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Identification of Two Novel, Potent, Low-Liability Antinociceptive Compounds from the Direct *In Vivo* Screening of a Large Mixture-Based Combinatorial Library

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Abstract. Synthetic combinatorial methods now make it practical to readily produce hundreds of thousands of individual compounds, but it is clearly impractical to screen each separately *in vivo*. We theorized that the direct *in vivo* testing of mixture-based combinatorial libraries during the discovery phase would enable the identification of novel individual compounds with desirable antinociceptive profiles while simultaneously eliminating many compounds with poor absorption, distribution, metabolism, or pharmacokinetic properties. The TPI 1346 small-molecule combinatorial library is grouped in 120 mixtures derived from 26 functionalities at the first three positions and 42 functionalities at the fourth position of a pyrrolidine bis-cyclic guanidine core scaffold, totaling 738,192 compounds. These 120 mixtures were screened *in vivo* using the mouse 55°C warm water tail-withdrawal assay to identify mixtures producing antinociception. From these data, two fully defined individual compounds (TPI 1818-101 and TPI 1818-109) were synthesized. These were examined for antinociceptive, respiratory, locomotor, and conditioned place preference effects. The tail-withdrawal assay consistently demonstrated distinctly active mixtures with analgesic activity that was blocked by pretreatment with the non-selective opioid antagonist, naloxone. Based on these results, synthesis and testing of TPI 1818-101 and 1818-109 demonstrated a dose-dependent antinociceptive effect three to five times greater than morphine that was antagonized by mu- or mu- and kappa-opioid receptor selective antagonists, respectively. Neither 1818-101 nor 1818-109 produced significant respiratory depression, hyperlocomotion, or conditioned place preference. Large, highly diverse mixture-based libraries can be screened directly *in vivo* to identify individual compounds, potentially accelerating the development of promising therapeutics.

KEY WORDS: analgesia; *in vivo*; mixture-based libraries; opioid; testing.

INTRODUCTION

Opioid agonists such as morphine are still the "gold standard" for producing a relief of pain (antinociception), but their clinical use is complicated by significant side effects, including respiratory distress, slowing of intestinal motility, tolerance, and abuse (1), spurring the search for novel opioid analgesics with fewer liabilities of use.

Mixture-based synthetic combinatorial libraries are effective tools for generating novel lead compounds in a fraction of the time and cost of equivalent individual compound arrays (see (2,3) for review). These include large libraries of peptides, peptidomimetics, and heterocycles. Such mixture-based combinatorial libraries may be composed of hundreds of thousands of different compounds (4–9). Importantly, it is impractical for the vast majority of laboratories to screen such large numbers of individual compounds, and the harsh reality is that the majority of drug candidates lack desirable drug-like properties at later stages of testing, resulting in a high rate of attrition in the traditional drug discovery process (10).

To circumvent the limitations of existing screening methods, we tested samples from a mixture-based combinatorial library directly *in vivo* for analgesic properties. Guiding this objective, we initiated our study using the scaffold ranking technique (3). Five small-molecule combinatorial libraries in our collection, TPI 1343, 1344, 1345, 1346, and

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ABBREVIATIONS: BBB, Blood–brain barrier; CNS, Central nervous system; CPP, Conditioned place preference; DAMGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; DOR, Delta-opioid receptor; DPDPE, [D-Pen^2,Phe^4,D-Pen^5]enkephalin; i.c.v., Intracerebroventricular; i.p., Intraperitoneal; KOR, Kappa-opioid receptor; MOR, Mu-opioid receptor; nor-BNI, Norbinaltorphimine; s.c., Subcutaneous; U50,488, (±)-*trans*-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; U69,593, (+)-(5α,7α,8β)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide.

1347, were tested as five single mixture-based samples each made up of 738,192 compounds (Fig. 1a). The most active of these sample mixtures in analgesic assays, the 1346 series, was then analyzed using the positional scan approach (11,12) to determine the most active antinociceptive mixtures in the library. The 1346 library is built around a core pyrrolidine bis-cyclic guanidine scaffold (Fig. 1a), with 26 different functionalities at each of the first three diversity positions and 42 functionalities at the fourth position (Table I), resulting in 120 mixtures in this library, varying from 17,576 to 28,392 compounds each, for a total of 738,192 compounds. The most active mixtures in the 1346 series were then used to guide the synthesis of compounds defined in two positions, the 1802 series, and subsequently determine the synthesis of two individual compounds, the 1818 series.

We hypothesize that the direct *in vivo* screening of mixture-based combinatorial libraries will yield therapeutically useful individual analgesics while simultaneously decreasing the failure rate inherent in the traditional drug discovery process. To this end, individual compounds were compared to morphine in a mouse assay of antinociception, the 55°C warm water tail-withdrawal test, while also evaluated for effects on respiration rate, locomotor activity, and conditioned place preference as models for respiratory distress and substance abuse.

MATERIALS AND METHODS

Synthesis of the Libraries TPI 1343, 1344, 1345, 1346, and 1347, with Notes Regarding the TPI 1346 Library

Small-molecule mixture-based libraries 1343 to 1347 were all derived from the same resin bound *N*-acylated peptide 1 (Fig. 1a) as described earlier (13) utilizing the libraries from libraries approach (8,14). Briefly, the five scaffolds share the same R group functionalities (Table I), but differ in the structure of the core scaffold (Fig. 1a). The R1 through R3 functionalities are derived from 26 amino acids and the R4 functionalities are derived from 42 carboxylic acids such that each of the five libraries contains 738,192 ($26 \times 26 \times 26 \times 42$) compounds systematically arranged into 120 ($26+26+26+42$) mixture samples. Utilizing the scaffold ranking method (3), a single sample was made for each library that contained an approximately equal molar mixture of all 738,192 compounds in that library.

The synthesis of the pyrrolidine bis-cyclic guanidine library, 1346, is described in Fig. 1a and elsewhere (13). Notably, this library has been used by our group to identify individual compounds with antimicrobial activity against drug-resistant Gram-positive pathogens (13) as well as affinity for the mu-opioid receptor (MOR) (3). Briefly, the first 26 samples (Table I and Fig. 1a) permit the assessment of the activity of the 26 different functionalities used at the R1 position. In these 26 mixtures, each of the samples has a fixed functionality at the R1 position and an equal molar mixture of the 26 functionalities at R2, 26 functionalities at R3, and 42 functionalities at R4. For example, sample 1 contains an equal molar mixture of 28,392 ($26 \times 26 \times 42$) compounds all fixed with *S*-methyl at the R1 position, whereas sample 2 contains an equal molar mixture of 28,392 compounds all fixed with *S*-benzyl at the R1 position. In this way, samples 1 to 26 scan the

first position by fixing each of the 26 samples with a different functionality at the R1 position. The next 26 samples (samples 27 to 52) assess the functionalities at R2 by fixing the R2 position and having equal molar mixtures at the other three positions. Likewise, samples 53 to 78 assess the R3 functionalities, and samples 79 to 120 assess the R4 functionalities.

Synthesis of the TPI 1802 Library

To confirm the activities of the constituent groups in combination, we used an iterative approach and synthesized 25 mixtures. The 25 mixtures were synthesized using the same synthetic scheme described for 1346 in Fig. 1 (13). The 25 mixtures were designed by fixing the R1 and R2 positions with the combination of the top five functionalities in R1 and R2 from the *in vivo* screening of 1346. The R3 and R4 positions in these samples contain equal molar mixtures of the 26 R3 functionalities and 42 R4 functionalities present in 1346 (Table II). Each sample in the 1802 series contains an equal molar mixture of 1,092 compounds (26×42).

