ACCELERATED COMMUNICATION

3,4-Methylenedioxymethamphetamine (MDMA, “Ecstasy”) Induces Fenfluramine-Like Proliferative Actions on Human Cardiac Valvular Interstitial Cells in Vitro

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ABSTRACT

Recent findings have implicated the 5-hydroxytryptamine 2B (5-HT2B) serotonin receptor in mediating the heart valve fibroplasia [valvular heart disease (VHD)] and primary pulmonary hypertension observed in patients taking the now-banned appetite suppressant fenfluramine (Pondimin, Redux). Via large-scale, random screening of a portion of the receptorome, we have discovered that the amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, “Ecstasy”) and its N-demethylated metabolite 3,4-methylenedioxyamphetamine (MDA) each preferentially bind to and activate human recombinant 5-HT2B receptors. We also demonstrate that MDMA and MDA, like fenfluramine and its N-deethylated metabolite norfenfluramine, elicit prolonged mitogenic responses in human valvular interstitial cells via activation of 5-HT2B receptors. We also report that pergolide and dihydroergotamine, two drugs recently demonstrated to induce VHD in humans, potently activate 5-HT2B receptors, thus validating this assay system for its ability to predict medications that might induce VHD. Our discovery that MDMA and a major metabolite, MDA, induce prolonged mitogenic responses in vitro similar to those induced by fenfluramine and norfenfluramine in vivo (i.e., valvular interstitial cell fibroplasia) predict that long-term MDMA use could lead to the development of fenfluramine-like VHD. Because of the widespread abuse of MDMA, these findings have major public health implications. These findings also underscore the necessity of screening current and future drugs at 5-HT2B receptors for agonist actions before their use in humans.

In September of 1997, the highly effective appetite suppressant fenfluramine (Pondimin), a component of the drug combination “Fen-Phen”, and the optically pure (+)-isomer dexfenfluramine (Redux) were voluntarily removed from the marketplace at the urging of the United States Food and Drug Administration because of their association with heart valve fibroplasia and dysfunction, a condition known as valvular heart disease (VHD). Since then, several independent echocardiographic studies of patients who received long-term fenfluramine therapy revealed an increased prevalence of valvular heart disease (Connolly et al., 1997; Jick et al., 1998; Weissman et al., 1998; Weissman, 2001). Histopathological examination of resected valves has revealed proliferative foci containing interstitial cells and increased levels of extracellular matrix (Steffee et al., 1999). Identical pathology has been seen in resected valves harvested from persons undergoing

ABBREVIATIONS: VHD, valvular heart disease; 5-HT, 5-hydroxytryptamine; h, human; PPH, primary pulmonary hypertension; MDMA, 3,4-methylenedioxymethamphetamine; HEK, human embryonic kidney; VIC, interstitial valvular cells; DMEM, Dulbecco’s modified Eagle’s medium; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; DA, dopamine; NE, norepinephrine; GBR12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine; IP, inositol phosphate; RTI-228, 3β-(4-iodophenyl)tropane-2β-pyrrolidine carboxamide; MDA, 3,4-methylenedioxyamphetamine; SB206553, 5-methyl-1-(3-pyridylcarbamoyl)-1H,2,3,5-tetrahydropyridol[2,3-f]indole.
long-term administration of certain ergot derivatives (e.g., ergotamine and methysergide) and from those suffering from carcinoid syndrome (Steffee et al., 1999).

Recently, we proposed that drugs (and/or their metabolites) associated with VHD should preferentially bind with high affinity to a single, proximal molecular target (receptor, channel, or transporter), whereas similar medications (e.g., fluoxetine, phentermine) not associated with VHD would not (Rothman et al., 2000). Via screening of VHD-associated and non-VHD-associated drugs at a limited number of recombinant receptors, transporters, and ion channels, we discovered that VHD-associated drugs shared high affinity for only the human 5-HT$_{2B}$ (h5-HT$_{2B}$) receptor (Rothman et al., 2000). In functional assays, we demonstrated that VHD-associated drugs were all h5-HT$_{2B}$ receptor agonists (Rothman et al., 2000). Fitzgerald et al. (2000) also reported that fenfluramine and a major metabolite, norfenfluramine, were agonists at recombinant h5-HT$_{2B}$ receptors and independently suggested that the 5-HT$_{2B}$ receptor was responsible for fenfluramine-induced VHD. Launay et al. (2002) subsequently discovered that activation of the 5-HT$_{2B}$ receptor is also responsible for fenfluramine-induced primary pulmonary hypertension (PPH).

