Cannabinoid Type 2 Receptor as a Target for Chronic - Pain

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Abstract: Availability of selective pharmacological tools enabled a great advance of our knowledge of cannabinoid receptor 2 (CB2) role in pathophysiology. In particular CB2 emerged as an interesting target for chronic pain treatment as demonstrated by several studies on inflammatory and neuropathic preclinal pain models. The mechanisms at the basis of CB2-mediated analgesia are still controversial but data are pointing out in two main directions: an effect on inflammatory cells and/or an action on nociceptors and spinal cord relay centers. In this review will be described the second messenger pathways activated by CB2 agonists, the data underpinning the analgesic profile of CB2 selective agonists and the mechanisms invoked to explain their analgesic action. Finally the ongoing clinical trials and the potential issues for the development of a CB2 agonist drug will be examined.

Key Words: Analgesia, agonist, neuropathic pain, signalling pathways.

INTRODUCTION

Chronic pain is a multifarious disease that affects a large cohort of patients. Its etiology is frequently classified based on the most relevant component (i.e. inflammatory, neuropathic, neoplastic, etc.), even though more components could be simultaneously present.

During the years several drugs, ranging from morphine to lidocaine from Gabapentin to Duloxetine, were developed to treat different painful pathological states. Despite their commercial success these medicines are falling short to fulfill the medical need due to either a quite partial pain relief or relevant side effects. In certain cases side effects could be severe enough to force patients to discontinue the use of the medication. Furthermore, beyond opioid analgesics, no agent achieves acceptable relief in more than 50% of treated patients, leaving considerable scope for improvement in efficacy. Thus the search for new drug targets to treat chronic pain has been fostered by all major pharmaceutical companies. Between the different targets under evaluation cannabinoid receptors occupy a significant position. Initially the interest on cannabinoid receptors as potential targets for chronic pain was restricted to cannabinoid receptor 1 (CB1), with the aim of identifying an agonist suitable to be developed into a drug. Unfortunately analgesia induced by CB1 agonists is associated to undesirable central side effects hampering the clinical development of these molecules. More recently the type 2 cannabinoid receptor (CB2) emerged as an interesting alternative to CB1. Its expression is upregulated in response to tissue or nerve injury and in pre-clinical pain models activation of this receptor induces significant analgesia without overt side effects.

In the last years a few reviews were published on CB2 and pain [1-3] that cover also aspects that are not treated in the present review and to which the reader could refer for additional information. The present review will focus on the following aspects: signal transduction pathways triggered by CB2 activation; evidence of analgesic activity of CB2 selective agonists in pre-clinical pain models; possible mechanisms underpinning CB2 agonist-induced analgesia; and finally ongoing clinical trials and critical issues that should be overcome to develop a CB2 agonist drug.

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CB2 RECEPTOR SIGNALLING PATHWAYS

The CB2 is a member of the G protein-coupled receptor (GPCR) superfamily. The main signal transduction pathways triggered by its stimulation relay on $G_{i/o}$ proteins activation, as demonstrated by inhibition of CB2 downstream signalling following pertussis toxin treatment [4]. Several second messenger pathways have been suggested to be modulated by CB2: cAMP, ERK1/2, p38, AKT/PKB, PI3K/AKT, NFkB, Ca^{2+}, JNK, PKA, NF-AT, CREB/ATF, JAK/STAT1, Caspase3 and 8, and ceramide. Some of them are well characterized (e.g. cAMP and ERK1/2), whereas for others data are still scanty (e.g. ceramide and NF-AT).

cAMP

One of the results of $G_{i/o}$ protein activation is the decrease of the cAMP level caused by adenylate cyclase inhibition. The decrease of cAMP intracellular level is, at present, considered the main downstream effect of CB2 activation, a claim supported by several studies showing inhibition of forskolin-induced cAMP production in CB2-transfected cell lines (CHO and HEK) [5-15]. However, in non recombinant systems, that is either immortalized cell line naturally expressing CB2 (HL60, Daudi, BV2, and HPB-ALL) or primary culture (splenocytes, T and B lymphocytes), inhibition was quite partial, reducing forskolin-induced cAMP accumulation by around 40-45%, or not detected at all [16-22]. In addition a shift in potency is also evident with maximal effect generally achieved only at quite high agonist concentration. The reason for this difference in efficacy and potency amongst recombinant and native systems still remains to be clarified. Nevertheless, a likely explanation of this discrepancy could reside in the higher expression level of the receptor in recombinant systems compared to native ones.

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MAP Kinase

Another important transduction pathway induced by CB2 stimulation leads to the activation of the MAP kinase cascade. MAP kinases are key signalling molecules that regulate many cellular functions such as cell growth, cell transformation and apoptosis. Two main pathways have been described to be triggered by CB2 agonists: the ERK1/2 (p42/p44 MAPK) and the p38 MAPK. CB2-induced MAP kinases activation cascade is pertussis toxin sensitive, indicating that this mechanism is $G_{i/o}$–mediated [16, 23].

