Synthesis and Biological Evaluation of 1-[1-(2-Benzo[b]thienyl)cyclohexyl]piperidine Homologues at Dopamine-Uptake and Phencyclidine- and σ-Binding Sites

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Piperidine and cyclohexyl ring homologues of the high-affinity dopamine (DA) uptake inhibitor 1-[1-(2-benzo[b]thienyl)cyclohexyl]piperidine (BTCP, 3) were each prepared in four steps from the appropriate cycloalkanones. These compounds were tested for their ability to displace [3H]BTCP and [3H]cocaine and to inhibit [3H]DA uptake in rat striatal homogenates. The ratios IC₅₀([3H]cocaine)/IC₅₀([3H]BTCP) ranged from 62 for BTCP to 1.5 for 1-[2-(benzo[b]thienyl)cyclopentyl]homopiperidine (17); cocaine gave a ratio of 0.6. This indicates that BTCP is the most selective of all the compounds tested for sites labeled by [3H]BTCP whereas cocaine is most selective for sites labeled by [3H]cocaine. The wide differences in the relative abilities of these compounds to displace [3H]BTCP and [3H]cocaine suggests that these two radioligands are labeling different sites on the transporter. In general, the compounds structurally related to BTCP exhibited greater selectivity for sites labeled by [3H]BTCP. However, several of the BTCP-related derivatives showed greater (compared with BTCP and cocaine) ability to displace [3H]cocaine. Most notably, 1-[1-(2-benzo[b]thienyl)cyclohexyl]pyrrolidine (7) exhibited a 3.4-fold greater affinity for these sites compared with BTCP and a 9-fold greater affinity at these sites than cocaine. Most of the BTCP homologues displayed greater ability to inhibit [3H]DA uptake in rat forebrain synaptosomes than cocaine. BTCP and 7 were the most potent of all the compounds tested in terms of their ability to inhibit uptake of [3H]DA. IC₅₀ ratios for [3H]cocaine binding/[3H]DA uptake ranged from 0.47 for 1-[1-(2-benzo[b]thienyl)cyclopentyl]homopiperidine (11) to 8.8 for 1-(2-benzo[b]thienyl)cyclohexylamine (4). The importance of this ratio remains unclear in terms of identification of potential cocaine antagonists. As for BTCP, all of the compounds tested showed Kᵢ values >10,000 nM for displacement of [3H]cocaine from rat brain homogenates. These compounds were able to displace the highly selective σ receptor probe [3H]-(+)-pentazocine from guinea pig brain homogenates with Kᵢ values ranging from 125 to 9170 nM. The significance of their σ-binding activity in light of their dopaminergic properties is unclear. The diverse binding properties of these compounds at the DA-uptake site and their spectrum of inhibitory activities for [3H]DA uptake identifies them as a useful base for the development of subtype selective probes at this site. These compounds will allow further study of the structure and function of the "cocaine" receptor as well as the development of potential cocaine antagonists.

Introduction

Cocaine, a major drug of abuse in the United States, exerts its pharmacological effects through interaction with several neurotransmitter systems. It has been shown to inhibit with equal potency the uptake of 5-hydroxytryptamine (5HT), norepinephrine (NE), and dopamine (DA). However, the reinforcing and behavioral effects of cocaine are mediated primarily through its interaction with DA-transporter sites in the central nervous system (CNS). Cocaine exerts its effects by markedly increasing levels of synaptic DA via a robust inhibition of its reuptake into presynaptic nerve terminals. Similarly, some of the behavioral effects of the abused drug phencyclidine (PCP, 1) (Chart I) have also been attributed to its interactions with DA transporters. Modification or substitution of the aromatic ring of PCP was shown to change its relative potencies at DA-uptake and PCP-binding sites. For example, incorporation of a thienyl ring gave TCP (2) (Chart I), which exhibited increased affinity for PCP sites and a corresponding decrease in affinity at DA-uptake sites. In contrast, substitution with a benzo[b]thienyl ring gave BTCP (3, Chart I), which showed a considerable reduction in affinity at PCP-binding sites and a large increase in affinity at DA-uptake sites. Comparison of the behavioral effects of BTCP with cocaine, PCP, and MK-801 indicated that its behavioral activity correlates best with inhibition of...
Chemistry