Recently, we pioneered the use of large-scale screening of psychoactive drugs at a huge panel of recombinant receptors (i.e., “receptorome”) to identify the κ-opioid receptor as the site of action of the novel hallucinogen Salvinorin A (Roth et al., 2002; Sheffler and Roth, 2003). We now report the results of a receptorome screen of the club drug 3,4-methylendioxymethamphetamine (MDMA, “Ecstasy”) that we performed to identify novel molecular targets responsible for the actions of MDMA in humans. These studies reveal that MDMA, like fenfluramine and other drugs known to be associated with heart valve fibroplasia, preferentially binds to and activates h5-HT$_{2B}$ receptors. Additionally, we demonstrate that MDMA induces prolonged mitogenic responses in primary cultures of human cardiac interstitial valve cells. Because drugs that activate h5-HT$_{2B}$ receptors induce VHD and PPH in humans, these findings have major public health implications.

Materials and Methods

HEK293 and COS-7 Cell Culture and Transfection. Stably and transiently transfected cells were maintained as detailed previously (Roth et al., 2002). For transfections with the h5-HT$_{2B}$ receptor, HEK293 or COS-7 cells were seeded in 100-mm dishes and transfected using 6 μg of plasmid and 36 μL of Fugene (Roche, Indianapolis, IN) according to the manufacturer’s protocol as described previously (Rothman et al., 2000).

Radioligand Binding Assays and Phosphatidylinositol Hydrolysis Assays. Radioligand binding assays were performed as described previously using the resources of the National Institute of Mental Health Psychoactive Drug Screening Program (Rothman et al., 2000). Phosphatidylinositol hydrolysis assays were performed using HEK293 cells transiently expressing human 5-HT$_{2B}$ receptors as reported previously (Rothman et al., 2000).

VIC Isolation and Culture. Human heart valves were obtained from donor hearts deemed unsuitable for transplantation, or from hearts that were removed from transplant recipients at the Cleveland Clinic Foundation. All Cleveland Clinic patients who have tissue surgically removed have authorized its subsequent use for research purposes (protocols approved by the CCF IRB 2378). To remove the cells from the tissue, the specimens were placed into sterile containers, immersed in a solution of collagenase-II (2 mg/ml; Worthington Biochemicals, Freehold, NJ) in serum-free medium, then digested in an incubated shaker (140 rpm, 20 min, 37°C). After return to the sterile low bowl, the surfaces were rubbed with a sterile cotton swab to remove the endothelial cells. The valve specimens were then finely minced and then digested with collagenase-III (1 mg/ml) in an incubated shaker (4 h, 140 rpm, 37°C). Each resulting cell suspension was filtered (70 μm) to remove debris, and the cell pellet was resuspended in DMEM/Ham’s F12 medium (1:1, containing low glucose with HEPES) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). The culture was incubated in a humidified atmosphere of 95% air/5% CO$_2$ at 37°C with changes of medium every 48 h.

[3H]Thymidine Deoxyribose Incorporation Assay. Subconfluent VIC seeded in 24-well clusters were incubated overnight in serum-free DMEM (Invitrogen). Cells were then treated over the course of 3 days with various concentrations of test agents. Twelve hours before the end of the treatment period, cells were pulsed with 2 to 5 μCi/ml [3H]thymidine deoxyribose (PerkinElmer Life Sciences, Boston, MA). After treatment, the medium was removed and the cells were washed thoroughly with ice-cold phosphate-buffered saline, pH 7.4. Ice-cold 10% trichloroacetic acid was then added and the cells were incubated for 30 min at 4°C. The cells were again washed thoroughly with ice-cold phosphate-buffered saline, pH 7.4, and then lysed in 0.5 N NaOH. After neutralization with glacial acetic acid, samples were assayed for [3H]thymidine deoxyribose incorporation by liquid scintillation counting. Values are reported as the mean ± S.E.M. of triplicate determinations and are representative of three independent experiments.