Synthesis of TPI 1818-101 and 1818-109

Based on the data obtained from the *in vivo* screening of 1802 and 1346, we synthesized the individual pyrrolidine bis-cyclic guanidine compounds TPI 1818-101 and 1818-109 (Fig. 1b) utilizing the same synthetic strategy outlined in Fig. 1 for 1346. TPI 1818-101 combines the following functionalities from 1346; R1 functionality 14 *S*-2-hydroxymethyl, R2 functionality 51 *S*-cyclohexyl, R3 78 *R*-cyclohexyl and R4 91 2-ethoxybenzyl, while TPI 1818-109 combines the functionality 14 *S*-2-hydroxymethyl (at the R1 position), functionality 51 *S*-cyclohexyl (at the R2 position), functionality 78 *R*-cyclohexyl (at the R3 position), and functionality 93, 3,4-dichlorobenzyl (at the R4 position).

Animals

All experiments used male C57Bl/6J mice (20–32 g each, Jackson Laboratories, Bar Harbor, ME). Mice were housed four per cage in a temperature-controlled room. Cages were kept in a room with 12-h light/dark cycle with the lights on from 0700 to 1900 hours and food and water available *ad libitum*. All procedures with mice were approved by the local IACUC.

Chemicals

In all assays, TPI compounds were dissolved in 10% dimethylsulfoxide, a concentration that did not produce any detectable behavioral effect. Morphine sulfate, naloxone, beta-funaltrexamine (β-FNA), nor-binaltorphimine (NorBNI), and naltrindole were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.9% sterile saline.

Opioid Receptor Binding to Murine Brain Membranes

Murine brain membranes were prepared as described previously (3). The affinity and selectivity of TPI 1818-101 and TPI 1818-109 were determined by incubating the membranes with radiolabeled ligands and six different concentrations of TPI
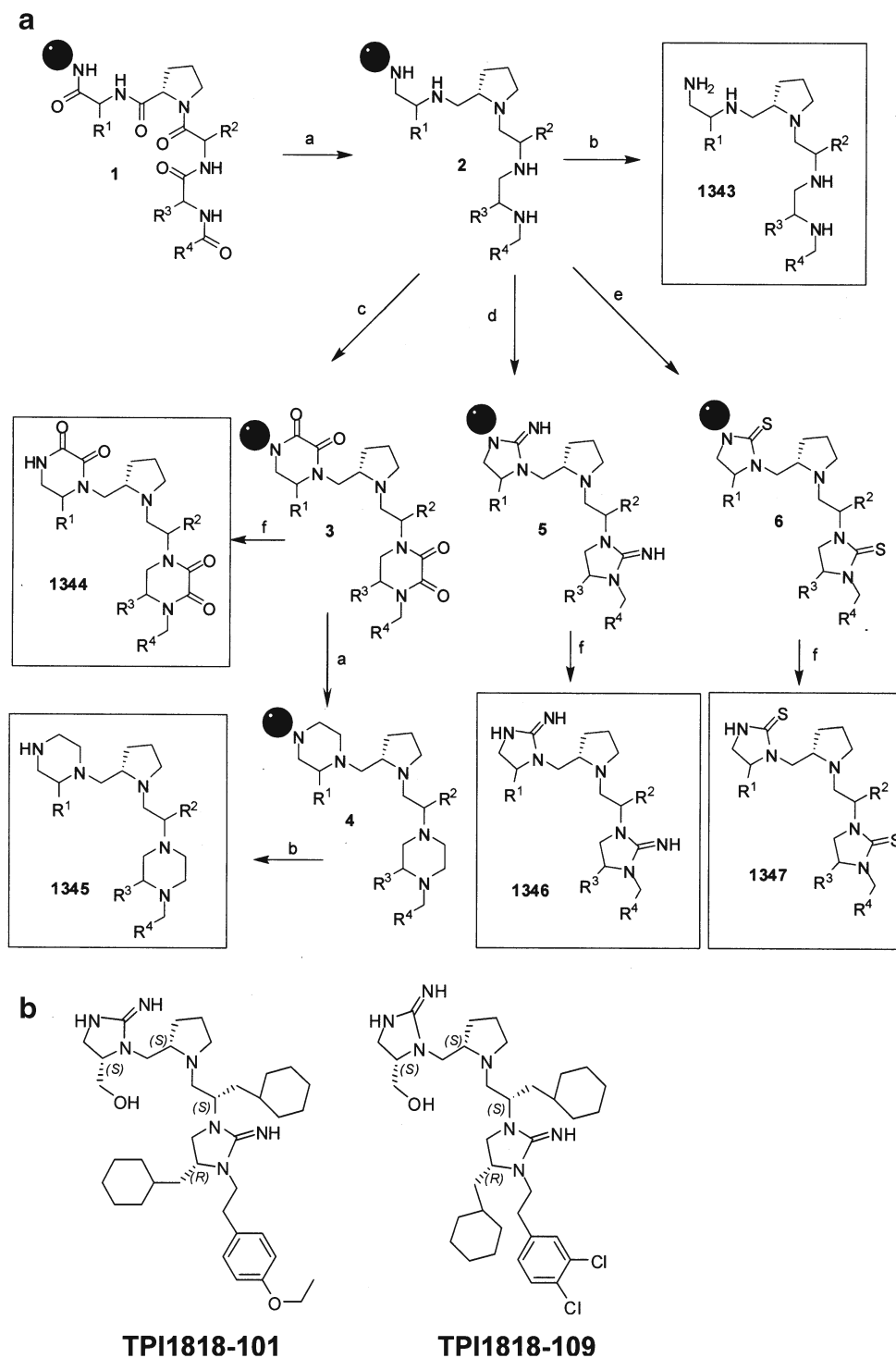
Fig. 1. a Five different synthetic combinatorial libraries (1343-1347) are derived from the same resin bound *N*-acylated tetrapeptide 1. The *N*-acylated tetrapeptide 1 is synthesized on MBHA resin using standard Boc chemistry. **a** Borane-THF 50°C, 72 h followed by Piperidine at 50°C, 24 h. **b** HF/anisole 7 h. **c** Oxalyldiimidazole/DMF, 24 h. **d** CNBr/DCM, 24 h. **e** Thiocarbonyldiimidazole/DCM, 24 h. **f** HF/anisole 1.5 h. Compounds from 1346, 1802, and 1818 libraries all have the same 1346 framework and are synthesized using the same synthetic pathway. **b** Samples 1818-101 (*left*) and 1818-109 (*right*) were synthesized using the synthesis strategy described above. 1818-101 combines the following functionalities from 1346: R1 functionality 14 *S*-2-hydroxymethyl, R2 functionality 51 *S*-cyclohexyl, R3 78 *R*-cyclohexyl and R4 91 2-ethoxybenzyl, while 1818-109 combines the following functionalities from 1346: R1 functionality 14 *S*-2-hydroxymethyl, R2 functionality 51 *S*-cyclohexyl, R3 78 *R*-cyclohexyl, and R4 93 3,4-dichlorobenzyl

Table I. Identities of 1346 Library Functionality Groups: R1, R2, R3, and R4 Correspond to Positions on the Pyrrolidine Bis-cyclic Guanidine Core Scaffold, 1346 (see Fig. 1)

