was diluted to 10 mL with methanol and the crude product was purified by preparative HPLC (gradient b) to yield the pure product as a yellow solid (16 mg, 50% yield). HPLC analysis: R_t = 5.11 min (gradient a). MS analysis: HRMS (MALDI) *m/z* calcd for $C_{57}H_{76}N_5O_{15}S_1$ 1102.5053; found 1102.506 (see Supporting Information for analyses).

Method 7: General Procedure for Flow-Cytometry Screening of Microsphere Mixtures. Individual microsphere-ligands were counted using a hemacytometer for a better distribution of the ratios in the set-mixtures. A mean of 5 × 105 microspheres of individual ligands per ml were necessary for each well. Thus the different ligand-microspheres were mixed to obtain the corresponding mixture sets. The different sets were dispersed in PBS in FACS tubes, and analyzed by FACS to evaluate their exact initial ratio. Then, 1% BSA was added to the suspensions, which were added in triplicates onto a six-well plate containing PC-3 cells seeded to 75% confluence and where incubated for 30 min at 370 C. Subsequently, the cells were washed with cold PBS (1 mL x10) and lysed using a solution of 0.3 M NaOH for 7 min. The remaining microspheres were centrifuged at 5700 rpm (4 min) and washed three times with buffer PBS/2% SDS. Microspheres were suspended on buffer PBS/2% SDS and were shaken for 3 h. The ratio between the different populations was measured again by FACS analysis and each compound was ranked using eq 1. Experiments were repeated 3 times.

Method 8: General Procedure for Hit Validation by a Cell Based Flow Cytometry Assay. PC-3 cells were grown in the incubator to 70%-80% confluence for 3 days at 370C and 5% CO_2. Then, cells were washed with 3 mL PBS and detached with 5 mL of 1 mM EDTA in PBS for 40 min in the incubator. Mixtures were transferred to tubes and centrifuged for 8 min at 1200 rpm. The cells were counted, resuspended at a density of 10^6 cells/mL in RPMI (without FCS), and centrifuged for 5 min at 1200 rpm, and the supernatant was discarded. Cells were placed in 1.5 mL tubes. In parallel, unlabeled DUP-1, 15-mer and pegylated Rak-2 were dissolved in fresh RPMI containing 1% BSA at different concentrations

(from 50 nM to 0.6 mM). 250 μL of each concentration were transferred to the 1.5 mL tubes containing the cells and preincubated for 40 min at 37 °C in the orbital shaker. Then, 250 μL of a 2 μM solution of FITC-labeled DUP-1 in 1% BSA was added to each tube and incubated for 10 min. The cells were transferred to FACS tubes and analyzed by flow cytometry without further processing. The measurement stopped when 20000 gated events were counted. The geometric means of fluorescence in channel FL-1 of the gated cells were obtained using the Cell-Quest software. Mean values ± S.E.M., $n = 3-5$.

Method 9: Cell Binding and Penetration Validation of Rak-2 as Compared to DUP-1 Using Microscopy. PC-3 cells were trypsinized, counted and cultured subconfluently (4 × 105 cells per dish) onto 35 mm glass-bottom culture dish (MatTek Co., Ashland, MA) for 24 h. On the day of experiment, the medium (RPMI 1640) was discarded and replaced by 2 mL of fresh medium. FITC-13Lys-DUP-1 or FITC-Rak-2 and sulforhodamine B were dissolved in RPMI-1640 (without FCS) at 100 μM. Twenty microliters of each compound was added to the cells to reach a final concentration of 1 μM in the medium. Cells are incubated for 1 h. Then, the cells were washed with fresh medium (without FCS) (5 × 2 mL). For measurement of fluorescence, 2 mL medium was added to the cells, and they were immediately transferred to the microscope. Then, 100 μM (20 μL) of sulforhodamine B were added to the dish and the cells were analyzed by confocal laser scanning microscopy. The analysis of cells was conducted at 400× magnification along their z-axis at 2 μm intervals.

Acknowledgment. We are indebted to The Marcus Center of Medicinal Chemistry, to the Israel-USA Binational Science Foundation (BSF 1998378) and to the AFIRST Israel/France (programs of the French Ministry of Foreign Affairs and the Israeli Ministry of Sciences). We wish to thank also Mr. Uri Karo for expert advise and help in flow-cytometry, to Dr. Hugo Gotlieb and Dr. Rachel Persky for structural analysis, and to Dr. Tamara Byk for expert advise in FACS, cell culture, and critical reading of the manuscript.

Supporting Information Available. Physical characterization of microspheres, physical properties of **DUP-1**, physical properties of Ugi molecules, and full NMR spectra of UGI compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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CC900156Z