Immunoblot Analysis of Erk 1/2 MAPK Phosphorylation. VIC seeded in 24-well clusters were incubated overnight first in DMEM containing 5% dialyzed fetal bovine serum and then in serum-free DMEM. Cells were treated over the course of 15 min with 10 μM fenfluramine, norfenfluramine, MDMA, or MDA. After treatment, the medium in each well was replaced with 200 μL of 1× Laemmli sample buffer and collected. Samples were resolved on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were probed for phospho-Erk 1/2 MAPK immunoreactivity using a 1:1000 dilution of polyclonal primary antibody (Cell Signaling Inc., Beverly, MA) and a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) according to the manufacturer’s recommendations. Immunoreactivity was revealed using LumiLight horseradish peroxidase substrate (Roche) and imaged on a Kodak Digital Science Image Station 440CF (Eastman Kodak, Rochester, NY). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD). Samples were similarly analyzed for total Erk 1/2 MAPK immunoreactivity, and the resulting values were used to correct phospho-Erk 1/2 MAPK measurements for slight differences in sample protein content. Values are reported as the mean ± S.E.M. of duplicate determinations and are representative of three independent experiments.

[3H]DA, [3H]NE, and [3H]5-HT Release Assays. Following published procedures (Rothman et al., 2001), rat caudate (for [3H]DA release) or whole brain minus cerebellum and caudate (for [3H]NE and [3H]5-HT release) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. Nomifensine (100 nM) and GBR12935 (100 nM) were also added to the sucrose solution for [3H]5-HT release experiments to block any potential [3H]5-HT reuptake into NE and DA nerve terminals. After 12 strokes with a Potter-Elvehjem homogenizer, homogenates were centrifuged at 1000g for 10 min at 0 to 4°C and the supernatants were retained on ice (synaptosomal preparation). Each rat brain (approximately 1200 mg) produced enough tissue for 250 test tubes for the [3H]DA and [3H]5-HT release assays and for 125 test tubes for the [3H]NE release assay.
Synaptosomal preparations were incubated to steady state with 5 nM [3H]DA (30 min), 7 nM [3H]NE (60 min), or 5 nM [3H]5-HT (60 min) in uptake buffer without bovine serum albumin, plus 1/9262 M reserpine, in a polypropylene beaker with stirring at 25°C. Nomifensine (100 nM) and GBR12935 (100 nM) were added to the buffer for [3H]5-HT release experiments, whereas RTI-229 (5 nM) was added to the buffer for [3H]NE release experiments. After incubation to steady state, 850/9262 l of synaptosomes preloaded with [3H]neurotransmitter were added to 12/11003 75-mm polystyrene test tubes that contained 150/9262 l of test drug in uptake buffer. After 5 min ([3H]DA and [3H]5-HT) or 30 min ([3H]NE), the release reaction was terminated by dilution with 4 ml of wash buffer (10 mM Tris-HCl, pH 7.4, containing 0.9% NaCl at 25°C) followed by rapid vacuum filtration over Whatman GF/B filters using a Brandel Harvester (Brandel Inc., Gaithersburg, MD). The filters were rinsed twice with 4 ml of wash buffer using the Brandel Harvester, and the retained tritium was counted by a Taurus liquid scintillation counter at 40% efficiency after an overnight extraction in 3 ml of Cytoscint (ICN Biomedicals Inc., Costa Mesa, CA).

**Results**

Screening the Receptorome Reveals the h5-HT2B Receptor As a Primary Molecular Target for MDMA and MDA. To discover novel molecular targets for the effects of psychoactive compounds (see Roth et al., 2002, for a recent example), we screened the club drugs MDMA and MDA at a