There is a general consensus that CB2 activation elicits ERK1/2 phosphorylation in both recombinant and native expression systems [11,16,23-29] with the only exception of Daudi cells [16]. Interestingly activation of MAP kinases was reported not only in cells of the immune system (HL-60 and Jurkat), but also, using the selective agonist HU308, in cells of the central nervous system (CNS) [29]. Similarly microglia/macrophage cells (RTMGLI and RAW264.7) when stimulated with either 2-arachidonylglycerol (2-AG), suggested to be the endogenous ligand for the CB2, [25] or JWH133 [26] showed an increase of ERK1/2 phosphorylation. Taken together these results suggest the presence of functional CB2 in neural cells, even though its expression and function could be ontogenetically restricted or confined to specific cellular populations.

The molecular events located downstream to $G_{i/o}$ protein and leading to MAP kinase activation are currently not completely elucidated. However, it was shown that treatment with GF109203X (a protein kinase C inhibitor) abolished CB2-mediated MAP kinase activation suggesting that a PKC lies on the route between $G_{i/o}$ and MAP kinase [16], probably linked by RAS activation. In human prostate epithelial PC-3 cells another possible mechanism of MAP kinase induction by CB2 was described. This mechanism relaying on PI3K/PKB pathway would induce translocation of Raf1 to the membrane and subsequent MAP kinase activation [30].

It has been also shown that activation of MAP kinase through CB2 plays a role in the control of gene transcription by phosphorylating transcription factors, such as Krox-24, which modulate expression of target genes [16].

NF-kB

A third intracellular pathway that has been linked to CB2 activation is NF-kB. This pathway plays a relevant role in the modulation of immune system function, but recently it was reported to be involved in various patho-physiological aspects of cell activity also outside the immune system. Data on the effect of CB2 stimulation on this pathway are, however, discordant since some indicate an inhibition and others an activation or a biphasic effect [17, 24, 31, 32]. In mouse spleenocytes and thymocytes cannabidiol was reported to inhibit the NF-kB/c-Rel pathway at early time post-administration [17]. Similar results were obtained with THC in the macrophage cell line RAW264.7 [33]. On the other hand in HL-60 cells transfected with human CB2 and stimulated by CP55,940 a biphasic regulation of IkB-α expression was observed [24]. An early decrease of IkB-α protein, due to degradation, was followed by an increase of IkB-α gene expression and protein levels with ensuing changes in NF-kB activity. Finally in human coronary artery endothelial cells, natively expressing CB2, the use of either CP55940 or JWH015 elicited an activation of the NF-kB pathway [32]. It remains unclear if inconsistencies are originated by differences in experimental procedures, could be related to the use of different agonists, are linked to the cell type, or could be due to dissimilarity between pathways activated by CB2 stimulation in human and rodent.

Calcium

Recently was reported that CB2 activation could lead also to changes in intracellular calcium level [11, 34-37]. This hypothesis is supported by two lines of evidence coming from calcium imaging studies on dorsal root ganglia (DRG) cells and calcium assay on CB2 transfected cells. Calcium imaging studies performed on adult DRG neurons obtained from normal, neuropathic (spinal nerve ligation: SNL) and sham-operated rats, showed that JWH133 attenuated capsaicin-evoked calcium responses. This effect was inhibited by the CB2-selective antagonist SR144528, but not by the CB1 antagonist SR141716A [36,37]. Consistently with an initial report showing that in CHO cells transfected with human CB2 the mixed agonist WIN55212-2 was unable *per se* to modulate intracellular [Ca^{2+}] [6], also JWH133 when applied alone on DRG neurons did not modify intracellular calcium level [37]. However, more recent data obtained either in calf pulmonary artery endothelial cells [35] or in recombinant CHO cells expressing the human receptor [11], suggest that CB2 agonists (Anandamide, 2-AG, CP55949 and Noladin ether) increase intracellular Ca^{2+}. At present it is not clear if this inconsistency is linked to the use of different agonists, as would be suggested by the agonist direct trafficking of receptor (ADTR) theory [11], or different experimental protocols. Because of the importance that Ca^{2+} modulation could have in the role played by CB2 agonists in intracellular signalling, further studies clarifying this aspect of CB2 pharmacology would be useful.

Other pathways such as JAK/STAT1 [38], JNK [28], ceramide [39], caspsase [28], PI3K/Akt [40], etc. have been associated to CB2 activation. However the relevance of these pathways in CB2 signalling is still poorly known and additional studies would be essential to fully appreciate their role in the intracellular events induced by CB2 stimulation.

Finally it has also been reported that HSP90 could directly interact with CB2 and influence CB2-mediated cell migration. HSP90 may serve as a scaffold protein to keep CB2 and its signalling components in proximity, thus facilitating CB2-mediated signalling [41]. If this observation will be confirmed this interaction could be an extremely relevant system in the control of CB2 action.

