Neurochemical Neutralization of Methamphetamine With High-Affinity Nonselective Inhibitors of Biogenic Amine Transporters: A Pharmacological Strategy for Treating Stimulant Abuse

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ABSTRACT   The abuse of methamphetamine (METH) and other amphetamine-like stimulants is a growing problem in the United States. METH is a substrate for the 12-transmembrane proteins which function as transporters for the biogenic amines dopamine (DA), serotonin (5-HT), and norepinephrine (NE). Increased release of CNS DA is thought to mediate the addictive effects of METH, whereas increased release of NE in both the peripheral and CNS is thought to mediate its cardiovascular effects. The neurotoxic effects of METH on both dopaminergic and serotonergic nerves requires the transport of METH into the nerve terminals. Thus, transport of METH into nerve terminals is the crucial first step in the production of METH-associated pharmacological and toxicological effects. A single molecular entity which would block the transport of METH at all three biogenic amine transporters might function to neurochemically neutralize METH. This agent would ideally be a high-affinity slowly dissociating agent at all three transporters, and also be amenable to formulation as a long-acting depot medication, such as has been accomplished with an analog of GBR12909. As a first step towards developing such an agent, we established an in vitro assay which selectively detects transporter substrates and used this assay to profile the ability of a lead compound, indatraline, to block the releasing effects of METH and MDMA at the DA, 5-HT, and NE transporters. The major finding reported here is that indatraline blocks the ability of METH and MDMA to release these neurotransmitters. **Synapse 35:222–227, 2000.**   Published 2000 Wiley-Liss, Inc.[†]

INTRODUCTION

The abuse of methamphetamine (METH) and other amphetamine-like stimulants is a growing problem in the United States (Anonymous, 1995). This abuse continues to spread to geographical areas not previously exposed concurrent with dramatic increases in the number of illicit METH laboratories seized by authorities (DEA, 1998). The use of these substances produces a number of adverse effects, including paranoid and psychotic behavior, myocardial infarction, and stroke (Anonymous, 1995), and long-term depletion of brain biogenic amines, such as dopamine (DA) and serotonin (5-HT) (Ricaurte and McCann, 1992), the clinical implications of which remain to be elucidated.

METH is a substrate for the 12-transmembrane proteins which function as transporters for the biogenic amines DA, 5-HT, and norepinephrine (NE). METH is transported by these proteins into the nerve terminal, where the drug promotes the release of neurotransmitter by a two-pronged mechanism: 1) increased cytoplasmic neurotransmitter by interfering with the accumulation of neurotransmitter in storage vesicles, and 2) a process of carrier-mediated exchange (Rudnick and Clark, 1993). Increased release of CNS DA is thought to

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mediate the addictive effects of METH (Wise, 1996), whereas increased release of NE in both the peripheral and central nervous systems is thought to mediate its cardiovascular effects. The neurotoxic effects of METH on both dopaminergic and serotonergic nerves requires the transport of METH into the nerve terminals (Seiden et al., 1993). Thus, transport of METH into nerve terminals is the crucial first step in the production of METH-associated pharmacological and toxicological effects.

Compounds that bind to biogenic amine transporters but are not themselves transported, i.e., reuptake inhibitors, block the effects of amphetamine-type releasing agents. For example, the high-affinity DA reuptake inhibitor GBR12909 attenuates amphetamine-induced increases in extracellular DA (Baumann et al., 1994; Villemagne et al., 1999) and fluoxetine, a selective inhibitor of 5-HT reuptake, blocks fenfluramine-induced increases in extracellular 5-HT (Gundlah et al., 1997). Although a selective DA reuptake inhibitor might block the reinforcing effects of METH, such an agent would not protect against the other adverse effects mediated by NE and 5-HT transporters. A single molecular entity which would block the transport of METH at all three biogenic amine transporters might function to neurochemically neutralize METH. Such an agent would ideally be a high-affinity slowly dissociating agent at all three transporters and also be amenable to formulation as a long-acting depot medication, such as has been accomplished with an analog of GBR12909 (Glowa et al., 1996). We anticipate that a medication meeting these requirements would not only neutralize the neurochemical effects of METH, but would also contribute anticraving and antidepressant effects, facilitating relapse prevention.