Fig. 1. Large-scale screening of the receptorome reveals that MDMA preferentially interacts with the human 5-HT2B serotonin receptor. Top, $K_i$ values for various drugs screened at a large number of mainly human recombinant receptors, ion channels, or transporters using the resources of the National Institute of Mental Health Psychoactive Drug Screening Program. For these studies, test compounds were initially screened at 10 μM. When greater than 50% inhibition of radioligand specific binding was obtained, $K_i$ values were determined in quadruplicate. A three-dimensional mesh plot of the data was made in which the $K_i$ values were color-coded. The red arrow indicates that MDMA has preferentially high affinity for h5-HT2B receptors. Bottom left, representative isotherms showing radioligand displacement from h5-HT2B receptors expressed in COS-7 cells, the nonlinear regression of which was used to determine IC50 values. $K_i$ values were calculated using the Cheng-Prusoff approximation. Bottom right, $K_i$ values for MDMA in bar chart format; the arrow shows the $K_i$ value for the h5-HT2B receptor; $K_i$ values >10,000 nM are set to zero for clarity. Red arrow, $K_i$ value for MDMA.
large number of recombinant (mostly human) neurotransmitter and hormone receptors, ion channels, and transporters. To our surprise, MDMA exhibited preferentially high affinity for the h5-HT$_{2B}$ receptor (Fig. 1A), a receptor previously implicated in fenfluramine-induced VHD (Fitzgerald et al., 2000; Rothman et al., 2000) and PPH (Launay et al., 2002). As shown in Fig. 1A and Table 1, other valvulopathic drugs are also characterized by preferentially high affinities for h5-HT$_{2B}$ receptors (Fig. 1A; Table 1). We also discovered that two additional commonly prescribed medications bind to and activate 5-HT$_{2B}$ receptors: 1) pergolide, a drug used in treating Parkinson’s disease that was recently associated with VHD of the fenfluramine-type (Pritchett et al., 2002) and 2) dihydroergotamine, a drug used in treating migraine headaches, which was reported several years ago to induce VHD (Creutzig, 1992). As is shown in Table 1, both pergolide and dihydroergotamine have high affinities for h5-HT$_{2B}$ receptors.

We subsequently examined the abilities of MDMA and its N-demethylated metabolite (MDA) to activate human, recombinant 5-HT$_{2B}$ receptors. These studies identified MDA as a more potent and efficacious agonist than MDMA (Fig. 2A; Tables 1 and 2). In this regard, we reported previously that the N-dealkylated metabolites of drugs known to induce either VHD or PPH (e.g., norfenfluramine and methylergonovine) are also more potent and efficacious 5-HT$_{2B}$ receptor agonists than their respective parent compounds (Table 2; Rothman et al., 2000). Importantly, the $EC_{50}$ values for activating phosphorysitol hydrolysis at h5-HT$_{2B}$ receptors for MDMA (2000 nM) and MDA (190 nM) are nearly identical to the plasma concentrations found in humans after a single recreational dose (150 mg) of MDMA in humans. For instance, after a single 150-mg dose of MDMA, de la Torre et al. (2000) reported a $C_{\text{max}}$ for MDMA of 2000 nM and a $C_{\text{max}}$ for MDA of 150 nM. Table 2 also demonstrates that both pergolide and dihydroergotamine, drugs recently demonstrated to induce VHD of the fenfluramine-type in humans (Pritchett et al., 2002), are also potent h5-HT$_{2B}$ agonists.

Because the (+)-stereoisomer of fenfluramine, dexfenfluramine, also used as an anorexigenic (Redux), was associated with VHD and PPH, we evaluated optically pure preparations of MDMA and MDA for potency and efficacy at human 5-HT$_{2B}$ receptors. We detected no significant difference in efficacy between the R- and S-stereoisomers of either MDMA or MDA; with respect to potency, the S-stereoisomer of MDMA was slightly more potent than the R-stereoisomer, whereas the R- and S-stereoisomers of MDA exhibited no statistically significant difference in potency (Fig. 2B and Table 2).

MDMA and MDA are widely appreciated to release the biogenic amine neurotransmitters from nerve terminals via a carrier-mediated exchange mechanism see (Baumann et al., 2000; Rothman and Baumann, 2002). We thus determined the $EC_{50}$ values of the compounds under consideration for releasing $[^{3}H]$5-HT, $[^{3}H]$NE, and $[^{3}H]$DA from rat brain synaptosomes. Norfenfluramine is more potent than fenfluramine at releasing $[^{3}H]$NE and $[^{3}H]$DA. MDMA is most potent at releasing $[^{3}H]$5-HT, but still potently releases $[^{3}H]$NE and $[^{3}H]$DA (Table 2). MDA differs from MDMA in that its most potent action is in releasing $[^{3}H]$NE (Table 2). There is a pronounced enantioselectivity in the actions of MDMA and MDA as indicated by the more potent effects of (S)-MDA and (S)-MDMA compared with (R)-MDA and (R)-MDMA, respectively (Table 2). Perhaps the key feature to emerge from this analysis is that the potency of (R)-MDMA, MDA and its stereoisomers, in the biogenic amine release assays is similar to their potency at 5-HT$_{2B}$ receptors, indicating that MDMA will activate 5-HT$_{2B}$ receptors at typical pharmacological doses.

