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In vivo pharmacological actions of two novel inhibitors of anandamide cellular uptake

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Abstract

Two inhibitors of the cellular uptake of the endocannabinoid anandamide, (*R*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine and (*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine (OMDM-1 and OMDM-2, respectively), were recently synthesized, and their in vitro pharmacological activity described. Here we have assessed their activity in two typical pharmacological responses of cannabimimetic compounds. We first examined whether these compounds exert any effect per se on locomotion and pain perception in rats, and/or enhance the effects of anandamide on these two processes. We compared the effects of the novel compounds with those produced by a previously developed selective inhibitor, *N*-arachidonoyl-(2-methyl-4-hydroxyphenyl)amine (VDM-11). When assayed alone, OMDM-1 and OMDM-2 (1–10 mg/kg, i.p.) did not affect any of the five motor parameters under investigation, although the former compound exhibited a trend for the inhibition of ambulation, fast movements, and speed in rats. OMDM-2 and, to a lesser extent, VDM-11 (5 mg/kg, i.p.) enhanced the motor-inhibitory effects of a noneffective dose (2 mg/kg, i.p.) of anandamide, while OMDM-1 did not. In a typical test of acute analgesia, OMDM-2 and VDM-11 (1–10 mg/kg, i.p.), but not OMDM-1, significantly enhanced the time spent by rats on a "hot plate." However, the same compounds (5 mg/kg, i.p.) did not enhance the analgesic effect of a subeffective dose (2 mg/kg, i.p.) of anandamide, whereas OMDM-1 exerted a strong trend towards potentiation ($P = 0.06$). We next explored the possible use of the two novel compounds in a pathological condition. Thus, we determined if, like other previously developed anandamide reuptake inhibitors, OMDM-1 and OMDM-2 inhibit spasticity in an animal model of multiple sclerosis—the chronic relapsing experimental allergic encephalomyelitis in mice. As previously shown with a higher dose of VDM-11, both novel compounds (5 mg/kg, i.v.) significantly reduced spasticity of the hindlimb in mice with chronic relapsing experimental allergic encephalomyelitis. We suggest that OMDM-1 and, particularly, OMDM-2 are useful pharmacological tools for the study of the (patho)physiological role of the anandamide cellular uptake process, and represent unique templates for the development of new antispastic drugs.
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1. Introduction

Endocannabinoids are endogenous compounds capable of binding to, and functionally activating, the two cannabinoid CB_1 and CB_2 receptors for marijuana's active principle, Δ^9-tetrahydrocannabinol (Di Marzo and Fontana, 1995: Pertwee, 1997). Five prototypical endocannabinoids have been described to date: *N*-arachidonoylethanolamine (anandamide) (Devane et al., 1992), 2-arachidonoylglycerol (Mechoulam et al., 1995; Sugiura et al., 1995), 2-arachidonylglyceryl ether (noladin) (Hanus et al., 2001; Fezza et al., 2002); *O*-arachidonoylethanolamine (virodhamine) (Porter et al., 2002), and *N*-arachidonoyl-dopamine (Bisogno et al., 2000; Huang et al., 2002). A function as neuromodulators has been proposed for the endocannabinoids (Di Marzo et

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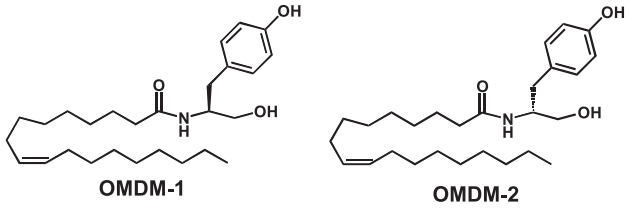

Fig. 1. Chemical structures of OMDM-1 and OMDM-2.

al., 1998), which requires for these substances specific biosynthetic and metabolic mechanisms subject to regulation during physiological and pathological conditions.