R1	R2	R3	R4	R1,2,3: amino acid R4: carboxylic acid	Functionality
1	27	53		Boc-L-Ala	*S*-methyl
2	28	54		Boc-L-Phe	*S*-benzyl
3	29	55		Boc-Gly	Hydrogen
4	30	56		Boc-L-Ile	*S*-2-butyl
5	31	57		Boc-L-Leu	*S*-isobutyl
6	32	58		Boc-L-Ser(Bzl)	*R*-hydroxymethyl
7	33	59		Boc-L-Thr(Bzl)	(*R,R*)-1-hydroxyethyl
8	34	60		Boc-L-Val	*S*-isopropyl
9	35	61		Boc-L-Tyr(BrZ)	*S*-4-hydroxybenzyl
10	36	62		Boc-D-Ala	*R*-methyl
11	37	63		Boc-D-Phe	*R*-benzyl
12	38	64		Boc-D-Ile	*R*-2-butyl
13	39	65		Boc-D-Leu	*R*-isobutyl
14	40	66		Boc-D-Ser(Bzl)	*S*-hydroxymethyl
15	41	67		Boc-D-Thr(Bzl)	(*S,R*)-1-hydroxyethyl
16	42	68		Boc-D-Val	*R*-isopropyl
17	43	69		Boc-D-Tyr(BrZ)	*R*-4-hydroxybenzyl
18	44	70		Boc-L-Phenylglycine	*S*-phenyl
19	45	71		Boc-L-Norvaline	*S*-propyl
20	46	72		Boc-D-Norvaline	*R*-propyl
21	47	73		Boc-L-Norleucine	*S*-butyl
22	48	74		Boc-D-Norleucine	*R*-butyl
23	49	75		Boc-L-Naphthylalanine	*S*-2-naphthylmethyl
24	50	76		Boc-D-Naphthylalanine	*R*-2-naphthylmethyl
25	51	77		Boc-L-Cyclohexylalanine	*S*-cyclohexyl
26	52	78		Boc-D-Cyclohexylalanine	*R*-cyclohexyl
			79	1-Phenyl-1-cyclopropanecarboxylic acid	(1-Phenyl-cyclopropyl)-methyl
			80	2-Phenylbutyric acid	2-Phenylbutyl
			81	3-Phenylbutyric acid	3-Phenylbutyl
			82	*m*-Tolylacetic acid	*m*-tolylethyl
			83	3-Fluorophenylacetic acid	2-(3-Fluoro-phenyl)-ethyl
			84	3-Bromophenylacetic acid	2-(3-Bromo-phenyl)-ethyl
			85	(α-α-α-Trifluoro-m-Tolyl) acetic acid	2-(3-Trifluoromethyl-phenyl)-ethyl
			86	*p*-Tolylacetic acid	*p*-tolylethyl
			87	4-Fluorophenylacetic acid	2-(4-Fluoro-phenyl)-ethyl
			88	3-Methoxyphenylacetic acid	2-(3-Methoxy-phenyl)-ethyl
			89	4-Bromophenylacetic acid	2-(4-Bromo-phenyl)-ethyl
			90	4-Methoxyphenylacetic acid	2-(4-Methoxy-phenyl)-ethyl
			91	4-Ethoxyphenylacetic acid	2-(4-Ethoxy-phenyl)-ethyl
			92	4-Isobutyl-α-methylphenylacetic acid	2-(4-Isobutyl-phenyl)-propyl
			93	3,4-Dichlorophenylacetic acid	3,4-Dichlorophenethyl
			94	3,5-Bis(Trifluoromethyl)-phenylacetic acid	2-(3,5-bis-trifluoromethyl-phenyl)-ethyl
			95	3-(3,4-Dimethoxyphenyl)-propionic acid	3-(3,4-Dimethoxy-phenyl)-propyl
			96	Phenylacetic acid	Phenethyl
			97	3,4,5-Trimethoxybenzoic acid	3,4,5-Trimethoxy-benzyl
			98	Butyric acid	Butyl
			99	Heptanoic acid	Heptyl
			100	Isobutyric acid	Isobutyl
			101	2-Methylbutyric acid	2-Methylbutyl
			102	Isovaleric acid	3-Methylbutyl
			103	3-Methylvaleric acid	3-Methylpentyl
			104	4-Methylvaleric acid	4-Methylpentyl
			105	*p*-Toluic acid	4-Methyl-benzyl
			106	Cyclopentanecarboxylic acid	Cyclopentyl-methyl
			107	Cyclohexanecarboxylic acid	Cyclohexyl-methyl
			108	Cyclohexylacetic acid	Cyclohexyl-ethyl
			109	Cyclohexanebutyric acid	Cyclohexyl-butyl
			110	Cycloheptanecarboxylic acid	Cycloheptyl-methyl
			111	2-Methylcyclopropanecarboxylic Acid	(2-Methyl-cyclopropyl)-methyl
			112	Cyclobutanecarboxylic Acid	Cyclobutyl-methyl
			113	3-Cyclopentylpropionic Acid	3-Cyclopentyl-propyl
			114	Cyclohexanepropionic Acid	Cyclohexyl-propyl

Table I. (continued)

R1	R2	R3	R4	R1,2,3: amino acid R4: carboxylic acid	Functionality
			115	4-Methyl-1-Cyclohexanecarboxylic Acid	4-Methyl-1-cyclohexyl-methyl
			116	4-*tert*-Butyl-Cyclohexanecarboxylic Acid	4-*tert*-butyl-cyclohexyl-methyl
			117	4-Biphenylacetic Acid	2-Biphenyl-4-yl-ethyl
			118	1-Adamantanecarboxylic Acid	Adamantan-1-yl-methyl
			119	1-Adamantaneacetic Acid	2-Adamantan-1-yl-ethyl
			120	2-Norbornaneacetic Acid	2-Bicyclo[2.2.1]hept-2-yl-ethyl

1818-101 and TPI 1818-109 as described previously (3). Incubation times of 60 min were used for the MOR selective peptide [^{3}H][D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin ([^{3}H] DAMGO) and 120 min for the delta-opioid receptor (DOR) selective peptide [^{3}H][D-Pen^2,Phe^4,D-Pen^5]enkephalin ([^{3}H] DPDPE) and the kappa-opioid receptor (KOR) selective ligand [^{3}H]U69,593 at final concentrations of 0.87, 2, and 2 nM, respectively.

Antinociceptive Testing Using the 55°C Warm Water Tail-Withdrawal Assay

Male C57Bl/6J mice ($n=8$) were used in all antinociceptive testing. A water bath heated to 55°C acted as a nociceptive stimulus with the latency to withdraw the tail taken as the endpoint (15). Mice showing no response within 5 s during the determination of baseline responses were excluded from the experiment. After determining baseline control responses, mice were administered graded doses of morphine or a TPI sample. All compounds were each given as single intraperitoneal (i.p.) injections with tail withdrawal latencies measured 0.5, 1, 2, 3.5, 5, 8, and 24 h post-administration unless otherwise stated. In the receptor selectivity studies, either the MOR-selective antagonist β-FNA (5 mg/kg, i.p.) or the KOR-selective antagonist nor-BNI (10 mg/kg, i.p.) were injected 24 h before TPI sample administration, whereas the DOR-selective antagonist naltrindole (20 mg/kg, i.p.) was administered 20 min prior to agonist administration. A cutoff of 15 s was used to avoid tissue damage; those mice failing to withdraw their tails within this time were assigned a maximal antinociceptive score of 100%.

For scaffold and positional screening studies (1346 and 1802 series results), results are presented as the sum of average responses at each time point across all seven time points tested. For more detailed analysis across time (TPI 1818-101 and 1818-109), antinociception at each time point was calculated as follows: % antinociception = $100 \times$ (test latency − control latency)/(15 − control latency).