Valvulopathic Drugs Induce Prolonged Mitogenic Responses in Human Heart Valve Interstitial Cells. Because much of the evidence implicating 5-HT$_{2B}$ receptor activation in drug-induced VHD is inferential, we set out to directly test the mitogenic activity of valvulopathic drugs using primary cultures of human heart valve interstitial cells (hVICs). In preliminary studies, we established that hVICs express functional 5-HT$_{2B}$ receptors coupled to phosphoinositide hydrolysis (data not shown). We next evaluated the abilities of selected VHD-associated drugs to elicit mitogenic responses from hVICs. For these studies, we incubated serum-starved hVICs for 48 h with fenfluramine, norfenfluramine, MDMA, MDA, MDMA, MDA, SB206553 (a 5-HT2B/2C antagonist), or 5-HT and measured $[^{3}H]$thymidine incorporation into newly-synthesized DNA.

The VHD-associated drugs fenfluramine and norfenfluramine each induced statistically significant mitogenic responses in hVICs (Fig. 3A). MDMA, MDA, and 5-HT, but not...
the 5-HT$_{2B/2C}$ receptor antagonist SB206553, each caused similar responses (Fig. 3A). The mitogenic response elicited by each drug was abrogated by coincubation with the 5-HT$_{2B/2C}$ receptor antagonist SB206553, demonstrating that the mitogenic response was caused by 5-HT$_{2B}$ receptor activation (Fig. 3B), because heart valve cells do not express 5-HT$_{2C}$ receptors (Roy et al., 2000).

Immunoblot analysis of vehicle- and drug-treated hVIC lysates revealed that short-term (10-min) treatment of serum-starved cells with either norfenfluamine, MDMA, MDA, or 5-HT induced an increase (statistically significant for all drugs but MDMA) in Erk 1/2MAPK phosphorylation, an early mitogenic marker, compared with vehicle-treated cells (Fig. 4). Interestingly, the 5-HT$_{2B/2C}$ receptor antagonist SB206553 caused a statistically significant decrease in [3H]thymidine deoxyribose incorporation and no increase in Erk 1/2MAPK phosphorylation compared with vehicle-treated cells, suggesting that 5-HT$_{2B}$ receptors regulate basal mitogenesis in hVICs. In fact, we have observed that basal Erk 1/2MAPK phosphorylation, which is quite high in serum-starved VICs compared with serum-starved HEK cells (data not shown), hinders the detection of a statistically significant mitogenic response to drug treatment.

**Discussion**

The major finding of the present study is that MDMA and MDA, in a manner identical to drugs demonstrated to induce VHD and PPH in humans, bind to and activate human recombinant 5-HT$_{2B}$ receptors and induce mitogenesis in human heart valve interstitial cells in vitro. Importantly, MDMA and MDA activate h5-HT$_{2B}$ receptors within the same concentration ranges at which they 1) occur in plasma