As a first step towards developing a METH-neutralizing agent, we established an in vitro assay which selectively detects transporter substrates and used this assay to profile the ability of a lead compound, indatraline (Hyttel and Larsen, 1985), to block the releasing effects of METH and MDMA at the DA, 5-HT, and NE transporters.

MATERIALS AND METHODS
[³H]DA, [³H]5-HT, and [³H]NE reuptake assays

The effect of test agents on [³H]DA and [³H]5-HT reuptake was evaluated using published methods (Rothman et al., 1993). Briefly, synaptosomes were prepared from rat caudate for [³H]DA reuptake, or from whole rat brain minus cerebellum for [³H]5-HT reuptake. Fresh tissue was homogenized in ice-cold 10% sucrose using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1,000g for 10 min at 4°C and supernatants were retained on ice and used in the reuptake assays as described previously (Rothman et al., 1993). [³H]NE reuptake proceeded with minor modifications of the above procedure: the tissue source was whole rat brain

TABLE I. Ki values of test agents for inhibiting biogenic amine reuptake*

Drug	[³H]DA Reuptake Ki (nM ± SD)	[³H]5-HT Reuptake Ki (nM ± SD)	[³H]NE Reuptake Ki (nM ± SD)
Reuptake Inhibitors			
Indatraline	1.90 ± 0.10	3.11 ± 0.16	11.4 ± 1.3
Fluoxetine[1]	>5000	9.58 ± 0.88	688 ± 97
GBR 12935[1]	3.70 ± 0.4	289 ± 29	277 ± 23
Desipramine	5946 ± 355	350 ± 29	8.32 ± 1.19
Substrates			
Methamphetamine	114 ± 11	2137 ± 214	48.0 ± 5.1
MDMA	1571 ± 130	239 ± 22	462 ± 76

*[³H]DA, [³H]NE and [³H]5-HT reuptake assays were conducted as described in Methods. Each value is the mean ± sem (n = 3). Under these conditions, the Km values for [³H]DA and [³H]5-HT reuptake are 38.3 ± 1.6 nM and 17.4 ± 0.8 nM, respectively (mean ± SD, n = 3) (Rothman et al., 1993).
[1]Data are from (Rothman et al., 1993).

minus caudate, 5 nM RTI-229 was used to block reuptake of [³H]NE into DAergic nerves, the incubations proceeded for 10 min at 25°C, the concentration of [³H]NE was 5 nM, and nonspecific binding was defined using 10 µM indatraline or 10 µM tyramine.