affinity at PCP and synaptosomes. of these compounds to inhibit the uptake of \([3H]DA\) into structure, it was necessary to evaluate their in vitro binding in good yield, 4, 17, sequence of reaction of cyclohexanone with 2-benzo[bl- and potential cocaine antagonists by measuring the ability of cyclohexyl and piperidine ring homologues patent literature. By comparison, structure-activity requirements (SAR) of BTCP at the receptor complex have been studied in great detail, and of PCP and related compounds at the PCP/NMDA of the highly selective DA-uptake inhibitor BTCP (1) were synthesized (Table I) to determine the SAR requirements of the binding of these two ring systems of BTCP to the DA transporter (i.e. "cocaine receptor") and to possibly identify subtype-selective agents and cocaine antagonists. The results (Table II) suggest that \([3H]cocaine and \([3H]BTCP\ are binding to different sites on the DA transporter since the relative abilities of the compounds to displace these radioligands show a great deal of variation.

In the final dialkylation step (Scheme I) for formation of the target compounds 3 and 7–14, treatment of the crude reaction product mixture with AcO followed by acid/base extraction proved to be a very useful method for the separation of the desired products (tertiary amine) from unreacted (primary amine) starting material and secondary amine byproducts. As expected from steric and thermodynamic considerations, the formation of 8, 11, and 14 (homopiperidines) from the corresponding primary amines proceeded in low (12–26%) yield.

Results and Discussion

A series of cyclohexyl and piperidine ring homologues of the highly selective DA-uptake inhibitor BTCP (1) were synthesized (Table I) to determine the SAR requirements of the binding of these two ring systems of BTCP to the DA transporter (i.e. "cocaine receptor") and to possibly identify subtype-selective agents and cocaine antagonists. The results (Table II) suggest that \([3H]cocaine and \([3H]BTCP\ are binding to different sites on the DA transporter since the relative abilities of the compounds to displace these radioligands show a great deal of variation.

In all of the compounds tested (Table II), BTCP (3) displayed the highest affinity for sites labeled by \([3H]BTCP\ (283-fold greater than cocaine), indicating that the ring sizes of the other compounds are nonoptimal for binding. However, in terms of binding to sites labeled by \([6H]cocaine, the cyclohexylpiperidolines 7 displayed a 3-fold improvement in binding over BTCP and a 9-fold greater affinity for this site over cocaine. Similarly, BTCP derivatives containing the cycloalkyl ring/cyclic tertiary amine ring sizes 6/5, 5/5, and 6/7 all showed improved affinity (over BTCP) for sites labeled by \([3H]cocaine. All (except 14) of the compounds containing a cyclic tertiary amine, as opposed to a primary amine, exhibited greater affinity for the site labeled by \([3H]cocaine compared with cocaine itself.

Compounds 4, 17, and 20 containing a primary amine instead of the piperidine ring showed a 17–93-fold reduced affinity for sites labeled by \([6H]cocaine and a 190–630-fold reduced affinity for sites labeled by \([3H]BTCP, suggesting that replacement of the tertiary with a primary amine is detrimental to the binding interaction at these sites. Interestingly, 4, 17, and 20 proved to be much more effective at inhibiting uptake of \([3H]DA\) into synaptosomes than their binding affinity would suggest (see binding to uptake ratios in Table II). In contrast, compound 11

Chemistry

1-(2-Benzo[b]thiencyclohexylamine (4) was obtained in good yield, as described previously, through the sequence of reaction of cyclohexanone with 2-benzo[b]-

thienyllithium in ether (to alcohol 5), followed by treatment with NaN₃/C₆H₅COOH (to give azide 6) and LiAlH₄ reduction (Scheme I). Treatment of 4 with 1,5-dibromopentane afforded BTCP (3); the homologues 7 and 8 (Scheme I) were similarly obtained by treatment of 4 with 1,4-dibromobutane and 1,6-dibromohexane, respectively. Reaction between cyclopentanone and 2-benzo[b]thienyllithium afforded crystalline alcohol 15, which was similarly transformed to 9–11. Treatment of cycloheptanone with 2-benzo[b]thienyllithium gave crystalline alcohol 18, which was transformed to 12–14. Alcohols 5, 15, and 18 could be readily dehydrated to the corresponding cyclohexenes 21 and 22 (Scheme I). During treatment of cyclopentanol and cycloheptanol intermediates 15 and 18 with NaN₃ in the presence of C₆H₅COOH, large amounts of elimination byproducts 21 and 22 were formed whereas no significant elimination was observed (1H NMR of the crude reaction product) after identical treatment of the cyclohexanol intermediate 5.

