**BMS-192548, a Tetracyclic Binding Inhibitor of Neuropeptide Y Receptors,
from *Aspergillus niger* WB2346**

I. Taxonomy, Fermentation, Isolation and Biological Activity

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During the screening of microbial fermentation extracts for their ability to inhibit the binding of ^{125}I -peptid YY (PYY) to the neuropeptide Y (NPY) receptor using the scintillation proximity assay (SPA), BMS-192548 was isolated from the extract of *Aspergillus niger* WB2346 by bioassay-guided fractionation. BMS-192548 showed the inhibitory activity against ^{125}I -PYY binding to SK-N-MC and SMS-KAN cells, which express NPY₁ and NPY₂ receptors, respectively, with IC₅₀ values of 24 μM in Y₁ and 27 μM in Y₂ receptor binding. BMS-192548 demonstrated weak cytotoxicity against murine tumor cell line M-109 with an IC₅₀ value of 240 μM .

Neuropeptide Y (NPY), is a 36-amino acid peptide neuro regulator and belongs to a family of homologous peptides including the gut peptide YY (PYY) and the pancreatic polypeptide.^{1,2)} NPY is the most abundant of peptides discovered in the mammalian brain and widely distributed in the catecholamine-containing neurons in both central and peripheral nervous systems of many animal species, including humans.^{3,4)} A large number of studies have demonstrated that NPY can produce a multitude of biological effects in the brain and periphery. For example, NPY is a potent stimulator of food and water intake when administered into the brain, and the peptide is also associated with vasoconstriction, anxiolysis/sedation and modulation of pituitary hormone release.^{3,5)} Since its discovery at least three subtypes of NPY receptors, termed as Y₁, Y₂, and Y₃, have been proposed based on the rank order of differential activity of peptide agonists.⁶⁾ The Y₁ receptor, the only cloned subtype thus far, appears to be the major vascular NPY (PYY) receptor present on smooth muscle cells, and involved in centrally evoked hypertension, sedation/anxiolysis and contraction of smooth muscles; the Y₂ receptor, predominantly present in the brain and particularly in the hippocampus, inhibits the release of neurotransmitters in the central and peripheral nervous systems. While Y₁ and Y₂ receptors are the best

established receptor subtypes for NPY, considerable evidence supports the existence of additional receptor subtypes such as Y₃ and possibly Y_{1a} (or Y₄) receptors. The later is presumed to be present in the paraventricular nucleus of hypothalamus and is similar but not identical to the Y₁ receptor, and this atypical Y₁ receptor appears to mediate the feeding behavior.^{7,8)} The NPY receptors have recently emerged as potential targets of drug development in the area such as eating disorder, anxiety and depressive illness, and hypertension.³⁾ Several full-length and centrally truncated NPY analogs and their derivatives were demonstrated to have antagonistic effects against NPY.^{3,9)} Only two non-peptidic NPY antagonists, benextramine analogs¹⁰⁾ and PP56¹¹⁾, have been reported thus far. The most studied compound PP56, however, only antagonizes a minority of NPY effects, and the antagonistic effects are noncompetitive. Moreover, PP56 does not bind to NPY receptors, hence it probably acts at a post-receptor level.¹¹⁾ Clearly, a specific, high-affinity and highly potent non-peptidic antagonist of NPY receptor will be very useful in the assessment of the physiological and pathological role of NPY and the research on such an antagonist may ultimately lead to the discovery of a useful therapeutic agent for a variety of disease states. A high-throughput screen for identifying NPY₁ and NPY₂ receptor binding

inhibitors was therefore developed. The screen was based on the measurement of binding of ^{125}I -PYY to the two human neuroblastoma cell lines, SK-N-MC and SMS-KAN, which express high levels of NPY_1 and NPY_2 receptors, respectively.

During the screening of microbial fermentation extracts for their ability to inhibit the binding of ^{125}I -PYY to the $\text{NPY}_1/\text{NPY}_2$ receptor, an extract of *Aspergillus niger* WB2346, was found to be active. When subjected to bioassay-guided fractionation, the extract yielded one active compound, BMS-192548. This report describes the fermentative production and isolation of this compound, the inhibitory effect of NPY receptor binding, and other biological activities.