While the enzymes responsible for endocannabinoid biosynthesis have not been characterized yet, proteins for endocannabinoid metabolism have been cloned. As all endocannabinoids are metabolised by intracellular enzymes, in order to become substrates for these enzymes, they need to be taken up by cells. A membrane transporter (for a review, see Hillard and Jarrahian, 2000) has been suggested to facilitate the uptake of all endocannabinoids, according to the gradient of concentration across the cell membrane. This suggestion was based on several observations that: (1) anandamide, 2-arachidonoyl-glycerol, noladin, and *N*-arachidonoyl-dopamine are rapidly taken up by both neuronal and nonneuronal cells in a saturable, temperature-dependent and, in the case of anandamide, energy-independent manner (Di Marzo et al., 1994; Hillard et al., 1997; Beltramo and Piomelli, 2000; Bisogno et al., 2001; Fezza et al., 2002; Huang et al., 2002); (2) 2-arachidonoyl-glycerol, noladin, *N*-arachidonoyl-dopamine, and virodhamine compete with anandamide cellular uptake, and anandamide competes with 2-arachidonoyl-glycerol, noladin, and *N*-arachidonoyl-dopamine uptake (Beltramo and Piomelli, 2000; Bisogno et al., 2000, 2001; Fezza et al., 2002); and (3) anandamide and 2-arachidonoyl-glycerol uptake is subject to selective inhibition by some anandamide analogues and to stimulation by nitric oxide (Beltramo et al., 1997; Bisogno et al., 2001; Maccarrone et al., 1998, 2000).

Unlike the enzymes for anandamide and 2-arachidonoyl-glycerol hydrolysis, that is, the fatty acid amide hydrolase (see Ueda et al., 2001 for review) and the monoacylglycerol lipase (Dinh et al., 2002), the putative endocannabinoid transporter has not been cloned yet. This and the fact that fatty acid amide hydrolase and monoacylglycerol lipase play an important role in determining the rate of anandamide and 2-arachidonoyl-glycerol cellular uptake (Bisogno et al., 2001; Deutsch et al., 2001) led some authors to suggest that endocannabinoid transport across the plasma membrane is not carrier-mediated and occurs uniquely via passive diffusion dependent on the rate of intracellular metabolism (Glaser et al., 2003). Accordingly, some inhibitors of anandamide cellular uptake are also fatty acid amide hydrolase inhibitors (Jarrahian et al., 2000) and vice versa (Deutsch et al., 2001; Glaser et al., 2003). However, several indirect observations support the existence of an endocannabinoid transporter and, in particular: (1) some substances selectively inhibit anandamide cellular uptake without inhibiting fatty acid amide hydrolase (Di Marzo et al., 2002; Ortar et al., 2003; Lopez-Rodriguez et al., 2003); (2) fatty acid amide hydrolase inhibitors enhance, and anandamide uptake inhibitors inhibit, anandamide accumulation in cells (Kathuria et al., 2003); (3) cells that do not express fatty acid amide hydrolase still rapidly take up anandamide (Di Marzo et al., 1999; Deutsch et al., 2001); (4) inhibitors of anandamide cellular uptake enhance those effects of anandamide that are mediated by cannabinoid receptors, but inhibit those effects that are exerted on the cytosolic side of membrane proteins, such as the stimulation of vanilloid VR1 receptors (De Petrocellis et al., 2001) or the inhibition of T-type Ca^{2+} channels (Chemin et al., 2001); (5) *N*-arachidonoyldopamine and noladin are still rapidly taken up by cells, and yet they are either very stable or refractory to enzymatic hydrolysis, respectively (Fezza et al., 2002; Huang et al., 2002); (6) lipopolysaccharide inhibits fatty acid amide hydrolase expression without affecting anandamide cellular uptake (Maccarrone et al., 2001); conversely, nitric oxide, peroxynitrite, and superoxide anions stimulate anandamide cellular reuptake (Maccarrone et al., 2000), while acute or chronic ethanol inhibits this process (Basavarajappa et al., 2003) without affecting fatty acid amide hydrolase activity.

Although the existence of the endocannabinoid transporter is still controversial, it is a fact that inhibitors of endocannabinoid cellular uptake have proven to be useful compounds for the manipulation of endocannabinoid levels both under physiological and pathological conditions where endocannabinoids exert a tonic beneficial action (for a review, see Baker et al., 2003). A few examples exist in the literature of the use of endocannabinoid uptake inhibitors to alleviate symptoms of some disorders in animal models. *N*-arachidonoyl-(4-hydroxyphenyl)-amine (AM-404) has been used against hyperkinesia in

Table 1
Dose-related effect of OMDM-1 on the five parameters of locomotor activity in rats, as measured in a computerized actimeter

Parameter	Vehicle ($n=6$)	OMDM-1 (1 mg/kg) ($n=6$)	OMDM-1 (5 mg/kg) ($n=6$)	OMDM-1 (10 mg/kg) ($n=6$)
Ambulation	1001.4 ± 226.8	867.4 ± 223.7	657.0 ± 203.8	516.1 ± 115.1
Inactivity	162.6 ± 27.7	162.6 ± 21.5	188.4 ± 30.1	190.9 ± 20.2
Fast movements	60.4 ± 20.8	58.8 ± 19.9	44.0 ± 15.6	38.6 ± 11.2
Speed	3.3 ± 0.7	2.9 ± 0.7	2.2 ± 0.7	1.7 ± 0.4
Exploratory activity	10.2 ± 2.6	10.8 ± 3.7	6.2 ± 1.9	7.8 ± 2.4

See details in the text. Values are mean ± S.E.M. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.