In synthesis and biological evaluation (Scheme I) for formation of the target compounds 3 and 7–14, treatment of the crude reaction product mixture with AcO followed by acid/base extraction proved to be a very useful method for the separation of the desired products (tertiary amine) from unreacted (primary amine) starting material and secondary amine byproducts. As expected from steric and thermodynamic considerations, the formation of 8, 11, and 14 (homopiperidines) from the corresponding primary amines proceeded in low (12–26%) yield.

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stimulatory effects than compounds such as BTCP. This suggests that cocaine is more effective than BTCP as a reinforcer and locomotor stimulant. Furthermore, cocaine is 15-fold less potent at inhibiting DA uptake in synaptosomes than BTCP. This suggests that the ratio of IC50 of ligand binding/IC50 of [3H]DA-uptake inhibition may not be a good criterion for identifying potential cocaine antagonists. The powerful behavioral effects of cocaine must be due to other factors. Such factors may include more effective in vivo bioavailability or faster access into the brain compared with BTCP. It is also likely that the combined interaction of cocaine with several different neurotransmitter systems is ultimately responsible for its greater behavioral efficacy compared to BTCP. For instance, mazindol inhibits both DA and NE transporters, yet it does not support self-administration behavior in humans.

Examination of the relative affinities of the compounds for sites labeled by [3H]cocaine and [3H]BTCP indicates that all of the BTCP-related compounds show higher affinity for sites labeled by [3H]BTCP. This is to be expected in view of the structural similarities. In terms of subtype selectivity, cocaine is more selective for the [3H]cocaine-binding site and BTCP more so for the [3H]BTCP site. Primary amine 17 is the most selective of the BTCP-related compounds for the [3H]cocaine site.

The structural similarity of these compounds to the abused drug PCP (1) suggests that they would be more potent members of this series at inhibiting [3H]DA uptake. It will be interesting to know if compounds such as 7 also have significant cocaine-like behavioral effects in animals. In making the assumption that cocaine exerts its powerful reinforcing effects by binding to the [3H]cocaine site on the DA transporter and thereby causing DA-uptake inhibition, one would expect that compounds such as BTCP with a larger IC50 of [3H]-cocaine displacement/IC50 of [3H]DA-uptake inhibition ratio would cause more potent reinforcing and locomotor stimulatory effects than compounds such as cocaine with a smaller ratio (Table II). However, previous studies have indicated that cocaine is more effective than BTCP as a reinforcer and locomotor stimulant. Furthermore, cocaine proved to be more effective at displacing [3H]cocaine than at inhibiting DA uptake.

BTCP and its cyclohexa-pyrrrolidine derivative proved to be the most potent members of this series at inhibiting [3H]DA uptake. It will be interesting to know if compounds such as 7 also have significant cocaine-like behavioral effects in animals. In making the assumption that cocaine exerts its powerful reinforcing effects by binding to the [3H]cocaine site on the DA transporter and thereby causing DA-uptake inhibition, one would expect that compounds such as BTCP with a larger IC50 of [3H]-cocaine displacement/IC50 of [3H]DA-uptake inhibition ratio would cause more potent reinforcing and locomotor stimulatory effects than compounds such as cocaine with a smaller ratio (Table II). However, previous studies have indicated that cocaine is more effective than BTCP as a reinforcer and locomotor stimulant. Furthermore, cocaine proved to be more effective at displacing [3H]cocaine than at inhibiting DA uptake.
as in 8, 11, 12, 13, and 14, exhibit weak $\sigma$-binding activity. However, much greater affinity is observed in compound 9 containing two 5-membered rings. No correlation is evident between the binding affinity of these compounds at $\sigma$ sites and their activity at DA reuptake sites, suggesting that these two sites are not coupled or related structurally.