From this overview on second messenger pathways emerges a complex, and still only partially understood, downstream signalling cascade linked to CB2 activation. The diversity of cell types and agonists (few of which are CB2 selective) used in the different studies leave open the question if part of this complexity could be associated to cell specific or agonist specific mechanisms. Particularly interesting in this respect is the ADTR theory [11]. This theory is based on the assumption that GPCRs exist in multiple active conformational states and that distinct active conformations may

couple the receptor to different intracellular effectors. Each agonist potentially could have a different affinity for each conformational state of the receptor and could thus ultimately facilitate the activation of a specific effector pathway, different from that activated by another agonist that preferentially bind to a different conformational state. From a drug development perspective it would be of paramount importance to confirm published data that suggest that this theory hold true for CB2 [11]. The fallout would be that selective activation of different second messenger pathways could lead from one side to a higher specificity of intervention and from the other to an improved therapeutic activity.

CB2 AGONISTS *IN VITRO* PROFILE AND ANALGESIC PROPERTIES

The analgesic effects of CB2 stimulation were studied with pharmacological tools that, based on their chemical structure (Fig. **1**), could be divided into several groups: bicyclic cannabinoids that lack the pyrane ring (HU308 and Cannabinor, **1** and **2**, Fig. **1**); 1-deoxy-3-(1'-1'-dimethylbutyl)-Δ^8-THC derivatives (JWH133, **3**, Fig. **1**); aminoalkylindoles (AM1241, L768242, A-796260, **4, 5** and **6,** Fig. **1);** pyrimidine derivatives (GW842166X, **7**, Fig. **1**), thiazole derivatives (A-836339, **8**, Fig. **1**), carboxiamide derivatives (Taisho, **9**, Fig. **1**) and thiazine derivatives (Shonogi, **10**, Fig. **1**). Not all of these compounds have been completely profiled *in vitro*. For some of them data are still quite partial and in particular some relevant information, such as selectivity, efficacy at the rat CB2 and PK data, is missing. Considering that CB2 ligands could show pharmacological differences at rat and human receptor [10,14,15,42], it is important to use caution when drawing inferences from the human to the rat and *viceversa*. Available information on selectivity, potency and *in vitro* efficacy of these compounds are reported in Tables **1** and **2**. In the following part of the review published evidence of analgesic action will be described for each compound.

BICYCLIC CANNABINOIDS

HU308

The interest on CB2 as potential target for pain treatment was triggered by an article published in 1999 [43] presenting, for the first time, evidence of an analgesic effect induced by CB2 selective activation (Table **1**, **2**). In this study the second phase of the nociceptive behaviours induced by intraplantar formalin injection was significantly reduced following i.p. administration of the potent and selective CB2 agonist HU308 (**1**, Fig. **1**, Table **3**) [43]. HU308 reduced also intestinal motility and lowered blood pressure, but, on the other hand, was inactive in the tetrad of test (locomotor activity, catalepsy, hypothermia and acute analgesia) normally