[³H]DA, [³H]NE, and [³H]5-HT "release" assays

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 1,000g for 10 min at 0–4°C and the supernatants were retained on ice (synaptosomal preparation). Each rat brain (approximately 1,200 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 Krebs-phosphate buffer (pH 7.4) which contained 154.4 mM NaCl, 2.9 mM KCl, 1.1 mM CaCl₂, 0.83 mM MgCl₂, 5 mM glucose, 1 mg/ml ascorbic acid, 50 µM pargyline, and 1 µM reserpine in a polypropylene beaker with stirring at 25°C. Nomifensine (100 nM) and GBR12935 (100 nM) were added to the Krebs-phosphate buffer for [³H]5-HT release experiments. RTI-229 (5 nM) was added to the Krebs-phosphate buffer for [³H]NE release experiments to prevent [³H]NE reuptake into dopamine nerve terminals. At the appropriate time, 900 µl of synaptosomes preloaded with [³H]neurotransmitter were added to 12 × 75 mm polystyrene test tubes which contained 100 µl test drug made up in the Krebs-phosphate buffer which had added to it 1 mg/ml bovine serum albumin. After 5 min ([³H]DA and [³H]5-HT) or 30 min ([³H]NE) the release reaction was terminated by dilution with
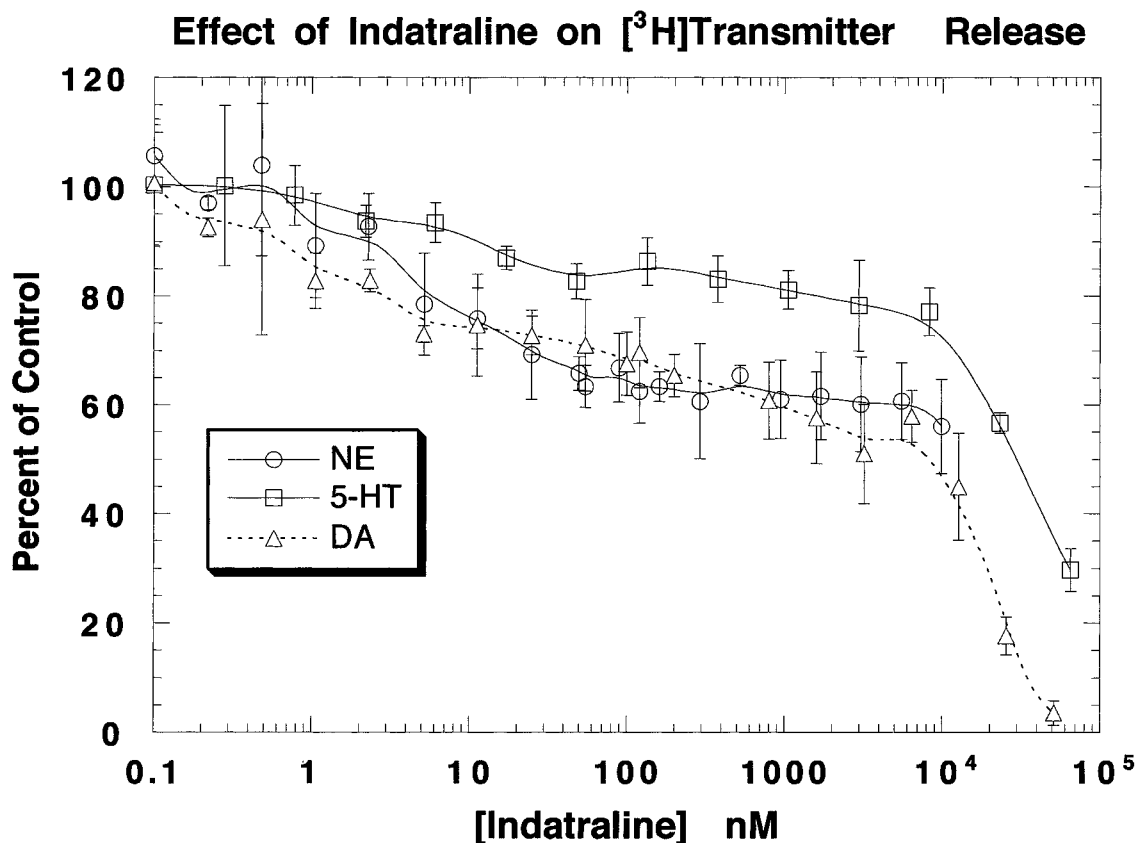
Effect of Indatraline on [^{3}H]Transmitter   Release

Fig. 1.   Effect of indatraline on [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT release. Each point is the mean ± SEM (n = 3).

4 ml 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. The filters were rinsed twice with 4 ml 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 Cytoscint (ICN). Nonspecific binding was measured by incubations in the presence of 100 µM tyramine for [^{3}H]5-HT release and 10 µM tyramine for [^{3}H]DA and [^{3}H]NE release.

Data analysis and statistics

As previously described (Rothman et al., 1993), IC50 values were determined using the nonlinear least-squares curve-fitting program MLAB-PC (Civilized Software, Bethesda, MD). In reuptake experiments, Ki values were calculated according to the formula: Ki = IC50/(1+L/Km) where L is the concentration of the radiolabeled drug ([^{3}H]DA, [^{3}H]NE, or [^{3}H]5-HT). In release experiments, the apparent Ki of indatraline was calculated according to the following formula: Ki = [Indatraline]/ (IC50$_2$/IC50$_1$ -1) where IC50$_1$ is the IC50 in the absence of indatraline and IC50$_2$ is the IC50 in the presence of indatraline. Statistical significance of

the differences between mean values were determined using ANOVA with Student-Newman-Keuls post-hoc analysis or the F-test.