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**TABLE 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>pEC$<em>{50}$ for 5-HT$</em>{2B}$-Mediated PI Hydrolysis</th>
<th>Relative Efficacy for 5-HT$_{2B}$-Mediated PI Hydrolysis</th>
<th>Release EC$_{50}$</th>
<th>5-HT</th>
<th>DA</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>1 (9.0 ± 0.1)</td>
<td>1.00 ± 0.06</td>
<td>44 ± 3</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>400 (6.4 ± 0.2)</td>
<td>0.13 ± 0.02</td>
<td>108 ± 4</td>
<td>&gt;10,000</td>
<td>740 ± 30</td>
<td></td>
</tr>
<tr>
<td>Norfenfluamine</td>
<td>60 (7.2 ± 0.1)</td>
<td>0.96 ± 0.03</td>
<td>104 ± 3</td>
<td>1900 ± 200</td>
<td>170 ± 10</td>
<td></td>
</tr>
<tr>
<td>Dihydroergotamine</td>
<td>30 (7.52 ± 0.09)</td>
<td>0.73 ± 0.02</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>(R,S)-MDMA</td>
<td>2000 (5.8 ± 0.1)</td>
<td>0.32 ± 0.02</td>
<td>72 ± 3</td>
<td>278 ± 7</td>
<td>110 ± 10</td>
<td></td>
</tr>
<tr>
<td>(R)-MDMA</td>
<td>900 (6.0 ± 0.2)*</td>
<td>0.27 ± 0.02</td>
<td>340 ± 20</td>
<td>3700 ± 100</td>
<td>560 ± 40</td>
<td></td>
</tr>
<tr>
<td>(S)-MDMA</td>
<td>6000 (5.2 ± 0.2)*</td>
<td>0.38 ± 0.03</td>
<td>74 ± 3</td>
<td>142 ± 4</td>
<td>136 ± 9</td>
<td></td>
</tr>
<tr>
<td>(R,S)-MDA</td>
<td>190 (6.73 ± 0.05)</td>
<td>0.80 ± 0.02</td>
<td>160 ± 7</td>
<td>190 ± 6</td>
<td>108 ± 7</td>
<td></td>
</tr>
<tr>
<td>(R)-MDA</td>
<td>150 (6.83 ± 0.05)</td>
<td>0.76 ± 0.02</td>
<td>310 ± 10</td>
<td>900 ± 30</td>
<td>290 ± 10</td>
<td></td>
</tr>
<tr>
<td>(S)-MDA</td>
<td>100 (6.9 ± 0.1)</td>
<td>0.81 ± 0.04</td>
<td>100 ± 4</td>
<td>98 ± 4</td>
<td>50 ± 5</td>
<td></td>
</tr>
<tr>
<td>Methysergide</td>
<td>150 (6.8 ± 0.1)</td>
<td>0.18 ± 0.02</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Methylergonovine</td>
<td>0.8 (9.2 ± 0.1)</td>
<td>0.40 ± 0.02</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined
* Significantly different (P < 0.05) from the other enantiomer by two-tailed t test.
† Significantly different from racemate (P < 0.05) by two-tailed t test.

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The structures are shown below the table, and the arrows indicate the dealkylated nitrogen. Drugs in bold are those known to induce VHD in humans.

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**Figure 2**

- Fenfluramine
- Norfenfluamine
- MDMA
- MDA
- Methysergide
- Methylergonovine
after a single recreational dose and 2) release biogenic amines, an activity widely accepted to be a major pharmacological effect of these agents. We also report that two commonly prescribed medications reported to induce VHD in humans, pergolide and dihydroergotamine (Creutzig, 1992; Pritchett et al., 2002), also activate h5-HT2B receptors in vitro. Previous studies suggested that VHD-associated drugs cause heart valve dysfunction via activation of heart valve interstitial cell 5-HT2B receptors. Our current report brings to five the number of medications known to activate 5-HT2B receptors (e.g., fenfluramine, ergotamine, dihydroergotamine, pergolide, and methysergide), each of which induces VHD of the fenfluramine-type in humans. With the exception of fenfluramine, all of the drugs currently reported to produce VHD in humans are ergot derivatives. Because of the widespread use of ergot derivatives for treating diseases such as migraine headaches and Parkinson’s disease, these findings are likely to have negative implications for drug discovery efforts that use ergolines or ergopeptides as lead candidates. Our finding that amphetamine derivatives (e.g., fenfluramine, MDMA, MDA, and norfenfluramine) also activate h5-HT2B receptors demonstrates that drugs of other classes also need to be screened for potential valvulopathogenic actions. In this regard, we are in the process of a large-scale screen of various drugs currently approved for use in humans to identify potential valvulopathogenic drugs by virtue of their ability to bind to and activate recombinant h5-HT2B receptors (V. S. Setola, S. J. Hufeisen, K. J. Grande-Allen, I. Vesely, R. A. Glennon, B. Blough, R. B. Rothman, B. L. Roth, manuscript in preparation).

Because there is no suitable animal model for predicting the valvulopathogenic actions of drugs, we evaluated the mitogenic effect of various drugs on hVICs, a novel in vitro model system. Because hVICs are the cells affected in drug-induced VHD, hVICs represent the most physiologically and pharmacologically relevant model system for VHD prediction. We report here that several drugs known to induce VHD in humans, as well as MDMA and MDA, elicit prolonged mitogenic responses in hVICs. Our results strongly suggest, therefore, that MDMA and MDA are valvulopathogenic; retrospective echocardiographic studies in human MDMA users are currently in progress to test this notion.