**Conclusion**

By changing the cyclohexyl and piperidine ring sizes of BTCP (3) or replacing the piperidine ring with a primary amine, we have demonstrated that the rings already present in BTCP are optimal for its high affinity at sites labeled by $[3H]$BTCP, but not those labeled by $[3H]$-cocaine. Compound 7, with a cyclohexyl and pyrrolidine ring, exhibited the best affinity for sites labeled by $[3H]$-cocaine. The observation that structural changes in BTCP cause differential changes in their affinity at sites labeled by $[3H]$BTCP and $[3H]$-cocaine suggests that the two radioligands are labeling different sites on the transporter. All of the compounds showed significant differences in their binding affinity versus their ability to inhibit DA uptake. Their lack of affinity for PCP receptors suggests that they will be useful tools for study of the DA transporter. These compounds will add to the SAR data of BTCP and eventually allow the construction of molecular models of the $[3H]$BTCP and $[3H]$-cocaine binding sites on the transporter.

**Experimental Section**

Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Elemental analyses were performed at Atlantic Microlabs, Atlanta, GA; where molecular formulae are indicated in Table I, elemental analyses were determined to be within $\pm 0.4\%$ of the theoretical values for C, H, and N or for non-nitrogen-containing compounds, C and H. Chemical ionization mass spectra were obtained using a Finnigan 1015 mass spectrometer. Electron ionization mass spectra were obtained using a VG-Micro Mass 7070F mass spectrometer. IR spectra were recorded from CHCl$_3$ solutions of compounds using a Bio-Rad FTS-45 FTIR spectrometer. $^1$H-NMR spectra were recorded from CDCl$_3$ solutions using a Varian FTIR spectrometer. 'H-NMR spectra were determined from CDCl$_3$ solutions using a VG-Micro Mass 7070F mass spectrometer. IR spectra were recorded from CHCl$_3$ solutions of compounds using a Bio-Rad FTS-45 FTIR spectrometer. $^1$H-NMR spectra were recorded from CDCl$_3$ solutions using a Varian XL-300 spectrometer; results are recorded as ppm downfield of the TMS signal. Spectral data ($^1$H NMR) for all amines is reported for the free base form. Thin-layer chromatography (TLC) was performed on 250 $\mu$m Analtech GHLF silica gel plates. TLC solvent system A refers to CHCl$_3$–MeOH, 19:1. TLC solvent system B refers to concentrated aqueous ammonia–MeOH–CHCl$_3$, 1:9:90. Solvent system C refers to ethyl acetate–hexane, 1:3. Solvent system D refers to concentrated aqueous ammonia–MeOH–CHCl$_3$, 0.1:1:100. No attempt was made to optimize the yields.

**Chemicals.** (+)-Pentazocine was provided by the National Institute on Drug Abuse (NIDA), Rockville, MD. ($^3$H)-(+)-Pentazocine was synthesized as described previously by us.$^{15}$TCP was synthesized in the Laboratory of Medicinal Chemistry, NIDDK.

**Synthesis of 1-(2-Benzothienyl)cycloalkanols. General Method A.** To a stirred solution of benzo[4]thiophene (0.25 mol) in dry ether (200 mL) was added dropwise, during 15 min at room temperature, a solution of n-butyl lithium in hexane (172 mL, 1.1 equiv of a 2.5 M solution). The reaction began to reflux vigorously during the addition and was controlled by periodic immersion in an ice bath. After complete addition, a solution of cycloalkanone (0.25 mol) in dry ether (200 mL) was added dropwise at room temperature to give a copious white precipitate of the lithium salt of the desired alcohol. The reaction mixture was poured into cold water (200 mL) and the aqueous layer was discarded. The organic layer was washed with saturated brine (100 mL) and dried (Na$_2$SO$_4$), and the solvent was evaporated in vacuo to give the desired alcohol. Further purification was achieved by crystallization from warm isooctane or hexane to give the products in 95–99% yield (see Table I).