Respiratory Effects

Respiration rates were recorded using the automated, computer-controlled Comprehensive Lab Animal Monitoring System (CLAMS) apparatus (Columbus Instruments, Columbus, OH). Male C57Bl/6J mice were placed in closed apparatus cages (23.5×11.5×13 cm) and habituated for a 30-min period. Mice were then administered a graded i.p. dose of morphine, saline, TPI 1818-101, or 1818-109. Follow-ing administration of the compounds, mice were returned to chambers for 90 min with respiration rate (breaths/min) measured in 30-s intervals. Using a pressure transducer built into the enclosed, sealed CLAMS cage, the respiration rate of each occupant mouse was counted. Each time the mouse breathed, the resultant change in pressure was recorded over the 90-min period to reveal the rate in breaths/min.

Locomotor Activity

Locomotor activity of mice was monitored for 90 min following a 5-min delay after administration of vehicle (10% DMSO in saline, 0.9%, i.p.), morphine (10 mg/kg, i.p.), TPI

Table II. Identities of 1802 Library Functionality Groups: R1, R2, R3, and R4 Correspond to Positions on the Pyrrolidine Bis-cyclic Guanidine Core Scaffold, 1346 (see Fig. 1)

1802	R1	R2	R3	R4
1	*R*-benzyl	*S*-isobutyl	X	X
2	*R*-benzyl	*R*-2-butyl	X	X
3	*R*-benzyl	*S*-2-napthylmethyl	X	X
4	*R*-benzyl	*S*-cyclohexyl	X	X
5	*R*-benzyl	*S*-hydroxymethyl	X	X
6	*S*-hydroxymethyl	*S*-isobutyl	X	X
7	*S*-hydroxymethyl	*R*-2-butyl	X	X
8	*S*-hydroxymethyl	*S*-2-napthylmethyl	X	X
9	*S*-hydroxymethyl	*S*-cyclohexyl	X	X
10	*S*-hydroxymethyl	*S*-hydroxymethyl	X	X
11	*R*-butyl	*S*-isobutyl	X	X
12	*R*-butyl	*R*-2-butyl	X	X
13	*R*-butyl	*S*-2-napthylmethyl	X	X
14	*R*-butyl	*S*-cyclohexyl	X	X
15	*R*-butyl	*S*-hydroxymethyl	X	X
16	*R*-cyclohexyl	*S*-isobutyl	X	X
17	*R*-cyclohexyl	*R*-2-butyl	X	X
18	*R*-cyclohexyl	*S*-2-napthylmethyl	X	X
19	*R*-cyclohexyl	*S*-cyclohexyl	X	X
20	*R*-cyclohexyl	*S*-hydroxymethyl	X	X
21	*S*-butyl	*S*-isobutyl	X	X
22	*S*-butyl	*R*-2-butyl	X	X
23	*S*-butyl	*S*-2-napthylmethyl	X	X
24	*S*-butyl	*S*-cyclohexyl	X	X
25	*S*-butyl	*S*-hydroxymethyl	X	X

The 1802 series of samples were designed by fixing the first two positions of the pyrrolidine bis-cyclic guanidine scaffold (1346) based on the results of the *in vivo* screening of the 1346 library. The R3 and R4 positions are equal molar mixtures of the 26 R3 functionalities and 42 R4 functionalities. Each sample in the 1802 series contains an equal molar mixture of 1,092 (26×42) compounds

1818-101 (5 mg/kg, i.p.), or TPI 1818-109 (10 mg/kg, i.p.). The doses of drug used in the locomotor activity assay were selected on the basis of maximal activity observed in the antinociceptive testing experiments. Locomotor activity was captured and digitized, with the distance traveled calculated by a Noldus Ethovision Pro locomotor tracking system. Before testing, mice were initially administered vehicle and confined to the locomotor chambers for 60 min to habituate the animals to the apparatus.

Conditioned Place Preference

Male C57Bl/6J mice were conditioned using a protocol similar to the previously established biased cocaine-conditioned place preference paradigm (16–18). The testing apparatus (San Diego Instruments, San Diego, CA) consisted of four identical compartmentalized boxes divided into two equal-sized outer compartments (25×25×25 cm) with distinct visual (horizontal or vertical alternating black and white lines, 1.5 cm wide) or tactile cues (smooth or mottled flooring), each joined to a small central section (8.5×25×25 cm) accessed through a single doorway (3 cm high). Each unit is fitted with infrared beams, the breaking of which allows an automated measure of the time animals spend in each chamber. Note that the biased place-conditioning protocol involves administration of the test sample (here, morphine, vehicle, TPI 1818-101, or 1818-109) to mice that were then restricted to the outer compartment opposite of their original preference response in an initial preconditioning preference test. The biased conditioned place preference protocol is a sensitive and consistent indicator of conditioned drug reward equivalent to alternative methods (e.g., the counterbalanced design; see (19) for a review).

On day 1, and prior to the start of conditioning, mice were placed inside the apparatus and allowed to move freely between all three chambers for a 30-min testing period. Initial preference was determined by measuring which of the two outer chambers, the left or the right, the mouse spent more time. On day 2, each animal was injected (i.p.) with saline (0.9%) 10 min prior to confinement in the initially preferred outer compartment for 45 min. After six more hours spent in the home cage, each mouse was then administered vehicle (10% DMSO in 0.9% saline), morphine (10 mg/kg), TPI 1818-101 (5 mg/kg), or TPI 1818-109 (10 mg/kg), 10 min prior to confinement in the appropriate opposite outer chamber for 45 min. The doses of drug used in the place conditioning assay were selected on the basis of maximal activity observed in the antinociceptive testing experiments. This conditioning cycle was repeated a second time on day 3, which has been demonstrated to be effective in producing morphine-conditioned place preference (CPP) (20). On day 4, the mice were again allowed to run freely between all three chambers to determine the final drug-conditioned place preference. Data are plotted as the difference in time spent in the eventual drug- and vehicle-paired compartments. By convention, the initial bias represents a negative value, whereas a positive value represents a conditioned preference for the drug-paired side. Note that conditioned place aversion, where animals avoid the drug-paired compartment, is also detectable under this method when animals spend significantly more time in the initially preferred side. However, this was not demonstrated in this study under any conditions.

Statistical Analysis

IC$_{50}$ values were calculated by least squares fit to a logarithm-probit analysis. The K_i values of TPI 1818-101 and TPI 1818-109 were calculated from the equation $K_i = IC_{50}/(1 + S)$, where S = (concentration of radioligand)/ (K_D of radioligand) by Prism 5.0 software (GraphPad Software, LaJolla, CA). All dose–response lines were analyzed by regression and D$_{50}$ (dose producing 50% antinociception) values and 95% confidence limits determined using each individual data point by Prism 5.0 software. Student's t tests comparing baseline and post-treatment tail-withdrawal latencies were used to determine statistical significance for all tail-withdrawal data. Student's t tests were also used to determine statistical significance of summarized antinociceptive effects of each individual sample against the same effect of morphine. Ranking of combined library samples (see Fig. 2) was performed with one-way ANOVA, with significant effects further analyzed by Tukey's multiple comparison post hoc testing using Prism 5.0 software. Data for respiration and locomotor effects were analyzed with one-way ANOVA using Prism 5.0, with significant effects further analyzed by Tukey's HSD post hoc testing. Data for conditioned place preference experiments were analyzed with two-way ANOVA using SPSS 14.0 (SPSS, Chicago, IL), with analyses examining the main effect of conditioned place preference phase (e.g., pre- or post-conditioning) and the interaction of drug pretreatment (morphine, TPI-1818-101 or 1818-109, or vehicle). Significant effects were further analyzed using Tukey's HSD post hoc testing. All data are presented as mean ± SEM, with significance set at $p \leq 0.05$.