These studies also showed that h5-HT2B receptor activation plays a critical role in the transduction of a mitogenic signal by VHD-associated drugs, strongly supporting the hypothesis that h5-HT2B Receptor agonists are likely to cause VHD. In this regard, we demonstrated that mitogenesis was abrogated by coincubation with a 5-HT2B/2C-selective antagonist (SB206553; see http://kidb.bioc.cwru.edu/pdsp.php for comprehensive pharmacological profile of SB206553). Because human cardiac valves express large quantities of 5-HT2B receptors and do not express appreciable amounts of 5-HT2C receptors (Fitzgerald et al., 2000), it is likely that the inhibition by SB206553 is principally caused by 5-HT2B receptor blockade. It is conceivable that the residual stimulation of [3H]thymidine incorporation not blocked by SB206553 might be caused by activation of mitogenic 5-HT2A receptors, because human heart valves express 5-HT2A mRNA [although sheep VICS apparently predominantly express 5-HT2A receptors (Xu et al., 2002)], and the drugs studied herein are low-affinity, low-efficacy 5-HT2A agonists (Nash et al., 1994; Rothman et al., 2000; Roy et al., 2000). Arguing against such a role for 5-HT2A receptors in the mitogenic response of hVICs is the observation that the genetic ablation of 5-HT2A receptors, but not of 5-HT2B receptors (J. Gingrich, personal communication), interferes with myocardial proliferation during embryonic development, suggesting that the activation of mitogenic pathways by 5-HT2A receptors in heart valves is not essential for cardiac development (Nebigil et al., 2000a,b). Taken together, these results imply that

![Fig. 3.](image-url)

*Fig. 3. MDMA and MDA induce mitogenesis in human heart valve interstitial cells in vitro. A, stimulation of [3H]thymidine deoxyribose incorporation in VICs treated for 48 h with either vehicle (V), 5-HT, the 5-HT2B antagonist SB206553 (SB), fenfluramine (F), norfenfluramine (NF), MDMA (X), or MDA (M) reveals a prolonged mitogenic response that is blocked by pretreatment with SB (B). All drugs used at 10 μM except SB206553, which was used at 1 μM. **, P < 0.05; *** P < 0.01; **** P < 0.001, significant difference from vehicle-treated cells by two-tailed t test. C, immunoblot analysis of Erk 1/2 phosphorylation in VICs treated for 10 min with norfenfluramine, MDMA, MDA, SB206553, or 5-HT reveals a short-term mitogenic response (i.e., increase in percent of total cellular Erk 1/2 phosphorylated; see Materials and Methods for details) after exposure to 5-HT2B receptor agonists. *, P < 0.05; **, P < 0.01, significant difference from vehicle-treated cells by two-tailed t test.
activation of mitogenic pathways by 5-HT$_{2A}$ receptors is
essential for cardiac development and that the 5-HT$_{2B}$ recep-
tor is most likely responsible for the mitogenic responses
induced by valprovapholic drugs. Other findings implicating
the h5-HT$_{2B}$ receptor as the proximal molecular target
responsible for fenfluramine-like VHD are the observations
that h5-HT$_{2B}$ receptors 1) are enriched in human heart
valves; 2) are essential for normal cardiac development; and
3) induce, upon activation, prolonged mitogenic responses in
heterologous expression systems (Fitzgerald et al., 2000; Nebigil et al., 2000b).

Our discovery that pergolide and dihydroergotamine, two
drugs reported to induce VHD in humans (Creutzig, 1992; Pritchett et al., 2002), also activate h5-HT$_{2B}$ receptors in
vitro validates the use of recombinant h5-HT$_{2B}$ receptors to
screen for valprovapholic potential. Of equal importance,
recent data have implicated the 5-HT$_{2B}$ receptor in the
pathogenesis of primary pulmonary hypertension, a severe
and frequently fatal illness (Launay et al., 2002). Impor-
tantly, in this regard, fenfluramine use increases the risk of
developing primary pulmonary hypertension (Abenhaim et
al., 1996). Thus, these data further highlight the necessity of
screening current and potential pharmacotherapies for ago-
nist potencies and efficiencies at human 5-HT$_{2B}$ receptors
and validate the use of 5-HT$_{2B}$ receptor-expressing cell lines as
models to do so. The data presented herein are thus of major
public health importance because they suggest that MDMA
abuse, which is at an all-time high, puts an expanding pop-
ulation at increased risk for developing VHD and primary
pulmonary hypertension.

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