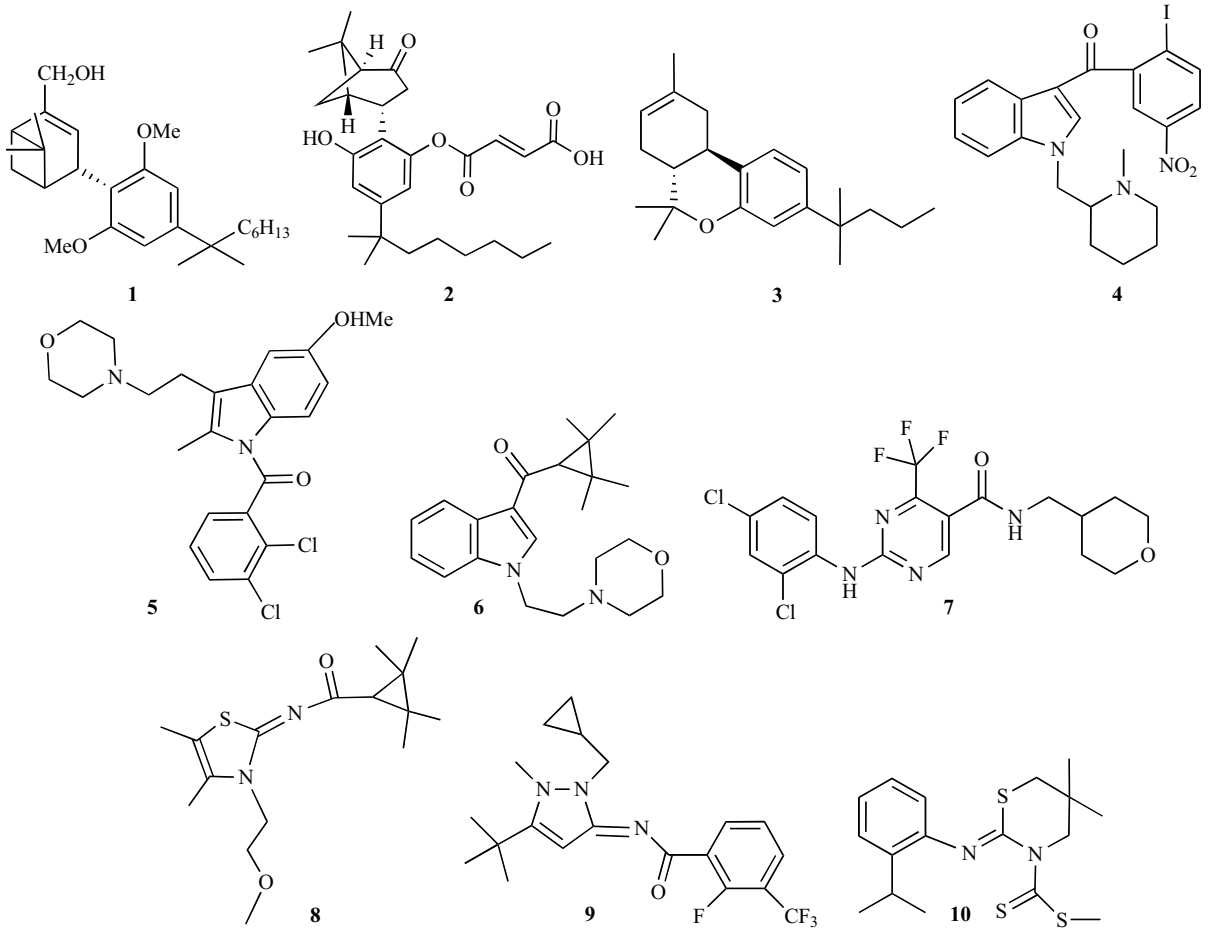

Fig. (1).

Table 1. CB2 Agonists Radioligand Binding Studies

Compound	K_i nM					
	Human Receptor			Rat Receptor		
	CB2	CB1	Reference	CB2	CB1	Reference
HU308	22.7[a]	NA	[43]	NA	>10,000[a]	[43]
Cannabinor (PRS211375)	1[b]	87[b]	[96]	NA	NA	-
JWH133	3.4[c] 20[c] 76[c]	677[c] NA 2718[c]	[47] [48] Unpublished data	300[c]	3210[c]	Unpublished data
AM1241 (racemic mixture)	7.1[c] 28.7[c] 11.5[c]	580[c] >1000[c] 1270[c]	[13] [15] [14]	3[d] (mouse) 2[d] (mouse) 3.4[c] (mouse)	240[d] 680[d] 280[c]	[64] [59] [54]
				26.7[c] 23.8[c] (mouse)	NA	[15]
				3.4[c]	115[c]	[14]
R(+)AM1241	6[c] 15[c]	240[c] 5000[c]	Unpublished data [15]	3.3[c]	279[c]	Unpublished data
				12[c] 13[c] (mouse)	NA	[15]
S(-)AM1241	658[c]	>1000[c]	[15]	893[c] 577[c] (mouse)	NA	[15]
L768242 (GW405833)	12[d] 4[c] 7.6[c] 5[c]	1900[d] 4772[c] 282[c] 904[c]	[66] [12] [14] Unpublished data	3.6[c] 11.1[c] 4[c]	273[c] NA 842[c]	[12] [14] Unpublished data
A-796260	0.8[c] 4.4[c]	330[c] 845[c]	[69] [14]	7.3[c] 13.0[c]	395[c] 395[c]	[69] [14]
GW842166X	2000[c] 2147[c]	>10000[c] 1157[c]	[14] Unpublished data	2580[c] 876[c]	>10000[c] 3464[c]	[14] Unpublished data
A-836339	0.4[c]	273[c]	[72]	0.7[c]	143[c]	[72]
Taisho 6b	2.9[c]	4000[c]	[76]	NA	NA	-
Shionogi 13	9[c]	>5000[c]	[77]	9[c] (mouse)	2020[c] (mouse)	[77]
GRC10622	10.6[d]	837[d]	[78]	3.4[d]	164.8[d]	[78]

[a] [^{3}H]HU243.

[b] The species was not reported.

[c] [^{3}H]CP55940.

[d] Radioligand unknown.

NA Not available.

used to evaluate CB1 activation [43]. This indicates the absence of the typical side effects associated to CB1 stimulation that can be linked to psychomimetic action in human. After this initial report HU308 has not been particularly exploited as a pharmacological tool to study CB2-induced analgesia. The only other published report showed that HU308 is an effective analgesic in an acute pain model, the hindpaw incision model of pain [44]. This analgesic effect was reverted by SR144528 confirming a CB2-mediated action [44].