Materials and Methods

Binding Assay

The SK-N-MC and SMS-KAN cells were obtained from Dr. J. BEIDLER (Memorial Sloan-Kettering Institute for Cancer Research, New York, NY). The SK-N-MC cells are a human neuroblastoma cell line (ATCC HTB10) expressing NPY_1 type receptor, they were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), 2 mM L-glutamine, 50 iu/ml penicillin G and 50 $\mu\text{g}/\text{ml}$ streptomycin. Media were changed every 2~3 days. The SMS-KAN cells expressing NPY_2 type receptor were cultured in MEM and Ham's F12 (1:1, v/v) media supplemented with all the non-essential amino acids, 15% BCS, 50 iu/ml penicillin G and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cells were grown, harvested and stored at -80°C at a concentration of $3.2 \times 10^8/\text{ml}$. On the day of use, an aliquot of cells was thawed and diluted to $6.4 \times 10^7/\text{ml}$ with 100 mM Hepes buffer (pH 7.4) containing 1.0 mM MgCl_2 , 2.5 mM CaCl_2 , 0.25 mg/ml bacitracin and 0.25 mg/ml NaN_3 . A 25 μl cell suspension was transferred into each well of a microtiter plate; additionally, each well contained 25 μl of ^{125}I -PYY (porcine, 4000 Ci/mmol, Amersham) at 0.08 nM, 25 μl of wheat germ agglutinin (WGA) scintillation proximity assay (SPA) beads (Amersham) at 20 mg/ml and 25 μl of sample to be tested or vehicle. The well contents were mixed and incubated on an orbital shaker at room temperature for 16 hours. Radioactivity bound to the cells was quantified by scintillation counting per well (Microbetaplate, Wallac). Non-specific binding of a control well, defined as that occurring in the presence of 100 nM unlabeled PYY (Sigma, 25 μl), was subtracted from the total binding to yield specific binding.

Taxonomy

The BMS-192548-producing culture, strain WB2346, was isolated from a soil sample collected in Honolulu, Hawaii. Taxonomy of the strain WB2346 was studied

according to the method of RAPER and FENNEL as described in "The Genus *Aspergillus*."¹²⁾

Fermentation

A. niger WB2346 was grown on slants of potato dextrose agar (PDA). The culture was transferred from the PDA slant into a 500-ml flask containing 100 ml of fresh medium which consisted of the following (amounts per liter of de-ionised water): dextrose 40.0 g, yeast extract 5.0 g, FeSO_4 0.01 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KCl 0.5 g, KH_2PO_4 1.0 g and NaNO_3 3.0 g. The medium was sterilized at 121°C for 20 minutes. Frozen vegetative preparations were made by mixing a culture grown for 3 days in liquid medium with an equal volume of glycerol 20%/sucrose 10%, and aliquots frozen in a dry ice/acetone bath and stored at -80°C . From the frozen stock, 4 ml was used as an inoculum into 100 ml of the vegetative medium described above. The culture was grown for 3 days at 28°C and at 250 rpm on a rotary shaker; then 4 ml was taken to inoculate 100 ml of the same medium which was used as the production medium. The production culture was grown at 28°C on a rotary shaker at 250 rpm for 6 days.

Isolation and Purification of BMS-192548

The isolation and purification of BMS-192548 from 20-liter of cultured broth of *A. niger* WB2346 is summarized in Fig. 1.

Cytotoxicity Assay

Cytotoxicity was assessed by the XTT-assay¹³⁾ using murine tumor cell line M-109, which is a Madison lung carcinoma derived cell line. Cells were seeded at 4,000 cells/well in 96-well microtiter plates, and 24 hours later, drugs at serially diluted concentrations were added. The cells were incubated at 37°C for 72 hours at which time the tetrazolium dye, XTT, was added. The results are expressed as an IC_{50} , which is the drug concentration required to inhibit cell proliferation (absorbance at 450 nm) to 50% of that of untreated control cells.

Results and Discussion

NPY Receptor Binding Assay

The subtype of the NPY receptor on SK-N-MC and SMS-KAN cells used in the screen were biochemically characterized. Binding of ^{125}I -PYY to SK-N-MC and SMS-KAN cells was inhibited by unlabeled PYY in a concentration-dependent fashion with the IC_{50} of 0.138 nM and 0.086 nM, respectively. The order of potency of inhibition of ^{125}I -PYY binding in SK-N-MC cells is $\text{PYY} > \text{NPY} > [\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY} \gg \text{NPY}_{13\sim36}$, which is typical for the Y_1 receptor;³⁾ in SMS-KAN cells the order is $\text{PYY} > \text{NPY} > \text{NPY}_{13\sim36}[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$, typical of the Y_2 receptor.³⁾ Further, the binding of ^{125}I -PYY to SK-N-MC and SMS-KAN cells was

Fig. 1. Isolation and purification procedures for BMS-192548.

specifically inhibited by the GTP analogs, Gpp(NH)p, GTP- γ -S and GDP, indicating the expressed NPY receptors may be coupled to G-proteins.³⁾ But the radio-ligand binding was not inhibited by a number of unrelated peptides such as angiotensin II, endothelin, bradykinin, and gp 120 (data not shown).