Table 2
Dose-related effect of OMDM-2 on the five parameters of locomotor activity in rats, as measured in a computerized actimeter

Parameter	Vehicle ($n=12$)	OMDM-2 (1 mg/kg) ($n=6$)	OMDM-2 (5 mg/kg) ($n=12$)	OMDM-2 (10 mg/kg) ($n=6$)
Ambulation	1183.1 ± 89.0	1183.6 ± 87.0	1324.4 ± 181.0	1145.3 ± 31.8
Inactivity	122.9 ± 12.3	106.6 ± 7.9	131.2 ± 16.8	123.8 ± 2.5
Fast movements	89.6 ± 8.3	96.2 ± 6.2	98.8 ± 15.4	93.1 ± 2.8
Speed	4.1 ± 0.3	4.1 ± 0.3	4.4 ± 0.6	3.9 ± 0.1
Exploratory activity	12.5 ± 2.2	15.5 ± 2.5	12.6 ± 2.3	14.5 ± 1.6

See details in the text. Values are mean ± S.E.M. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.

a model of Huntington's chorea, but its effects on motor behaviour are due to direct activation of vanilloid VR1 receptor, rather than indirect activation of cannabinoid CB_1 receptors (Lastres-Becker et al., 2003). *N*-arachidonoyl-(2-methyl-4-hydroxyphenyl)amine (VDM-11), which, unlike AM-404, does not activate vanilloid VR1 receptors (De Petrocellis et al., 2000), has been used successfully against: (1) spasticity, in a mouse model of multiple sclerosis (Baker et al., 2001); (2) striatal glutamatergic hyperactivity, in a rat model of Parkinson's disease (Maccarrone et al., 2003); and (3) excessive intestinal secretion, in a mouse model of cholera toxin intoxication (Izzo et al., 2003). In the present study, we have investigated the effects on locomotion and central acute pain perception (two typical pharmacological responses of cannabimimetic compounds) of two novel inhibitors of the putative endocannabinoid membrane transporters, (*R*)-(*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine and (*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine (OMDM-1 and OMDM-2), which are both more potent and more metabolically stable in vitro than AM-404 and VDM-11 (Ortar et al., 2003). Furthermore, we have assessed the effect of the two compounds in a mouse model of multiple sclerosis, a disease where cannabinoids have been proposed as therapeutic substances to alleviate symptoms as spasticity and pain (for a review, see Baker et al., 2003).

2. Materials and methods

2.1. Test substances

OMDM-1, OMDM-2, and VDM-11 (Fig. 1) were synthesized in our laboratories as described previously (De Petrocellis et al., 2000; Ortar et al., 2003).

2.2. Animals, treatments, and sampling

Male Wistar rats were housed in a room with controlled photoperiod (0800–2000 h light) and temperature (23 ± 1 °C). They had free access to standard food and water. Animals were used at about 2 months of age (250–350 g weight) in all experiments, which were always conducted according to European and local rules on the care of and research with experimental animals. In a first experiment, rats were injected intraperitoneally (i.p.) with three different doses (1, 5, and 10 mg/kg) of OMDM-1 or OMDM-2, or with vehicle (Tween 80–saline, 1:16). Ten minutes later, animals were assessed in the hot-plate test and, immediately afterwards, in a computerized actimeter (Actitrack system). The response of the two compounds was compared with that produced by the previously described anandamide uptake inhibitor, VDM-11. In a second experiment, rats were divided into three series (one for each compound) of four groups and subjected to the following i.p. injections: (i) vehicle (Tween 80–saline, 1:16); (ii) an intermediate dose (5 mg/kg) of OMDM-1, OMDM-2, or VDM-11; (iii) a subeffective dose of anandamide (2 mg/kg); and (iv) the combination of OMDM-1, OMDM-2, or VDM-11, and anandamide, administered at the same time. Ten minutes later, animals were assessed in the hot-plate test and, immediately afterwards, in the Actitrack system.