RESULTS

Ranking the Five Scaffolds for Antinociceptive Potency

The antinociceptive activity of the five library scaffolds (1343, 1344, 1345, 1346, and 1347; see Fig. 1) was evaluated. While the systematic arrangement of each library into 120 (26+ 26+26+42) mixture samples significantly reduced the screening
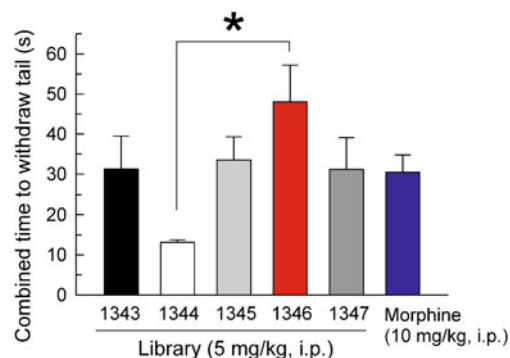

Fig. 2. Scaffold ranking of libraries 1343 (*black bar*), 1344 (*white bar*), 1345 (*gray bar*), 1346 (*red bar*), and 1347 (*dark gray bar*) by *in vivo* antinociception response in the mouse 55°C warm water tail-withdrawal test. Morphine (10 mg/kg, i.p., *right side*) was tested as a positive control. Data represent average (±SEM) summed tail-withdrawal latencies calculated by taking the sum of the average tail withdrawal latencies for each animal from each time point over a 24-h period. All samples were administered at a dose of 5 mg/kg, i.p. *Bars*, 8 mice each. *Significantly different, $p<0.05$, Tukey's test

Fig. 3. Positional scan screening of 1346-series OXXX samples: summed antinociception produced by 1346 series samples measured in the mouse 55°C warm water tail-withdrawal test across a 24-h period. **a** 1346 defined at position 1 (*red bars*). **b** 1346 defined at position 2 (*blue bars*). **c** 1346 defined at position 3 (*yellow bars*). **d** 1346 defined at position 4 (*green bars*). The combined time to withdraw tails (s; *y*-axis) was calculated by taking the sum of the average tail-withdrawal latencies from each time point. Samples (*x*-axis; see Table I for full identities) were administered at a dose of 5 mg/kg i.p. for testing. Functionalities of key samples are described in simplified form for convenience; see Table I for complete descriptions. Morphine (10 mg/kg, i.p., *far right bar*) was tested as a positive control. Data represent average (±SEM) summed tail-withdrawal latencies calculated by taking the sum of the average tail-withdrawal latencies for each animal from each time point over a 24-h period. Samples administered at dose of 5 mg/kg, i.p. *Bars*=8 mice each. *Significantly greater than morphine effect, $p<0.05$, Student's *t* test


set size (from 3,690,960 individual samples (5×738,192) to 600 mixture-based samples (5×120)), this number of samples was still somewhat prohibitive. Therefore, to prioritize the complete library to be screened, we employed the scaffold ranking method (3). For this method, we assayed each library represented by a single sample containing an approximately equal molar mixture of all 738,192 compounds contained in the particular library. Like morphine, administration of all five samples significantly increased the combined time mice required to remove their tails from a noxious stimulus of 55°C warm water (Fig. 2). However, the sample representing the 1346 library (Fig. 2, red bar) produced the greatest magnitude of activity ($F_{(5,42)}$=2.7, $p<0.05$), establishing this scaffold for further evaluation in this study.

Positional Scanning of the 1346 Series Library OXXX Defined Samples *In Vivo*

Utilizing the positional scanning format (11,12), the antinociceptive activity of each of the 120 mixture-based samples comprising the 1346 series library was evaluated after administration (5 mg/kg, i.p.) with the 55°C warm water tail-withdrawal assay (Fig. 3). The combined time mice demonstrated to withdraw their tail was summed over the seven time points examined for each sample tested and is reported by substitution position (Fig. 3a–d). Notably, a number of samples defined at each substitution position significantly increased the combined tail-withdrawal time as compared to the effect of morphine (10 mg/kg, i.p.; black bar on right of each graph in Fig. 3).

Iteration of the 1346 Series Results: 1802 Series Library OOXX Defined Samples *In Vivo*

Partial iteration of the 1346 library was accomplished with the 1802 library, which shares the scaffold with the 1346 library but which consists of compounds defined at the R1 and R2 substitution positions. *In vivo* evaluation of the antinociceptive activity of the 25 mixture-based samples in this library was performed with the 55°C warm water tail-withdrawal assay along with morphine as a reference standard (Fig. 4). While four of five samples containing D-Phe in the first substitution position demonstrated statistically greater combined tail withdrawal times than morphine, the greatest magnitude of response was
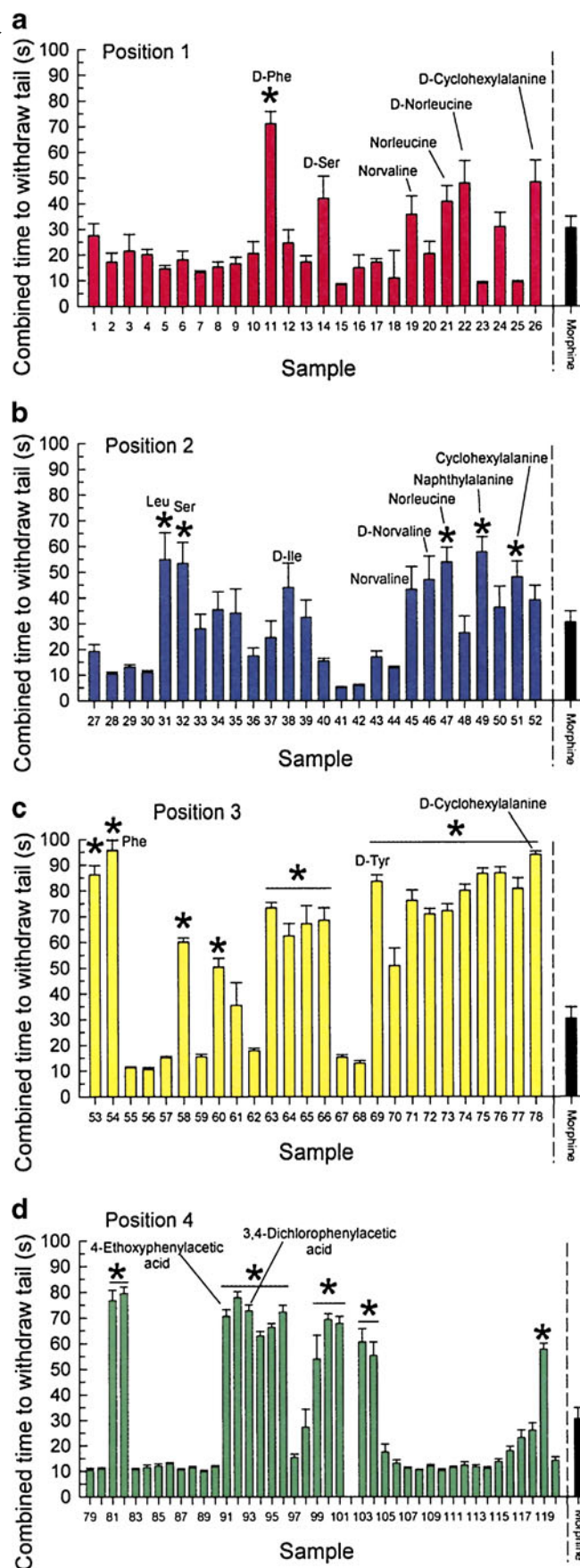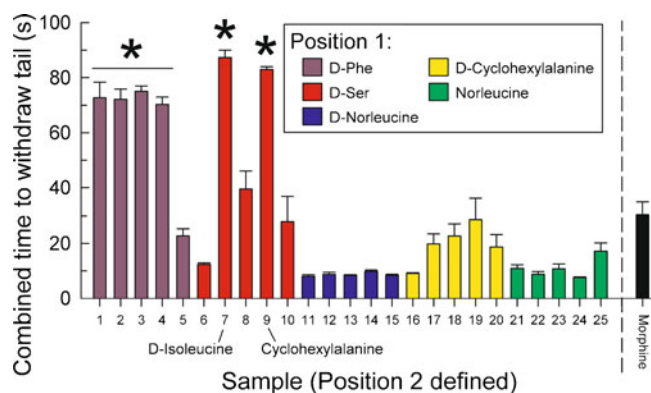
Fig. 4. Iterative screening of OOXX samples using *in vivo* antinociception: summed antinociception produced by 1802 series samples (5 mg/kg, i.p.) measured in the mouse 55°C warm water tail-withdrawal test across a 24-h testing period. Samples were defined in the first position (*inset key*) and second position (see Table II) identified on the *x*-axis. The combined time to withdraw tails (s; *y*-axis) was calculated by taking the sum of the average tail withdrawal latencies from each time point. Functionalities of key samples are described in simplified form for convenience; see Table II for complete descriptions. Note the inclusion of morphine (10 mg/kg, i.p., *far right bar*) as a positive control. Data represent average (±SEM) summed tail-withdrawal latencies calculated by taking the sum of the average tail withdrawal latencies for each animal from each time point over a 24-h period. Samples were administered at a dose of 5 mg/kg, i.p. *Bars*, 8 mice each. *Significantly greater than morphine effect, $p < 0.05$, Student's *t* test