PRS211375 (Cannabinor) and other Pharmos' Compounds

Pharmos group licensed HU308 from the Hebrew University and initiated a SAR study to improve its pharmacological profile. This effort generated a CB2 receptor agonist, PRS211375, called Cannabinor (**2**, Fig. **1**, Table **1**). In binding studies selectivity of Cannabinor over hCB1 was reported to be around 80-90 folds, conversely data on rCB2 affinity and selectivity over rCB1 are not available. In the last years results on the analgesic effect produced by Cannabinor in pain models were presented at various meeting [45, 46] (Table **3**). Cannabinor showed a broad antinociceptive spectrum producing analgesia in chronic neuropathic pain model (CCI), inflammatory (Carrageenan), as well as in visceral and chemotherapeutics induced pain models (taxol-induced neuropathy) [45, 46]. Conversely the compound was reported to have little analgesic effect in acute pain model (i.e. tail flick).

Pharmos has also synthesized and tested other CB2 agonists (PRS211096 and PRS211359, an ester of PRS211096);

Table 2. CB2 Agonist Potency and Efficacy *In Vitro.* If not Otherwise Specified Reported Data were Obtained with cAMP Functional Assay

Compound	Human Receptor					Rat Receptor				
	hCB2		hCB1		Reference	rCB2		rCB1		Reference
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}		EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	
HU308	5.57	109%[a]	> 1000	72 %[a] (@ 10000 nM)	[43]	NA	NA	NA	NA	
JWH133	18 29 146[‡]	NA 102%[b] 149%[c]	> 1000 NA NA	NA NA NA	[47] [50] [48]	63 (mouse) 48	106%[a] 82%[b]	2500 (mouse) NA	94%[a] NA	[49] [50]
AM1241 (Racemic mixture)	190 >1000	60%[d] NA	NA 2650	NA 101[b]	[15] [14]	216 >1000 NA	-30%[d] NA -76%[a] (mouse)	NA 1970 64	NA 81%[b] 81%[a] (mouse)	[15] [14] [49]
(+)AM1241	ND 118	ND 40%[c]	NA NA	NA NA	[50] [15]	4 315 341 (mouse)	-19%[b] -30%[c] 70%[c] (mouse)	NA NA NA	NA NA NA	[50] [15] [15]
(-)AM1241	131	80%[c]	NA	NA	[15]	785 2000 (mouse)	80%[c] 80%[c]	NA NA	NA NA	[15] [15]
L768242 (GW405833)	0.6 44.4 10	44.6%[b] -30%[b] -25%[b]	NA NA NA	NA NA NA	[12] [14] [50]	3.3 5	- 39%[b] -75%[b]	916 NA	70%[b] NA	[14] [50]
A-796260	0.7	78%[b]	983	118%[b]	[14]	1.6	69%[b]	286	97%[b]	[14]
GW842166X	63[§] 133 95	95%[e] 101%[b] 76%[b]	>30000 >25000 NA	NA NA NA	[70] [14] [50]	91[§] 96 33	100%[e] 95%[b] 105%[b]	NA 26.000 NA	NA 33%[b] NA	[70] [14] [50]
A-836339	1.6	102%[b]	>1000	NA	[72]	0.5	71%[b]	1137	130%[b]	[72]
Taisho 6b	1.8[‡]	85%[b]	NA	NA	[76]	NA	NA	NA	NA	
Shionogi 13	6	NA	>5000	NA	[77]	9 (mouse)	NA	2020 (mouse)	NA	[77]
GCR10622	0.6	NA	857	NA	[78]	NA	NA	>10000[‡]	NA	[78]

[‡] GTPγS assay

[§] Yeast expression system in which degradation of FDGlu to fluorescein due to yeast exogluconase is measured

[a] Unkonw reference for E_{max} % calculation

[b] vs CP55940

[c] Basal was set as 100% effect

[d] Of maximal response

[e] Versus HU210

NA Not available

ND Not determined

at present both *in vitro* and *in vivo* data on these compounds are very limited.

Δ8-THC DERIVATIVES

JWH133

This compound is derived from the classical cannabinoid structure (**3,** Fig. **1**)**:** it has been shown to have good selectivity over hCB1 (Table **1**) [47, 48] and full efficacy at rodent CB2 (Table **2**) [49,50]. Behavioural studies assessing JWH133 analgesic effects are quite limited (Table **3**). In the formalin model the analgesic activity of JWH133 was assessed after either i.c.v. or i.p. administration, but antinociceptive effects were observed only after i.p. administration [51]. In the carrageenan model the injection of the compound, either pre or post carrageenan administration, resulted in a reduction of tactile allodynia suggesting that JWH133 may reverse established inflammatory hypersensitivity [52]. Very interestingly JWH133 was able, after i.t. administration, to reverse mechanical allodynia induced by partial