2.3. Actimeter

Motor activity was analyzed in a computerized actimeter (Actitrack, Panlab, Barcelona, Spain). This consisted of a 45 × 45-cm arena, with infrared beams all around, spaced 2.5 cm, coupled to a computerized control unit, which allows the analysis, among others, of the following parameters: (i) distance run in the actimeter (ambulation); (ii) mean speed employed to run all distance; (iii) fast move-

Table 3
Effect of OMDM-1 on the action of anandamide (AEA) on the five parameters of locomotor activity in rats, as measured in a computerized actimeter

Parameter	Vehicle ($n=6$)	OMDM-1 (5 mg/kg) ($n=6$)	AEA (2 mg/kg) ($n=6$)	+Both ($n=6$)	Statistics (one-way ANOVA)
Ambulation	810.6 ± 81.9	958.9 ± 326.1	528.2 ± 142.6	489.4 ± 170.2	$F=1.11$, ns
Inactivity	214.0 ± 26.6	164.9 ± 36.5	237.5 ± 23.6	224.4 ± 22.3	$F=1.422$, ns
Fast movements	57.2 ± 8.3	72.9 ± 26.0	33.2 ± 12.2	28.7 ± 12.3	$F=1.545$, ns
Speed	1.7 ± 0.6	3.2 ± 1.1	1.1 ± 0.5	1.6 ± 0.6	$F=1.66$, ns
Exploratory activity	5.2 ± 3.0	7.8 ± 4.3	4.6 ± 2.8	1.4 ± 0.7	$F=0.854$, ns

See details in the text. Values are mean ± S.E.M. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.

Table 4
Effect of OMDM-2 on the action of anandamide (AEA) on the five parameters of locomotor activity in rats, as measured in a computerized actimeter

Parameter	Vehicle ($n=12$)	OMDM-2 (5 mg/kg) ($n=12$)	AEA (2 mg/kg) ($n=6$)	Both ($n=6$)	Statistics (one-way ANOVA)
Ambulation	1183.1 ± 89.0	1324.4 ± 181.0	814.3 ± 186.5	587.9 ± 152.2*	$F=4.08$, $P<0.05$
Inactivity	122.9 ± 12.3	131.2 ± 16.8	190.8 ± 16.5*	205.7 ± 23.1*	$F=5.46$, $P<0.005$
Fast movements	89.6 ± 8.3	98.8 ± 15.4	53.4 ± 13.7**	41.2 ± 8.1***	$F=3.75$, $P<0.05$
Speed	4.1 ± 0.3	4.4 ± 0.6	2.7 ± 0.6**	1.9 ± 0.5*	$F=4.247$, $P<0.05$
Exploratory activity	12.5 ± 2.2	12.6 ± 2.3	7.2 ± 1.3	3.6 ± 1.1**	$F=2.86$, $P=0.05$

See details in the text. Values are mean ± S.E.M. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.
* $P<0.01$ vs. vehicle and OMDM-2.
** $P<0.05$ vs. vehicle and OMDM-2.
*** $P<0.005$ vs. vehicle and OMDM-2.

ments (speed >5 cm/s); (iv) resting time (inactivity); and (v) number of head entries into the square holes in the arena (exploratory activity). The analysis of motor activity was done immediately after the hot-plate test, for a period of 8 min, although only the last 5 min were scored (the first 3 min served as the period of habituation to the novel environment, which reduced the influence of emotional aspects).

2.4. Hot-plate analysis

For the assay of central acute antinociception, we used the hot-plate procedure described by Girard et al. (2001). Rats were placed individually on a hot plate maintained at 52 °C, and the latency to exhibit the first sign of pain (i.e., licking the hind paws or jumping) was measured for each rat. Animals not responding were removed after 30 s (cutoff time to avoid tissue damage).

2.5. Statistical analysis of the actimeter and hot-plate tests

Data were assessed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test using the GraphPad® software.

2.6. Spasticity in chronic relapsing experimental allergic encephalomyelitis in mice

Induction of chronic relapsing experimental autoimmune encephalomyelitis following injection of spinal cord homogenate in Freund's adjuvant in 6- to 8-week-old Biozzi ABH mice, and assessment of spasticity against a strain gauge were carried out as described previously (Baker et al., 2000). Animals were injected intravenously (i.v.) with OMDM-1 or OMDM-2, dissolved in ethanol, cremophor (Sigma, Poole, UK), and phosphate-buffered saline (1:1:18). Injection of vehicle alone failed to inhibit spasticity over a 1-h observation period. Results were compared (pairwise) using one-way repeated-measures analysis of variance (Student–Newman–Keuls method) using SigmaStat v2.0 (Jandel, USA). The results represent the mean ± S.E.M. resistance to hindlimb flexion (Newtons). Each group contained a minimum of six animals and each limb was analysed as an individual result.