RESULTS

Table 1 reports the Ki values for indatraline, METH, MDMA, and some reference compounds inhibiting [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT reuptake. As reported previously (Hyttel and Larsen, 1985), indatraline has high affinity at all three transporters. Its rank order of potency for the transporters was DA (Ki = 1.9 nM) > 5-HT (Ki = 3.11 nM) > NE (Ki = 11.4 nM). METH was most potent at inhibiting [^{3}H]NE reuptake (Ki = 48 nM), followed by [^{3}H]DA (Ki = 114 nM), and then [^{3}H]5-HT (Ki = 2137 nM).

In the release assay (Fig. 1), indatraline generally had a biphasic effect on retained [^{3}H]neurotransmitter. Low concentrations in the range where indatraline blocks reuptake decreased retained [^{3}H]neurotransmitter by less than 20%. Concentrations of indatraline in excess of 10 µM were required to produce substantial decreases in retained [^{3}H]neurotransmitter. In contrast, METH caused a potent dose-dependent release of [^{3}H]DA (IC50 = 22.3 nM), [^{3}H]NE (IC50 = 11.5 nM) and [^{3}H]5-HT (IC50 = 881 nM) (Fig. 2, Table 2). METH was
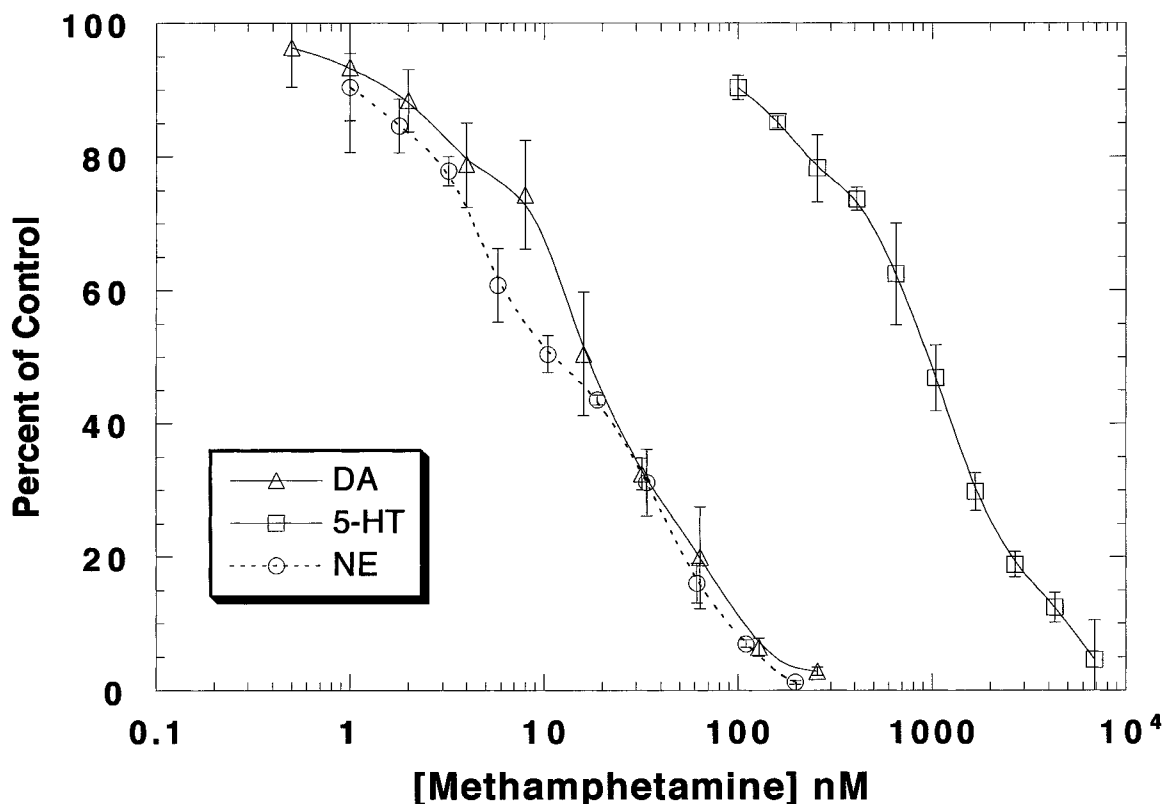
Effect of Methamphetamine on [³H]Transmitter   Release