Table 3. Analgesic Effect of CB2 Selective Agonists in Pre-Clinical Pain Models

Compound	Pain Model	Behavioral Readout	Route	Active Doses	Reference
HU308	Formalin	Nocifensive response (1^{st} and 2^{nd} phase)	i.p.	50 mg/kg (only 2^{nd} phase)	[43]
	Incision model of postoperative pain	Tactile allodynia (Von Frey hairs)	i.p.	30 mg/kg	[44]
Cannabinor (PRS211375)	Tail Flick	Withdrawal latency	i.p. or p.o.	unkonwn	[45]
	Carrageenan	Mechanical hyperalgesia	i.p. or p.o.	unkonwn	[45]
	Carrageenan	Thermal hyperalgesia	i.p. or p.o.	unkonwn	[45]
	Acute visceral pain (acetic acid)	Writhing	i.p. or p.o.	unkonwn	[45]
	Chronic constriction injury	Thermal allodynia	i.p. or s.c.	10 mg/kg	[46]
	Chronic constriction injury	Tactile allodynia	i.p. or s.c.	10 mg/kg	[46]
	Taxol-induced neuropathy	Thermal allodynia	i.p. or s.c.	10 mg/kg	[46]
	Taxol-induced neuropathy	Tactile allodynia	i.p. or s.c.	10 mg/kg	[46]
JWH133	Carrageenan	Mechanical hypersensitivity (Weight bearing)	i.p.	3 mg/kg	[52]
	Partial sciatic nerve ligation	Tactile allodynia (Von Frey hairs)	i.t.	50-100 nmol/mouse	[49]
	Partial sciatic nerve ligation	Tactile allodynia (Von Frey hairs)	i.t.	100 nmol/mouse	[49]
	Formalin	Nocifensive response (1^{st} and 2^{nd} phase)	i.p.	1^{st} phase 0.1-1-5 mg/kg 2^{nd} phase not active	[51]
	Formalin	Nocifensive response (1^{st} and 2^{nd} phase)	i.c.v.	inactive	[51]
A796260	Freund's complete adjuvant	Thermal allodynia (plantar test)	i.p.	1.1-3.5-11 mg/kg	[14]
	Chronic constriction injury	Tactile allodynia (Von Frey hairs)	i.p.	11-35 mg/kg	[14]
	Incision model of postoperative pain	Tactile allodynia (Von Frey hairs)	i.p.	11-35 mg/kg 35 mg/kg (5 days dosing)	[14]
	Knee joint osteoarthritic pain	Grip force	i.p.	35 mg/kg	[14]
GW842166X	Freund's complete adjuvant	Mechanical hypersensitivity (Weight bearing)	p.o.	0.1-0.3 mg/kg	[70]
	Freund's complete adjuvant	Mechanical hypersensitivity (Weight bearing)	p.o.	0.3 mg/kg	[71]
	Freund's complete adjuvant	Mechanical hypersensitivity (Weight bearing)	p.o.	15 mg/kg (5 days dosing)	[71]
	Chronic constriction injury	Mechanical hyperalgesia (paw withdrawal threshold)	p.o.	15 mg/kg (8 days dosing)	[71]
Taisho	Yeast induced inflammatory pain	Mechanical hyperalgesia (Randall-Selitto)	p.o.	10-30 mg/kg	[76]

(Table 3. Contd….)

Compound	Pain Model	Behavioral Readout	Route	Active Doses	Reference
Shionogi	Formalin	Nocifensive response (1^{st} and 2^{nd} phase)	i.pl.	500 μg	[77]
GRC10622	Freund's complete adjuvant	Mechanical hyperalgesia	p.o.	0.1-1-3-10 mg/kg	[78]
	Chronic constriction injury	Mechanical hyperalgesia	p.o.	3-10 mg/kg	[78]
	Partial sciatic nerve ligation	Mechanical hyperalgesia	p.o.	3-10 mg/kg	[78]

sciatic nerve ligation in wild type but not in CB2 $^{-/-}$ mice [49]. Electrophysiological recordings from spinal cord showed that JWH133 reduces noxious mechanical responses in non-inflammed, carrageenan-inflammed, sham operated and neuropathic rats [36, 53] suggesting that CB2 is involved in pain transmission and processing in presence or absence of tissue or nerve injury.

AMINOALKYLINDOLES

AM1241

The structure of AM1241 reproduced in Fig. **1** (**4**) is the same that was reported in the publication of Ibrahim *et al*. [54], however it should be mentioned that a different structure for the same compound has also been published [55].

Interestingly in *in vitro* recombinant system this compound behaves as a protean agonist [13-15,49, 50, 56], see Table **2**. A protean agonist is a compound that, depending on the constitutive activity of the system, may behave on the same receptor as a partial agonist, neutral antagonist or inverse agonist [57]. Unfortunately at present the study of this peculiar pharmacology of AM1241 has not been extended to *in vitro* native system. However, in *ex-vivo* study of capsaicin-induced CGRP release from lumbar spinal cord slices AM1241 has an effect that is reverted by the CB2 antagonist SR144528 [58]. In addition analgesic effects in various rodent pain models were also reversed by CB2 antagonists [54, 58-61], suggesting that in native system this compound should behave as an agonist as it was originally claimed.

AM1241 has a chiral center and most studies were performed using the racemate. However, *in vitro* was reported a different capacity of the (+) and (-) enantiomers to activate CB2 [15]. Thus this difference should be kept in mind when comparing data obtained with the racemate or with the enantiomers.