3. Results

3.1. Effect of OMDM-1, OMDM-2, and VDM-11 on locomotion in a computerized actimeter

When administered per se, OMDM-1 (1–10 mg/kg) did not exhibit any statistically significant effect on the five parameters of motor behaviour under study. However, a slight trend for a dose-dependent inhibition of ambulation and speed, and for a dose-dependent enhancement of time spent in inactivity was observed (Table 1). By contrast, OMDM-2 (1–10 mg/kg) or VDM-11 (5 mg/kg) did not affect in any way any of the five parameters under study (Table 2 and data not shown).

When OMDM-1 and OMDM-2 (5 mg/kg) were coadministered with a subeffective concentration of anandamide (2 mg/kg), only the latter compound enhanced the motor-inhibitory actions of the endocannabinoid (Tables 3 and 4). In particular, in the first experiment, anandamide

Table 5
Effect of VDM-11 on the action of anandamide (AEA) on the five parameters of locomotor activity in rats, as measured in a computerized actimeter

Parameter	Vehicle ($n=6$)	AEA (2 mg/kg) ($n=6$)	VDM-11 (5 mg/kg) ($n=6$)	Both ($n=6$)	Statistics (one-way ANOVA)
Ambulation	1234.1 ± 127.4	1047.2 ± 173.4	1120.7 ± 127.9	946.1 ± 77.8	$F=0.81$, ns
Inactivity	83.7 ± 15.8	127.0 ± 19.9	117.9 ± 10.2	140.1 ± 8.3	$F=2.87$, $P=0.06$
Fast movements	93.1 ± 14.1	74.8 ± 5.3	71.2 ± 11.1	70.6 ± 6.8	$F=1.18$, ns
Speed	4.1 ± 0.4	3.5 ± 0.6	3.8 ± 0.4	3.2 ± 0.3	$F=0.79$, ns
Exploratory activity	20.8 ± 5.3	16.2 ± 2.3	15.5 ± 2.8	14.0 ± 3.3	$F=0.67$, ns

See details in the text. Values are mean ± S.E.M. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.

alone did not exhibit any significant effect on any of the five parameters tested, and coadministration with OMDM-1 did not result in any effect of anandamide, except for a slight trend towards inhibition of the exploratory activity (Table 3). In the second experiment, anandamide exerted a statistically significant inhibitory action per se on fast movements and speed, and enhanced significantly the time spent in inactivity. In this case, coadministration with OMDM-2 led to a significant effect also on ambulation and exploratory activity, and appeared to enhance the effects of anandamide also on the other three parameters (Table 4). In a third experiment, anandamide was again inactive in all tests, and coadministration with VDM-11 (5 mg/kg) did not lead to a statistically significant effect in any of the five parameters under study, except for a very strong trend towards enhancing the time spent in inactivity ($P=0.06$) (Table 5).

3.2. Effect of OMDM-1, OMDM-2, and VDM-11 on analgesia in the "hot-plate" test

When administered alone, OMDM-1 (1–10 mg/kg) did not affect the latency time of rats on a "hot plate," whereas OMDM-2 produced a slight, albeit significant, analgesic effect only at the intermediate dose of 5 mg/kg. At this same dose, VDM-11 was also active per se (Table 6). When coadministered with anandamide (2 mg/kg), which exerted a significant analgesic action only in one out of three experiments, OMDM-1, but not OMDM-2 or VDM-11, exhibited an almost statistically significant ($P=0.06$) trend for the potentiation of anandamide analgesia (Table 7).

3.3. Effect of OMDM-1 and OMDM-2 on spasticity in chronic relapsing experimental allergic encephalomyelitis in mice

Both OMDM-1 and OMDM-2 demonstrated significant inhibition ($P<0.001$) of spasticity (Fig. 2A and B). These assays were performed on different groups of animals, which prevents direct comparisons of absolute activities of the compounds. However, the effect of OMDM-2 remained stable 60 min after the administration, and was still significant 90 min from injection (mean resistance to flexion = 0.143 ± 0.0119, $n=12$, $P<0.001$, and data not shown). The effect of OMDM-1 was instead only tested up to 60 min since it already started to decline at this time point.

Table 6
Dose-related effect of OMDM-1, OMDM-2, and VDM-11 on the latency to respond to a noxious stimulus measured in the hot-plate test

Compound	Vehicle	1 mg/kg	5 mg/kg	10 mg/kg
OMDM-1	8.7 ± 1.1 ($n=6$)	7.3 ± 1.0 ($n=6$)	7.0 ± 0.7 ($n=6$)	7.7 ± 1.7 ($n=6$)
OMDM-2	5.0 ± 0.3 ($n=12$)	6.0 ± 0.5 ($n=6$)	6.3 ± 0.6* ($n=12$)	7.2 ± 0.9 ($n=6$)
VDM-11	6.8 ± 0.4 ($n=6$)	8.3 ± 0.7 ($n=6$)	11.5 ± 0.8* ($n=6$)	7.5 ± 0.8 ($n=6$)

See details in the text. Values are mean ± S.E.M. with the number of determinations per group in parentheses. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.
* $P<0.05$ vs. vehicle.