demonstrated by samples defined in the first position with D-Ser and in the second position with D-isoleucine or cyclohexylalanine. In contrast, no other samples showed greater activity than morphine (Fig. 4).

Testing of Individual Compounds TPI 1818-101 and 1818-109

Based on the data obtained from the *in vivo* screening of 1346 and 1802, we synthesized a pair of individual pyrrolidine bis-cyclic guanidine compounds, TPI 1818-101 and TPI 1818-109 (Fig. 1b), to determine if individual compounds resulting from the analysis of the data collected from the 1346 and 1802 series samples might demonstrate favorable antinociceptive activity. Using radioligand competition binding assays, TPI 1818-101 and TPI 1818-109 demonstrated poor affinity for the multiple opioid receptors as established by the K_i values for the inhibition of MOR-, DOR-, and KOR binding (Table III).

However, both individual small-molecule compounds, TPI 1818-101 and 1818-109, produced a time- and dose-dependent antinociception after i.p. administration in the mouse 55°C warm water tail-withdrawal assay (Fig. 5a, b).

TPI 1818-101 and 1818-109 produced maximal antinociception 60 min after administration of 5 mg/kg of TPI 1818-101 and 10 mg/kg of TPI 1818-109 that lasted up to 5 and 2 h, respectively. The antinociceptive D_{50} values of TPI 1818-101 and 1818-109 (and 95% confidence intervals) were 1.09 (0.06–9.63) mg/kg and 4.63 (2.81–6.40) mg/kg, respectively (Fig. 5c). In comparison, the antinociceptive D_{50} value of morphine sulfate was 11.24 (2.4–20.1) mg/kg.

Receptor selectivity of both TPI 1818-101 and TPI 1818-109 after i.p. administration was determined by pretreating mice with selective opioid receptor antagonists prior to testing in the 55°C warm water tail-withdrawal assay as shown in Fig. 6a, b. Opioid receptor antagonists were administered at doses and in sufficient advance of TPI compounds to insure inhibition of only one type of opioid receptor. The pretreatment of mice with the MOR-selective antagonist β-FNA before the administration of TPI 1818-101 or 1818-109 significantly reduced the antinociceptive effects of both compounds. In contrast, pretreatment with the KOR-selective antagonist nor-BNI did not alter the antinociception produced by TPI 1818-101 (Fig. 6a), but significantly reduced the antinociception induced by TPI 1818-109 (Fig. 6b). Pretreatment with the DOR-selective antagonist naltrindole had no significant effect on antinociception produced by either compound. These results suggest that TPI 1818-101 produced antinociception through the MOR, whereas TPI 1818-109 induced antinociception through activity mediated by both the MOR and KOR.

The effects of TPI 1818-101 and TPI 1818-109 on respiration rate and locomotor activity were assessed. Mice were administered TPI compounds at doses corresponding to maximal effects observed in antinociceptive dose–response testing. Additional mice were treated with vehicle (10% DMSO in 0.9% sterile saline, i.p.) and morphine (10 mg/kg, i.p.) for comparison. As expected of a MOR agonist, this dose of morphine significantly reduced the respiration rate 25.1% ($F_{(3,26)} = 3.73$, $p < 0.05$; Fig. 7a) while increasing distance traveled fourfold ($F_{(3,36)} = 34.77$, $p < 0.01$; Fig. 7b) from the response of saline-treated mice. However, neither TPI 1818-101 (5 mg/kg, i.p.) or TPI 1818-109 (10 mg/kg, i.p.) significantly altered the respiration rate or locomotor activity from the saline-induced response.

The rewarding or aversive effects of TPI 1818-101 and TPI 1818-109 were determined by place conditioning mice with either compound at doses corresponding to the maximal effect observed in antinociceptive dose response testing. Morphine (10 mg/kg, i.p.) place conditioning over 2 days (Fig. 8a) resulted in a significant preference for the morphine-paired side ($F_{(3,49)} = 6.05$, $p < 0.01$; Fig 8b, white bars), but place conditioning with vehicle, TPI 1818-101, or TPI 1818-109

Table III. K_i Values for the Inhibition of Mu-, Delta-, and Kappa-opioid Receptor Binding to Murine Brain Membrane Protein by TPI 1818-101 and TPI 1818-109

	K_i (nM) ± SEM		
Radiolabeled ligands	[3H]DAMGO (MOR)	[3H]DPDPE (DOR)	[3H]U69,593 (KOR)
TPI 1818-101	2,790±741	1,930±474	3,910±1,640
TPI 1818-109	9,470±1,210	2,210±932	7,750±3,830

Murine brain membrane protein was incubated with six different concentrations of each TPI compound in the presence of 0.87 nM [3H] DAMGO, 2 nM [3H]DPDPE, or 2 nM [3H]U69,593 in 50 mM Tris–HCl, pH 7.5, at 25°C as described in "MATERIALS AND METHODS." Data are listed as the mean K_i values ± SEM from at least three experiments
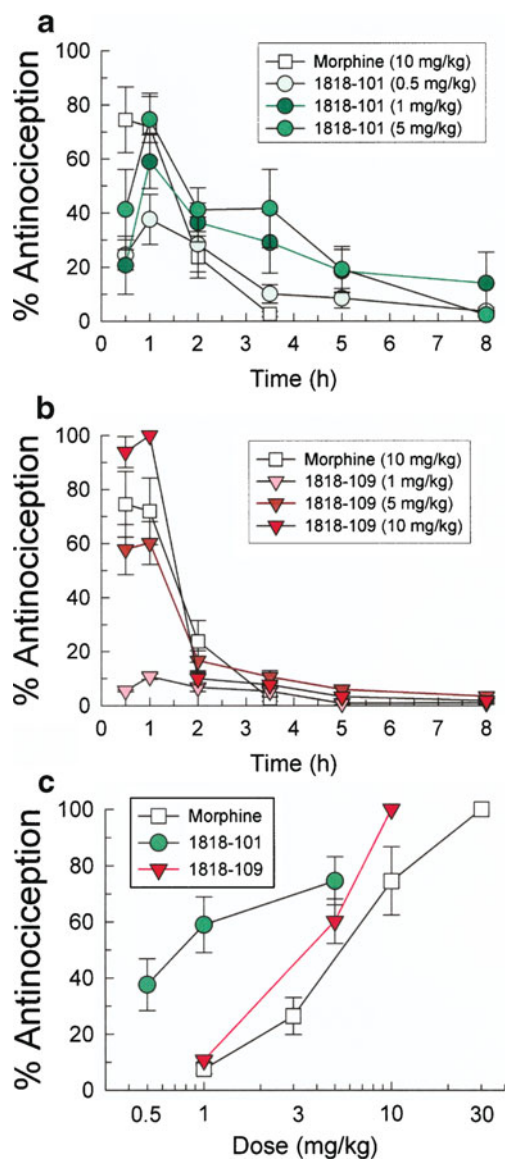
Fig. 5. Antinociception produced by TPI-1818-101 and 1818-109 in the mouse 55°C warm water tail-withdrawal test is dose- and time-dependent. **a** Time course of TPI 1818-101 antinociception. **b** Time course of TPI 1818-109 antinociception. **c** Dose–response lines of morphine, TPI 1818-101, and TPI 1818-109 given by i.p. injection 30 min before testing in the mouse 55°C tail-withdrawal assay. *Points,* 8–10 mice