AM1241 is the CB2 agonist that has been more extensively studied in pre-clinical models (Table **4**). Its analgesic efficacy was assessed in inflammatory models such as carrageenan [15, 59, 60, 62], Freund's complete adjuvant [14], and capsaicin [60, 61]; in neuropathic models such as spinal nerve ligation [54, 58]; in a mixed model such as the formalin [58, 63]. In all these models the compound was analgesic, even though with variable efficacy and potency. Comparison between efficacy and potency in the various pain models is quite difficult because of the use of different vehicles, different administration routes, and different experimental readouts used to evaluate pain. Nevertheless, as a general trend, it could be noted that to elicit significant analgesia after i.p. administration in the inflammatory pain models were necessary lower doses of the compound than in the neuropathic pain models.

In acute pain models the results were more variable with reported analgesic efficacy in plantar test, incision model of postoperative pain, and visceral pain [15, 44, 64, 65] and no activity in the tail flick and hot plate [15].

The analgesic effect of AM1241 administration were reverted by CB2 antagonist but not CB1 antagonist [15,54,58-61,64] and were still present in CB1$^{-/-}$ mice [54]. These data strongly support the claim that AM1241 produces its effects *via* CB2. On the other hand it was shown that in acute and inflammatory pain model [14, 65] also the μ opiod receptor antagonist naloxone was able to block the analgesic effect of AM1241. Since AM1241 was reported not to have binding affinity for μ opiod receptor this effect was attributed to an indirect action [65]. This aspect would be discussed further in the section dedicated to the mechanisms of CB2 analgesia.

L768242 (GW405833)

This compound (**5**, Fig. **1**) was the first published CB2 selective agonist [66] and is the second most used compound for *in vivo* evaluation of CB2 agonist analgesic effects. Its *in vitro* pharmacological profile (Table **1**) indicates a good affinity and selectivity for CB2. Nevertheless, in functional assays performed in recombinant systems expressing either hCB2 or rCB2 (Table **2**) L768242 acted as a partial agonist or even as an inverse agonist [12, 14, 50]. On the other hand SR144528 shifted the concentration response curve of L768242 on the release of capsaicin-induced CGRP release from spinal cord slice [58], suggesting that, like AM1241, also this compound could be a protean agonist [50].

L768242 has poor oral bioavailability, however when administered either i.p. (30 and 100 mg/kg) or i.v. (10 mg/kg) the compound achieved significant plasma levels that remain sustained for up to 5 hours [12]. Thus it was possible to assess its analgesic properties in *in vivo* models (Table **5**). It is important to notice that in the rat at 100 mg/kg i.p. the compound induces ataxia, loss of coordination and catalepsy that confound the analgesic effect [12, 58]. Considering the binding selectivity ratio versus rat CB1 (~75), these effects could probably be explained by the activation of CB1. Lower doses (0.1 to 30 mg/kg) did not induced side effects, nevertheless, they were analgesic in various pain models (Table **4**) (i.e. partial sciatic nerve ligation, spinal nerve ligation, formalin, carrageenan, CFA, and hindpaw incision) in both rat and mouse [12, 44, 58, 67, 68]. In general analgesic effects

Table 4. Analgesic Effect of AM1241 in Pre-Clinical Pain Models

Pain Model	Behavioral Readout	Administration Route	Active Doses	Reference
SNL (spinal nerve ligation)	Tactile allodynia (Von Frey hairs)	i.p.	0.3-3 mg/kg	[54]
		i.v.	3-6 mg/kg [(+)AM1241]	[58]
		i.p.	1 mg/kg	[54]
		i.p.	1 mg/kg	[54]
	Thermal allodynia (plantar test)	i.p.	0.3-3 mg/kg	[54]
		i.p.	3 mg/kg	[54]
		i.p.	3 mg/kg	[54]
Formalin	Nocifensive response (1^{st} and 2^{nd} phase)	i.pl.	0.3 mg	[63]
	Nocifensive response (2^{nd} phase)	i.v.	1-3 mg/kg [(+)AM1241]	[58]
Carrageenan (2%)	Thermal allodynia (plantar test)	i.p.	3-10 mg/kg [(R,S-AM1241)] 0.3-10 mg/kg [(S-AM1241)]	[15]
		i.p.	0.3-1 mg/kg	[60]
		i.pl.	2-4 mg/kg	[60]
Carrageenan (3%)	Tactile allodynia (Von Frey hairs)	i.p.	0.1-0.33 mg/kg	[59]
		i.pl.	0.033 mg/kg	[59]
	Mechanical hyperalgesia (Von Frey hairs)	i.p.	0.1-0.33 mg/kg	[59]
		i.pl.	0.033 mg/kg	[59]
	Thermal allodynia (plantar test)	i.p.	0.1-0.33 mg/kg	[59]
		i.pl.	0.033 mg/kg	[59]
Carrageenan (6%)	Tactile allodynia (Von Frey hairs)	i.pl.	0.033 mg/kg	[62]
	Mechanical hyperalgesia (Von Frey hairs)	i.pl.	0.033 mg/kg	[62]
	Thermal allodynia (plantar test)	i.pl.	0.033 mg/kg	[62]
Freund's complete adjuvant	Thermal allodynia (plantar test)	i.p.	15 mg/kg	[14]
Intradermal capsaicin (20 μg/ μl)	Nocifensive response	i.p.	0.1-0.3	[60]
	Thermal allodynia (plantar test)	i.p.	0.1-0.3	[60]
Intradermal capsaicin (10 μg/ μl)	Tactile allodynia (Von Frey hairs)	i.p	0.33 mg/kg	[61]
	Mechanical hyperalgesia (Von Frey hairs)	i.p	0.033-0.33 mg/kg	[61]
	Thermal allodynia (plantar test)	i.p	0.033-0.33 mg/kg	[61]
		i.pl.	0.033 mg/kg	[61]
	Nocifensive response	i.p	0.033-0.33 mg/kg	[61]