Table 7
Effect of OMDM-1, OMDM-2, and VDM-11 on the action of anandamide (AEA) on the latency to respond to a noxious stimulus measured in the hot-plate test

Compound	Vehicle	AEA (2 mg/kg)	Compound (5 mg/kg)	Both	Statistics (one-way ANOVA)
OMDM-1	6.4 ± 0.5 ($n=6$)	5.8 ± 0.4 ($n=6$)	4.8 ± 0.4 ($n=6$)	7.0 ± 0.8 ($n=6$)	$F=2.82$, $P=0.06$
OMDM-2	5.0 ± 0.3 (12)	6.3 ± 0.9 ($n=6$)	6.3 ± 0.6* ($n=12$)	4.2 ± 0.3 ($n=6$)	$F=3.182$, $P<0.05$
VDM-11	8.0 ± 0.7 ($n=12$)	10.7 ± 0.9* ($n=6$)	11.4 ± 0.7 ($n=12$)**	14.0 ± 3.3 ($n=6$)*	$F=3.154$, $P<0.05$

See details in the text. Values are mean ± S.E.M. with the number of determinations per group in parentheses. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test.
* $P<0.05$ vs. vehicle.
** $P<0.01$ vs. vehicle.

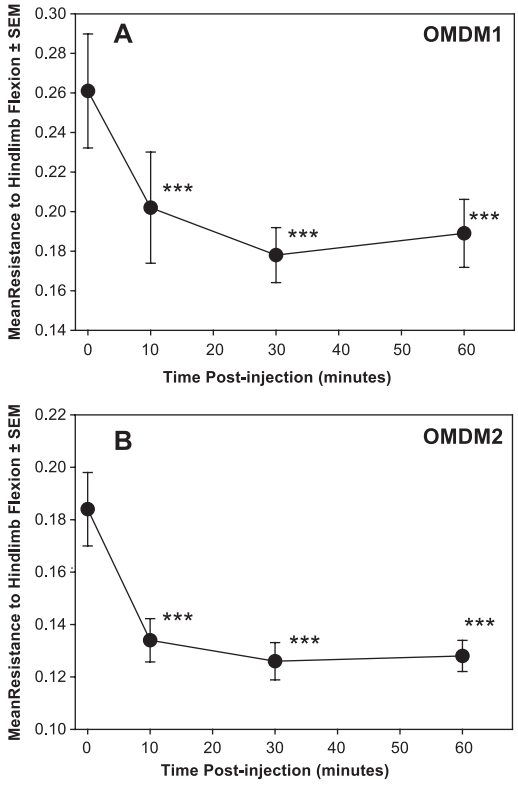

Fig. 2. Inhibition of spasticity by OMDM-1 and OMDM-2. Following the induction of chronic relapsing experimental allergic encephalomyelitis, mice developed spasticity 60–80 days postinduction. The resistance to hindlimb flexion against a strain gauge was measured before and after i.v. injection with OMDM-1 or OMDM-2. The results demonstrate the mean ± S.E.M. resistance forces after treatment ($n>10$ group).