**Synthesis of 1-(2-Benzothienyl)cycloalkyl Azides. General Method B.** To a mechanically stirred mixture of 1-(2-benzothienyl)cycloalkanol (0.366 mol) and NaN$_3$ (1.1 mol, 3 equiv) in hydrocarbon-stabilized chloroform (500 mL) was added dropwise, at 0°C during 30 min, trifluoroacetic acid (1.47 mol, 4 equiv). The solution was stirred overnight under a nitrogen atmosphere or until the reaction was deemed complete by TLC (solvent system A). Water (400 mL) was added to the solution followed by concentrated aqueous ammonia to pH 9. The chloroform layer was separated and the aqueous layer was washed further with 2 $\times$ 200 mL of chloroform. The combined organic extract was dried (Na$_2$SO$_4$) and the solvent was evaporated in vacuo to give the crude azides in 40–100% yield. Varying amounts of olefin, ranging from 0 to 60%, were formed in these reactions. No attempt was made to further purify or characterize these azido intermediates because of their lability; these compounds showed a very strong IR absorbance at 2100 cm$^{-1}$ (CHCl$_3$ solution) characteristic of the N$_3$ group.

**Synthesis of 1-(2-Benzothienyl)cycloalkylamines. General Method C.** To a stirred solution of azide (0.566 mol) in ether (500 mL) was added dropwise at room temperature as a solution of LiAlH$_4$ in THF (750 mL of a 1.0 M solution, 0.75 mol, 2 equiv). The reaction mixture began to reflux gently during the addition; stirring was continued overnight or until TLC (solvent system A) indicated that the reaction was complete. The reaction mixture was treated dropwise with water (28 mL) and 15% aqueous NaOH (26 mL) followed by water (85 mL). The reaction mixture was filtered and the filter cake was washed with little THF. The combined filtrate and washings were evaporated in vacuo, the residue was treated with 10% aqueous citric acid (600 mL), the aqueous solution was washed with ether (3 $\times$ 500 mL), and the combined ether washings were discarded. The aqueous solution was basified by the addition of excess concentrated aqueous ammonia solution and extracted with CH$_2$Cl$_2$ (500 mL).

The organic extract was washed with water (500 mL) and dried (Na$_2$SO$_4$), and the solvent was evaporated in vacuo to give the amines in 40–60% overall yield from the alcohols. These compounds were obtained in crystalline form either by recrystallization of the free base from hexanes or by suitable salt formation (see Table I).

**Synthesis of 1-(2-Benzothienyl)cycloalkylamines. General Method D.** To a stirred solution of 1-(2-benzothienyl)cycloalkylamine (157 mmol) in dry DMF (400 mL) was added a $\omega$-dibromo-$\omega$-alkane (173 mmol, 1.1 equiv), and the mixture was heated and stirred at 40°C for 14 h. Anhydrous K$_2$CO$_3$ (239.9 g, 173 mmol, 1.1 equiv) was added and the mixture was heated and stirred for a further 24 h when TLC (solvent system B) indicated that the reaction was complete. The reaction mixture was cooled and quenched into cold water (1.2 L). The solution was extracted with ether (3 $\times$ 400 mL), the combined organic extracts were back-washed with water (600 mL), and the combined etheral extract was reduced to 500 mL by evaporation in vacuo. The ethereal extract was extracted with 10% aqueous citric acid (1 L), the aqueous extract was washed with 3 $\times$ 300 mL of ether, and the combined organic extract was discarded. The aqueous layer was basified by addition of excess concentrated aqueous ammonia and extracted with ether (3 $\times$ 300 mL). The combined organic layer was back-washed with water (500 mL) and dried (Na$_2$SO$_4$), and the solvent was evaporated in vacuo to give the product. Further purification was achieved either by crystallization of the crude form of the product, by column chromatography eluting with solvent system C, or by crystallization of suitable salts from an appropriate solvent (Table I) to give the final products 3 and 7–14 in yields ranging from 12 to 71%.

In certain cases (see Table I), if TLC indicated the presence of substantial amounts of unreacted starting material and/or the presence of secondary azides and the solvent was evaporated in vacuo from above was evaporated in vacuo and the residue was treated with acetic acid anhydride (200 mL) for 1 h at room temperature. The reaction mixture was diluted to 500 mL with ether and the organic layer was then subjected to acid/base extraction as described above. This step could bypass the requirement for further chromatographic purification.
Dehydration of Alcohols 5, 15, and 18. General Method E. To a stirred solution of alcohol (40.6 mmol) in toluene (300 mL) over a period of 2 min, followed by the addition of water (20 mL), the reaction mixture was refluxed briefly (2-5 min), cooled, poured into saturated aqueous NaHCO₃ (100 mL), and washed thoroughly. The organic layer was separated, washed with water (2 × 100 mL), and evaporated in vacuo to give the olefinic products as oils (quantitative yield; >99% pure by 'H NMR). These were dissolved in hexanes (50 mL) and induced to crystallize by cooling and scratching with a glass rod. The crystallization mixture was cooled to 0°C and then filtered. The crystals were dried in vacuo at room temperature (see Table I).