The screen was carried out by making use of a novel binding methodology known as the scintillation proximity assay (SPA).^{14,15)} Using SPA, it is possible to use radioisotopes in binding assays without the need to separate free from bound components. It involves the use of fluoro microspheres coated with acceptor molecules, such as wheat germ agglutinin, that will bind to carbohydrate (N-acetyl β -D-glucosamine) residues on the cell surface. Thus, the interaction between labeled ligand and the receptor, which is in close proximity to the fluoro microspheres, could be measured without the need to separate the free ligand. Depending on the type of receptor being studied, the sensitivity of the method is variable compared to conventional binding assays, but is highly amenable to automation. It also uses considerably less radioisotope labeled material, hence minimizing the radioactive waste and its disposal.

During the testing of a large number of natural products in the high-throughput screen, an extract of the

fungal strain WB2346 was found to inhibit ¹²⁵I-PYY binding to SK-N-MC and SMS-KAN cells; further taxonomy, fermentation and bioassay-guided isolation studies of the strain or its active metabolites were thus pursued.

Taxonomy

The organism grew rapidly on various culture media forming conidiophores with the axis swelling up to form a vesicle, a series of biserial sterigmata are formed on the vesicle, where phialides are borne in clusters. The phialiospores are formed in long columnar chains. On Czapeck Dox agar, colonies grow rapidly at room temperature (24~26°C) to 5 cm diameter in 14 days, consisting of white to yellow basal mycelium that gives rise to dark purple-brown to black conidial heads, the reverse is uncolored giving rise to a very light yellow tint with age. No sclerotia or exudates are formed. Conidial heads are large and black, sometimes splitting into two or more compact columns. Conidiophores are smooth-walled in brownish shades; vesicles are globose measuring about 45 μ m in diameter with their lumen separate from the lumen of the conidiophore; sterigmata are in two series, primary sterigmata measure an average of 15.4 μ m by 5.5 μ m at onset of sporulation, secondary sterigmata

range from 7~10 μm by 3 μm ; conidia appear globose averaging of 4.4 μm in diameter with echinulate walls; On malt extract agar colonies grow slightly faster than on Czapeck's agar and the split in the columnar conidial heads is slightly more distinct. All other characteristics remain the same; On potato dextrose agar colonies grow as previously described but the spore color is jet black without the purple-brown shades. Based on the morphological and colonial characteristics described, we identified the producing strain WB2346 as *Aspergillus niger*, a member of the niger group.

Isolation

The fractionation of the culture broth WB2346 of *A. niger* was monitored by the NPY receptor binding assay, and the procedure is illustrated in Fig. 1. Briefly, the fermentation broth was separated into filtrate and mycelia. The filtrate was extracted with ethyl acetate. The mycelium cake was first percolated with methanol/acetone, the mixture was then concentrated to a reduced volume and then extracted with ethyl acetate. The ethyl acetate extracts of the filtrate and mycelia were processed in parallel by sequential solvent partition against per-equilibrated hexane and carbon tetrachloride. The two bioactive extracts, carbon tetrachloride fractions C and D, were combined, and purified on Sephadex LH-20 column chromatography, preparative HPLC (YMC ODS 120Å, 10 mm, 3 $\times$ 50 cm, YMC, Inc.) and finally by silica gel column to give BMS-192548.

Biological Activity

BMS-192548 showed the inhibitory activity against ^{125}I -PYY binding to SK-N-MC and SMS-KAN cells, which express Y_1 and Y_2 receptors, respectively, the IC_{50} values are 24 μM in Y_1 and 27 μM in Y_2 receptor binding (Fig. 2), showing lack of specificity against one of the receptor subtypes. Since the structure of BMS-192548 bears a tetracyclic ring system, which is being described in the accompanied paper,¹⁶⁾ several tetracycline analogs such as tetracycline (Aldrich), dactylocyclinone¹⁷⁾ and three derivatives synthesized from tetracycline¹⁸⁾, namely anhydrotetracycline, des-*N*-dimethyltetracycline and des-*N*-dimethyl-anhydrotetracycline, were tested to assess their inhibitory effect against NPY receptor binding; however, none of the compounds showed activity (data not shown). On the other hand, not surprisingly, due to a lack of key pharmacophores of tetracycline antibiotics¹⁹⁾ BMS-192548 showed no antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli* and *Saccharomyces cerevisiae* at a concentration of 100 $\mu\text{g}/\text{ml}$.

Fig. 2. Inhibition of NPY₁ and NPY₂ receptor binding by BMS-192548.

BMS-192548 demonstrated weak cytotoxicity with IC_{50} value of 240 μM against murine tumor cell line M-109.

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