4. Discussion

The potential therapeutic value of *Cannabis sativa* and its major components, Δ^9-tetrahydrocannabinol and cannabidol, is currently being reevaluated. In the case of Δ^9-tetrahydrocannabinol and its synthetic analogues capable of activating cannabinoid CB_1 receptors in the brain, the possible therapeutic applications are hindered by their psychotropic side effects. For this reason, the possibility that substances capable of activating cannabinoid CB_1 receptors indirectly (i.e., by enhancing endocannabinoid levels) might represent templates for the development of new therapeutic drugs is being investigated. These inhibitors are, in fact, more likely to increase the concentrations of endocannabinoids only at sites where their biosynthesis or inactivation has been altered with pathological consequences, without greatly altering the activity of cannabinoid receptors at other sites, at least when administered at the doses normally used for direct agonists. To date, several examples of the use of inhibitors of endocannabinoid inactivation with beneficial effects on symptoms of several human disorders in animal models have been reported. For example, VDM-11, a selective inhibitor of endocannabinoid uptake, was shown to ameliorate spasticity in the chronic relapsing experimental allergic encephalomyelitis model of multiple sclerosis (Baker et al., 2001), to reduce hyperactivity of glutamatergic neurons and ameliorate movement in a rat model of Parkinson's disease (Maccarrone et al., 2003), and to inhibit intestinal hypersecretion and diarrhea in cholera toxin-treated mice (Izzo et al., 2003). On the other hand, also genetic or acute inactivation of fatty acid amide hydrolase, with subsequent increase of the endogenous levels of anandamide, but not 2-arachidonoyl-glycerol, has been found to lead to analgesia (Martin et al., 2000; Cravatt et al., 2001) or inhibition of anxiety (Kathuria et al., 2003). To date, several more or less selective inhibitors of the putative endocannabinoid membrane transporter have been designed. In this study, we have aimed at investigating the effects in vivo of two very recently developed inhibitors, OMDM-1 and OMDM-2 (Ortar et al., 2003). These two compounds, unlike the widely used AM-404, are inactive as fatty acid amide hydrolase inhibitors and do not activate the vanilloid VR1 receptors. Unlike both AM-404 and VDM-11, they are also metabolically stable in vitro, and are among the most potent inhibitors ever characterized of anandamide cellular uptake by isolated intact cells (Ortar et al., 2003). Finally, like other similar inhibitors, OMDM-1 and OMDM-2 are almost inactive at cannabinoid CB_1 and CB_2 receptors. In general, the two compounds share several of the unique advantageous features of the recently developed UCM-707 (Lopez-Rodriguez et al., 2001, 2003), whose metabolic stability was not investigated, but whose effects in vivo, including the significant potentiation of anandamide actions on locomotion and central antinociception, appears to be very promising (De Lago et al., 2002).

When the spontaneous motor behaviour of rats in an open field was studied by analysing five parameters, we found that neither OMDM-1 nor OMDM-2 exerted any statistically significant effect per se, even at the highest of the three doses used (10 mg/kg), although the former compound did exhibit a trend towards a general hypokinetic action, which might be presumably ascribed to small increases in endogenous cannabinoid tone produced by the blockade of the transporter at that highest dose. In the case of OMDM-1, the testing of an even higher dose might have resulted in achieving a statistically significant effect, although this possibility could not be tested because of solubility problems. On the other hand, it is less likely that OMDM-2 would have exerted a significant action at a higher dose, since its effect appeared to peak (admittedly nonsignificantly) at the 5 mg/kg dose. By contrast, VDM-11 (Lastres-Becker et al., 2003) and UCM-707 (De Lago et al., 2002) were previously found to enhance the time spent in inactivity at the highest doses tested (5 and 10 mg/kg, respectively), possibly for the same reason. Interestingly, the effect of VDM-11 (5 mg/kg) on immobility was not confirmed in the present study, where a different protocol for the measurement of motor behaviour was used. In the case of AM-404, this compound was not tested here but it was found previously to induce very strong effects on rat motor behaviour even when different protocols are used (Gonzalez et al., 1999; Giuffrida et al., 2000), and even though it is less active on anandamide uptake than UCM-707. The effects of AM-404, however, appear to be mostly mediated by the vanilloid VR1 receptor (Lastres-Becker et al., 2003), whose activation can lead to both enhanced anandamide production and hypolocomotion (Di Marzo et al., 2001; Ahluwalia et al., 2003). Therefore, the lack of significant activity on motor behaviour of OMDM-1 and OMDM-2, if confirmed by studies carried out using also different experimental protocols, would suggest that these compounds are totally inactive as ''direct'' agonists of ''central'' cannabinoid CB_1 or vanilloid VR1 receptors also in vivo, at least at the doses used here, and would render them unique (in the 5–10 mg/kg, i.p. dose range) among the anandamide uptake inhibitors developed so far. Like other previously developed inhibitors of anandamide uptake, however, OMDM-2 did potentiate the negligible or weak hypokinetic effects of a low dose of anandamide, particularly on ambulation and exploratory activity, whereas OMDM-1 was inactive, and VDM-11 appeared to be generally less efficacious and exhibited a strong trend towards the potentiation only of the effect of anandamide on inactivity. The lack of efficacy of OMDM-1 or VDM-11 might be due, in part, to the fact that in the set of experiments where these compounds were tested, the effects of anandamide on locomotion did not achieve statistical significance. Indeed, some inconsistencies were observed between the various experiments as regards the effects of anandamide. These are probably a consequence of the use of a subeffective dose of the

endocannabinoid, whose effect, depending on variable factors and on the number of replicates, may or may not reach statistical significance. On the other hand, the use of a greater dose of anandamide would have prevented any potentiating effect of the uptake inhibitors from being observed.