Fig. 2.   Effect of METH on [³H]NE, [³H]DA, and [³H]5-HT release. Each point is the mean ± SEM (n = 3).

TABLE II. *Indatraline-antagonism of METH- and MDMA-induced release of biogenic amines**

Drug	[³H]DA Release IC50 (nM ± SD) [Indatraline Apparent Ki, nM]	[³H]5-HT Release IC50 (nM ± SD) [Indatraline Apparent Ki, nM]	[³H]NE Release IC50 (nM ± SD) [Indatraline Apparent Ki, nM]
METH			
Indatraline 0 nM	22.3 ± 4.5	881 ± 92	11.5 ± 1.6
Indatraline "x" nM	110 ± 20	1642 ± 120	34.2 ± 6.1
	[5.0 nM]	[3.5 nM]	[6.6 nM]
Indatraline "y" nM	438 ± 79	6223 ± 527	153 ± 19
	[1.3 nM]	[2.5 nM]	[5.3 nM]
MDMA			
Indatraline 0 nM	376 ± 28	55.7 ± 4.7	64.2 ± 6.1
Indatraline "x" nM	1971 ± 262	139 ± 15.4	394 ± 100
	[1.2 nM]	[2.0 nM]	[2.5 nM]
Indatraline "y" nM	7076 ± 1117	393 ± 33	1056 ± 111
	[1.4 nM]	[2.5 nM]	[4.2 nM]

*The effect of METH and MDMA on [³H]DA, [³H]NE and [³H]5-HT release was determined in the absence and presence of two concentrations of indatraline, chosen to be approximately 1-times and 5-times the Ki value of indatraline for the respective transporter system. The "x" concentration was 5 nM, 13 nM and 3 nM for [³H]DA, [³H]NE and [³H]5-HT and the "y" concentration was 25 nM, 65 nM and 15 nM for [³H]DA, [³H]NE and [³H]5-HT. Each value is the mean ± sem (n = 3). Apparent Ki values were calculated as described in the Methods.

more potent in the release assay than in the reuptake inhibition assay. MDMA caused a potent dose-dependent release of [³H]NE (IC50 = 64.2 nM), [³H]5-HT (IC50 = 55.7 nM) and [³H]DA (IC50 = 376 nM). As reported in Figure 3, indatraline, added at a concentration equal to approximately 1-times and 5-times its Ki value for inhibition of [³H]DA reuptake, caused a dose-dependent shift-to-the-right in the METH release curve. Indatraline alone at these concentrations had minimal effects on [³H]DA, [³H]NE, and [³H]5-HT release. Similar data were obtained for METH-induced release of [³H]NE and [³H]5-HT as well as MDMA-induced release of [³H]DA, [³H]NE, and [³H]5-HT (Table 2). The apparent Ki values of indatraline calculated from the degree of rightward-shift produced in the METH- and MDMA-release curves were close to the Ki