Biological Materials and Methods. Tissue Preparation for [3H]Dopamine-Uptake and [3H]Cocaine/[3H]BTCP-Binding Studies. Rats (Harlan Sprague-Dawley, Indianapolis, IN) (males, 2-4 months old) were decapitated using a guillotine, and the cerebellum was removed and placed in cold 0.17 g/mL saline. The whole brain minus the cerebellum was rapidly removed and disrupted with 40 volumes of 5 mM ice-cold Tris-HCl buffer (pH 7.4) for 50 μL of buffer, test compound, or 10 μM TCP for determination of nonspecific binding. After a 90-min incubation at 5°C, the reaction was terminated by rapid filtration using a Brandel cell harvester (Brandel, Inc., Gaithersburg, MD) through #32 Schleicher and Schuell glass-fiber filters which had been precleaned in 0.03% polysyline (Sigma Chemical Co., St. Louis, MO; molecular weight 150 000-300 000) for 2 h at 5°C. The filters were washed with three 5-mL aliquots of ice-cold assay buffer and placed in counting vials with 4 mL of Cytoscan ES (ICN Biomedicals, Irvine, CA) scintillation cocktail and allowed to stand overnight before counting in a Packard Tri-Carb 2200CA liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

The inhibition constant (Ki) was calculated using GraphPAD software (ISI Software, Philadelphia, PA) using a Ki for TCP of 16.5 nM as determined by Scatchard analysis.

Binding assay. Male Hartley guinea pigs (Charles River, Kingston, NY) were decapitated and the whole brains plus cerebellum were rapidly removed and disrupted with 40 volumes of 50 mM ice-cold Tris-HCl buffer (pH 8.0) using a Brinkman polytron (setting 6, 20 s). The homogenates were centrifuged at 20000 g for 20 min at 5°C. The pellet was washed in fresh buffer and recentrifuged a total of three times. The final resuspension was performed in 45 volumes of fresh assay buffer and kept on ice until needed.

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Binding assay. Male Hartley guinea pigs (Charles River, Kingston, NY) were decapitated and the whole brains plus cerebellum were rapidly removed and disrupted with 40 volumes of 50 mM ice-cold Tris-HCl buffer (pH 8.0) using a Brinkman polytron (setting 6, 20 s). The homogenates were centrifuged at 20000 g for 20 min at 5°C. The pellet was washed in fresh buffer and recentrifuged a total of three times. The final resuspension was performed in 45 volumes of fresh assay buffer and kept on ice until needed.

In a modification of our previously described method,16 a binding to homogenates was determined in a 1-mL incubation volume, consisting of 900 μL of tissue (containing approximately 0.8 mg of protein by Lowry analysis), 50 μL of [3H]TCP (40.8 Ci/mmol; New England Nuclear, Boston, MA) for a final concentration of 2 nM, and 50 μL of buffer, test compound, or 10 μM TCP for the inhibition of uptake of [3H]DA and the binding of [3H]cocaine and [3H]BTCP were constructed from six different concentrations of test compound, each run in triplicate. The concentrations of the test ligands were chosen to bracket the IC₅₀ values as predicted by preliminary binding assays. Each experiment was performed in duplicate on different preparations and analyzed using the SigmaPlot Scientific Graphing System Version 4.0S6 (Jandel Corp., San Rafael, CA) (Scatchard program). The tabulated IC₅₀ values represent the nanomolar concentration of drug inhibiting 50% of the total uptake or binding.

Phencyclidine-Binding Assay. Following decapitation from male Sprague-Dawley rats (Taconic Farms, Germantown, NY), the whole brains minus the cerebellum were rapidly removed and disrupted with 45 volumes of 5 mM ice-cold Tris-HCl buffer (pH 7.4) using a Brinkman polytron (setting 6, 20 s). The homogenates were centrifuged at 20000 g for 20 min at 5°C. The pellet was washed in fresh buffer and recentrifuged a total of three times. The final resuspension was performed in 45 volumes of fresh assay buffer and kept on ice until needed.
References


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