Another typical effect of endocannabinoids is the inhibition of nociception in several paradigms of pain models (Walker and Huang, 2002). When the latency to respond to an acute painful stimulus was measured (“hot-plate” test), OMDM-2 behaved differently from OMDM-1, and in a similar way to VDM-11. In fact, while OMDM-1 per se was inactive at all doses, both VDM-11 and OMDM-2 exhibited an antinociceptive effect that was not dose-related and statistically significant only at the dose of 5 mg/kg. The analgesic effect of drugs or other conditions that inhibit the inactivation of endocannabinoids has been already observed also with fatty acid amide hydrolase inhibitors (Martin et al., 2000; Cravatt et al., 2001; Kathuria et al., 2003), and is suggestive of a possible antinociceptive tonic action of endogenous cannabinoids, deserving further investigation in view of the development of novel analgesics. Indeed, although we did not investigate whether the effects of OMDM-2 and VDM-11 per se were due to an enhancement of anandamide endogenous levels, one should not forget that it is the capability of these compounds to indirectly activate cannabinoid CB_1 receptors, possibly only in peripheral tissues, which would justify their therapeutic use (see below). On the other hand, of the three compounds tested in this study, only OMDM-1 exerted an almost statistically significant potentiation of the analgesic action of exogenous anandamide. VDM-11 also appeared to potentiate the effect of anandamide, but this effect, which was not statistically significant, possibly because of the high S.E.M. value for this data point, might have arisen from a mere additive action of the two drugs. From the above data, it seems that, at least in the present study, a statistically significant potentiation of anandamide effects, or a very strong trend towards it, by anandamide uptake inhibitors is observed particularly in those cases when the inhibitors are totally inactive if administered alone. This suggests that a possible enhancement of anandamide effects might be masked by the effects of the inhibitors. On the other hand, the use of doses of inhibitors active per se might have prevented us to distinguish between a potentiation or a simple additive effect of the two compounds on anandamide actions. These considerations, and the possible interaction of high doses of OMDM-1 and OMDM-2 with cannabinoid receptors (see Ortar et al., 2003), advised us against testing doses of the inhibitors higher than 5 mg/kg.

Once we had determined that at least one of the two novel inhibitors was able to enhance at least some of the anandamide behavioural actions, and hence was suitable to be used in vivo, we next wanted to assess the effects of OMDM-1 and OMDM-2 on a typical sign of multiple sclerosis in a mouse model of this disorder—the chronic relapsing experimental allergic encephalomyelitis in Biozzi mice (Baker et al., 2000). In this model, other inhibitors of endocannabinoid inactivation, including VDM-11, were previously found to be very effective against spasticity (Baker et al., 2001). Their effect was blocked by a combination of cannabinoid CB_1 and CB_2 receptor antagonists, thus suggesting that these substances were indeed acting by enhancing the levels of endocannabinoids, which exert a tonic antispastic effect (Baker et al., 2000, 2001). AM-404 inhibited spasticity in this model, but at a much lower concentration (2.5 mg/kg), probably because also activation of vanilloid VR1 receptors contributes to antispastic effects in this model of multiple sclerosis (Brooks et al., 2002). In the present study, OMDM-1 and OMDM-2 exhibited, at the dose of 5 mg/kg, an antispastic effect that was as efficacious as that previously observed with a higher dose (10 mg/kg) of VDM-11 (Baker et al., 2001). Furthermore, while the antispastic effects of selective inhibitors of endocannabinoid inactivation and OMDM-1 start being reversed 60 min from their administration (Baker et al., 2001), the effect of OMDM-2 was still strong at 90 min from treatment (see Results). Our data suggest that OMDM-1 and, particularly, OMDM-2 represent potentially useful templates for the development of potent antispastic agents in multiple sclerosis. Due to its analgesic effect observed here with the same dose, OMDM-2 might be effective also in counteracting pain, which is another impairing symptom typical of this disorder.

In conclusion, we have provided for the first time data on the possible use in vivo of OMDM-1 and OMDM-2, two newly developed inhibitors of the putative endocannabinoid transporter, both as enhancers of actions of exogenously administered anandamide and as possible palliatives against spasticity in an animal model of multiple sclerosis, as an alternative to “direct” cannabinoid CB_1 receptor agonists. Although subtle differences in the pharmacological activities of the two compounds were observed, these might have been due to the fact that they were never compared in the same sets of experiments. Further studies are now to be fostered to fully assess the suitability of both compounds as pharmacological tools, as well as therapeutic drugs in disorders for which *Cannabis* and cannabinoids have been suggested to have a beneficial action.

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