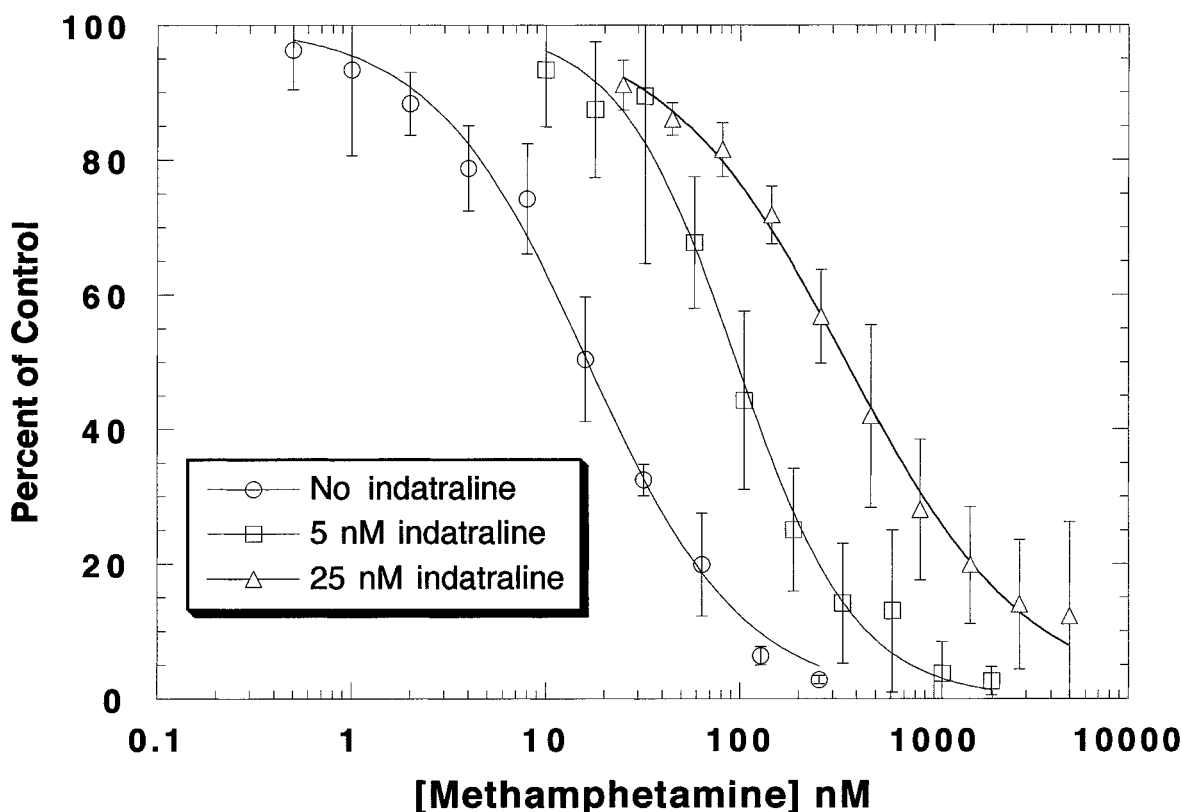## Effect of Indatraline on Methamphetamine-Induced [³H]DA Release

Fig. 3.   Effect of indatraline (5 nM and 25 nM) on METH-induced release of [³H]DA. Each point is the mean ± SEM (n = 3).

values of indatraline for the DA, 5-HT and NE transporters, as determined in the reuptake inhibition assays.

DISCUSSION

The major finding reported here is that indatraline, a reuptake inhibitor with high affinity for NE, DA, and 5-HT transporters (Hyttel and Larsen, 1985), blocks the ability of METH and MDMA to release these neurotransmitters. The results demonstrate that an indatraline, or a similarly acting agent, has the potential to neutralize the neurochemical effects of METH and MDMA, and therefore attenuate not only the addictive effects of these agents, possibly mediated via mesolimbic DA, but to also block their cardiovascular and neurotoxic effects. As noted in the Introduction, we anticipate that a high-affinity slowly dissociating agent at all three transporters, which would also be amenable to formulation as a long-acting depot medication, would not only neutralize the neurochemical effects of METH, but would also contribute anticraving and antidepressant effects, thereby facilitating relapse prevention.

Thus, our efforts in this direction will be to develop and characterize indatraline-like agents with the goal of developing a proof-of-principle medication to test in various animal models of METH addiction and toxicity.

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