produced no such conditioned-place preference (Fig. 8b, filled bars). Notably, the TPI compounds also did not demonstrate aversive properties as place conditioning with either TPI 1818-101 or TPI 1818-109 produced final place preference responses that were both statistically similar to matching pre-conditioning responses (Fig. 8b, open bars) and the final saline-conditioned place preference response.

DISCUSSION

Agonists activating MOR are generally regarded as the "gold standard" for analgesics (1). However, activation of the MOR produces significant well-established clinical liabilities, notably respiratory depression (21), the slowing of intestinal

motility (22), modulation of the immune response (23), antinociceptive tolerance (24,25), and physical and psychological dependence (24,26). Likewise, while agonists selective for both the DOR and KOR also induce potent antinociception, they also produce significant side effects (1), notably respiratory depression (27) and seizures (28) by DOR agonists, and diuresis (29) and dysphoria (30) by KOR agonists. The prevalence of deleterious side effects by the established opioid analgesic agents has encouraged the search for compounds that produce analgesia with fewer or no clinical liabilities.

We have pioneered two of the key methodologies currently utilized to generate and screen very large numbers of low-molecular-weight synthetic compounds. The first is commonly known as the "tea bag" approach (31), and the second is the practical synthesis and deconvolution of mixture-based combinatorial libraries made up of millions of compounds (2–5,8,14,32,33). These approaches enable millions of acyclic and heterocyclic compounds, as well as tens of millions to billions of peptides, to be prepared and screened in readily accessible formats in a fraction of the time and cost of equivalent individual compound arrays.

Two primary approaches are used to prepare and screen the large numbers of compounds produced by combinatorial libraries. These are: (1) the massive parallel synthesis and screening of large individual compound arrays and (2) the generation and screening of extremely large focused, but chemically narrower, mixture-based libraries. Once individual
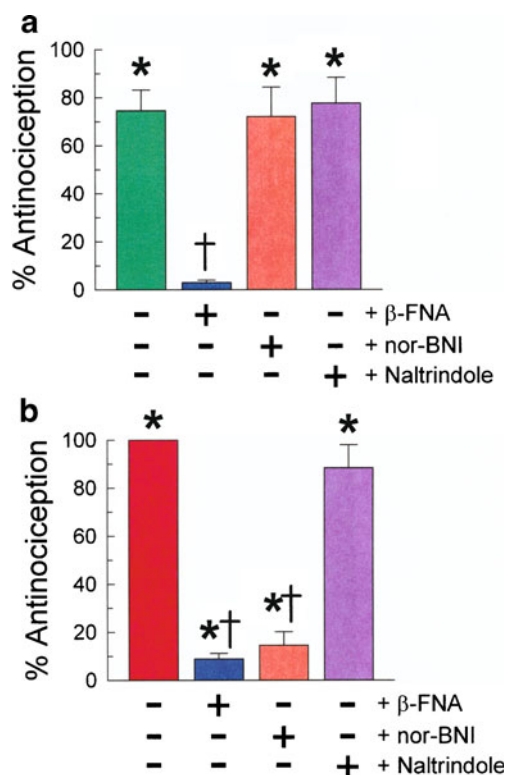

Fig. 6. Antinociceptive effects of **a** TPI 1818-101 (5 mg/kg, i.p., −30 min) and **b** TPI 1818-109 (10 mg/kg, i.p., −30 min) in mice with or without pretreatment with β-FNA (5 mg/kg, i.p., −24 h), nor-BNI (10 mg/kg, i.p., −24 h), or naltrindole (20 mg/kg, i.p., −15 min). *Bars,* 8–10 mice. *Significantly different from baseline latency. †Significantly less than matching 1818 compound alone, $p < 0.05$
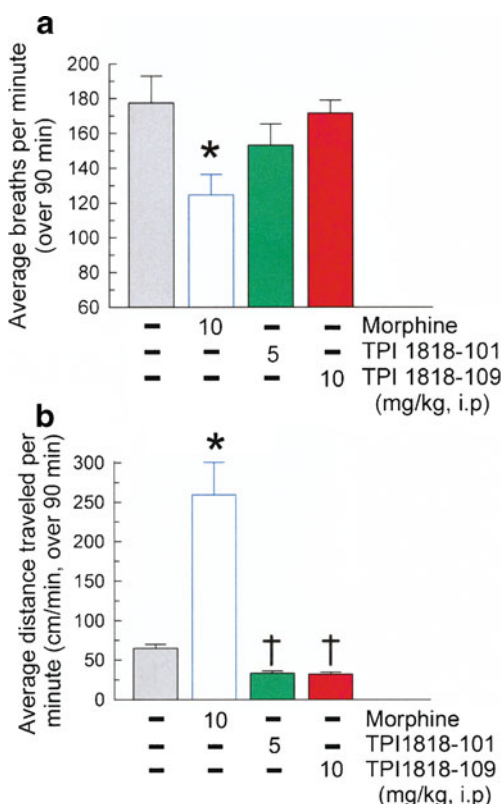
Fig. 7. TPI 1818-101 and 1818-109 do not induce morphine-like liabilities of use. Mice were administered vehicle (10% DMSO in 0.9% saline, *gray bar*), morphine (10 mg/kg, *white bar*), TPI 1818-101 (5 mg/kg, *green bar*), or TPI 1818-109 (10 mg/kg, *red bar*) and activity determined for 90 min on **a** respiration rate and **b** locomotor effects. TPI-treated mice do not show significant **a** slowed respiratory function (breaths/min) or **b** excess locomotor activity (cm/min) when compared to vehicle-treated mice, as produced by morphine. *Each bar* represents eight mice. *Significant difference from the vehicle treated control group ($p < 0.05$ for A, $p < 0.01$ for B). †Significant difference from morphine treated mice ($p < 0.01$)

compounds are identified as therapeutically useful, their general target activities are often further improved by classic structure–activity relationship approaches prior to testing *in vivo*. However, it remains impractical for the majority of academic and small research organizations to make and screen such large numbers of compounds. Moreover, most compounds initially found to be promising are rejected at the *in vivo* stage of the discovery process. Indeed, the inverse of this concept is also true: an exciting aspect of the present data stems from the promising *in vivo* activity of both TPI 1818-101 and TPI 1818-109 despite the poor opioid receptor affinity demonstrated by *in vitro* testing, which might otherwise have lessened enthusiasm for further examination of these compounds.

To circumvent the limitations of existing *in vitro* screening methods, we sought to directly test samples from a mixture-based combinatorial library *in vivo* for analgesic properties, thereby simultaneously increasing the evaluation of compounds while decreasing the failure rate inherent in the traditional drug discovery process (10). The *in vivo* screening and deconvolution of mixture-based combinatorial libraries has previously yielded therapeutically useful individual compounds in a cost-effective

manner. Previous screening of a library of 400 separate mixtures each of 130,321 hexapeptides by monitoring blood pressure and heart rate in rats (5) demonstrated the feasibility of this approach, identifying useful therapeutic candidates while simultaneously eliminating compounds with poor absorption, distribution, metabolism, and pharmacokinetic properties. Likewise, a mixture-based combinatorial library containing all D-amino acid hexapeptides was used previously to identify a novel agonist for the MOR, Ac-rfwink-NH$_2$ (34), which induced a potent, long-lasting antinociception in mice that was blocked by administration of the opioid antagonist naloxone (34).

While the present data again suggest the success of this approach, it is notable that the strategy is not without limitations. The evaluation of the 134X scaffolds and the mixture-based samples of the 1346 and 1802 libraries may be susceptible to false negative outcomes resulting from the inclusion of opioid receptor antagonists that reduce or mask the potency of opioid receptor agonists in the tested mixture. It is notable that opioid receptor antagonists have been identified from positional scanning and screening of synthetic peptide combinatorial libraries (11). While the end result of the present search for low-liability analgesic agents was successful, additional detailed screening of libraries based
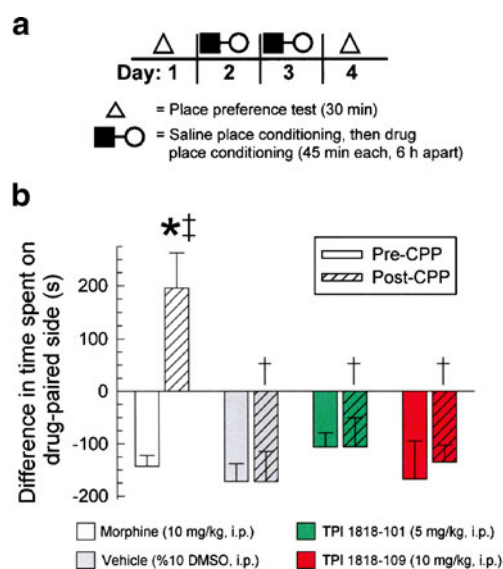

Fig. 8. TPI 1818-101 and 1818-109 do not induce morphine-like conditioned place preference. **a** Schematic of testing protocol. **b** Summary graph of place preference results. Initial preference for either the left or right compartment of the CPP system was determined on day 1 of testing for each group of mice (*solid bars*) and plotted as the difference in time spent on the eventual drug-paired side (s). On days 2 and 3, individual mice were place conditioned in the initially preferred chamber with vehicle (0.9% saline) followed 6 h later by place conditioning in the initially non-preferred chamber side with morphine (*white bars*), vehicle (10% DMSO in 0.9% saline, *gray bars*), TPI 1818-101 (*green bars*), or 1818-109 (*red bars*). On the fourth day of testing, mice were again tested to determine final place preference (*striped bars*). Final conditioned place preference did not significantly differ from the initial preference for mice treated with vehicle, TPI 1818-101, or 1818-109, but was significant for morphine. *Each bar* represents 8–16 mice. *Significant difference from the initial preference ($p < 0.05$). †Significant difference from final preference of morphine treated mice ($p < 0.05$). ‡Significant difference from saline place-conditioned animals ($p < 0.05$)

on other scaffolds can be expected to provide enhanced antinociceptive agents. It should be noted that the identification of selective, novel opioid receptor antagonists is itself of potential therapeutic value, especially given findings of the potential antidepressant and anxiolytic effects of KOR antagonists (35). While not within the scope of the present study, the lack of antinociceptive response from select mixture samples in the 1346 library suggests the strong likelihood of finding potential antagonists using this approach.

The potent antinociceptive effect of the individual compounds TPI 1818-101 and 1818-109 are conspicuous for their accompanying lack of respiratory depression, locomotor, or place conditioning effects. A mechanism accounting for this pattern of activity is unknown. It is possible that these effects are due to non-opioid-mediated interactions induced by the TPI compounds. Supporting this, TPI 1818-101 and TPI 1818-109 showed poor affinity for the MOR, with both compounds demonstrating micromolar affinity for the three opioid receptors in competition binding assays in the present study. Likewise, an earlier study of the 1346 library mixture-based samples with competition opioid radioligand binding assays found poor to low affinity for the mu opioid receptor (3). Moreover, it is important to note that the samples found to have higher affinity for the mu-opioid receptor in the previous binding study did not correlate well with the active compounds identified directly using the present *in vivo* testing approach. However, this possibility is depreciated by the present findings that the opioid-receptor selective antagonists β-FNA and nor-BNI antagonized the antinociceptive effects of the TPI compounds *in vivo*. Along a similar line of reasoning, it remains possible that the antinociceptive effects observed *in vivo* are due to a metabolite of either TPI compound tested. As these metabolites might not be expected to occur in the environment of an *in vitro* binding assay, it is possible that potential metabolites of TPI 1818-101 and 1818-109 could account for the opioid receptor activity, not unlike morphine-6β-glucuronide demonstrating higher potency at the MOR receptor over that of the parent substrate, morphine (36). Alternatively, it is also possible that the administration of TPI 1818-101 or 1818-109 may produce antinociception through the induced release of an endogenous opioid, such as β-endorphin or an enkephalin. A number of compounds induce the release of endorphins to produce opioid-receptor-mediated antinociception, such as cannabinoids (37) or the endothelin A receptor antagonist BQ-123 (38). However, as the endogenous opioids produce all the detrimental effects of opioid agonists (such as the respiratory depression induced by β-endorphin) (39), it is unclear why TPI 1818-101 or 1818-109 would produce opioid-mediated antinociception without the opioid-mediated liabilities. A simpler alternative possibility is that TPI 1818-101 and 1818-109 may not cross the blood–brain barrier, thereby restricting the activity of these compounds to the periphery after intraperitoneal administration. Peripherally restricted opioid agonists such as *N*-methylmorphine have been shown to produce relief from some types of pain (40,41) and have been generally proposed as novel opioid analgesics with fewer liabilities of use, as they would be expected to lack many of the detrimental clinical effects mediated by mechanisms in the CNS (42). However, additional work to confirm the inability of TPI 1818-101 or 1818-109 to cross the blood–brain barrier, or to

produce respiratory and psychostimulant effects after central administration, is required to determine the mechanism producing the effects of these compounds.

CONCLUSION

The *in vivo* screening of the 120 mixture samples comprising the 738,192 individual compounds making up the 1346 library yielded mixtures that produced robust opioid-mediated antinociception in the 55°C warm water tail-withdrawal assay. Deconvolution of these antinociceptive results resulted in the synthesis of individual compounds, TPI 1818-101 and TPI 1818-109. These compounds produced a dose-dependent antinociception equivalent to morphine that was blocked by MOR- and KOR-selective antagonists, respectively. Importantly, neither of these compounds demonstrated respiratory distress or psychostimulant effects, suggesting they might produce analgesia without many of the clinical liabilities presented by morphine. Moreover, the work demonstrates the validity of using mixture-based combinatorial libraries and deconvolution of *in vivo* testing results to identify individual compounds with enhanced potential therapeutic value.

ACKNOWLEDGMENTS

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