(Table 4. Contd….)

Pain Model	Behavioral Readout	Administration Route	Active Doses	Reference
Plantar test	Thermal sensitivity plantar test)	i.pl.	1 mg/kg	[64]
		i.p.	0.33 mg/kg	[64]
		i.p.	0.1 mg/kg	[65]
Tail flick	Withdrawal latency	i.p.	Not active up to 10 mg/kg	[15]
Hot plate	Nocifensive response	i.p.	Not active up to 10 mg/kg	[15]
Incision model of postoperative pain	Tactile allodynia (Von Frey hairs)	i.p.	10-30 mg/kg	[44]
Acute visceral pain (Paraphenylquinone)	Stretching movement	s.c.	10 mg/kg (S- and R-AM1241)	[15]

are achieved at lower doses in mechanical hyperalgesia compared to tactile allodynia, whereas no overt difference in active doses is observed comparing neuropathic and inflammatory pain models. Antagonism with SR144528 prevents these analgesic effects [58,67] that were strongly reduced also in the CB2 KO mice [12], further confirming a CB2-mediated mechanism. On the other hand, in acute model of pain (i.e. tail flick and hot plate) the analgesic effect were maintained in the CB2 $^{-/-}$ mice suggesting that this is probably a CB1-mediated action.

Interestingly μ opioid receptor antagonists, such as naloxone, have no effect on the L768242-induced analgesia [68]. This data suggests a clear difference from what observed with AM1241, where administration of naloxone blocked AM1241 analgesic effect [65].

A-796260

A-796260 (**6**, Fig. **1**) is an indole derivative CB2 selective agonist discovered by Abbott Laboratories [14] with an affinity for hCB2 in the low nanomolar or subnanomolar range (Table **1**). This compound has similar functional efficacy and potency on human and rat CB2 (Table **2**). On the other hand, it is also a full agonist at CB1 receptor (Table **2**). However selectivity ratio, as estimated in functional assay (>1000 and >150 over human and rat CB1 respectively), should assure a sufficient window to clearly separate CB2 from CB1-mediated actions. Actually up to the dose of 35 mg/kg i.p. the compound did not produce alteration of spontaneous locomotor activity in rat. Its analgesic effects were reverted by SR144528 or AM630, but not by SR141716A [14]. In addition Naloxone, a μ opiod receptor antagonist, had no effect on A-796260-induced analgesia, indicating a mechanism of action different from that of AM1241. Analgesic effects of this compound were evaluated in various pain models (Table **3**) and the emerging picture seems to point to a difference in efficacy in the various models [14, 69]. In particular in the CFA A-796260 is fully active at the dose of 11 mg/kg i.p. (ED_{50} 2.8 mg/kg i.p.), whereas in the knee joint osteoarthritic pain model full efficacy is not achieved even at the highest dose (35 mg/kg i.p.), which induced a 50-60% reversal. An intermediate situation is observed in the CCI (ED_{50} 15 mg/kg i.p.) and the incision model of postoperative pain (ED_{50} 18 mg/kg i.p.).

In acute pain models (incision model of postoperative pain) the analgesic effect of A-796260 was maintained following repeated treatment (up to 5 days), suggesting that this compound does not induce tolerance.

PYRIMIDINE

GW842166X

Another interesting CB2 agonist (**7**, Fig. **1**) has been discovered by GSK. This compound (Table **2**) was reported as full agonist at both h and rCB2 [14, 50, 70]. The compound was inactive at hCB1 up to 30 μM [14, 70] and had a modest efficacy on rCB1 at high concentration [14]. Binding studies (Table **1**) confirmed a lack of affinity for h and rCB1 but, surprisingly, also poor affinities for h and rCB2 [14].

The limited data on the analgesic profile of GW842155X (Table **3**) indicate that it was able to fully reverse hyperalgesia in the CFA model (ED_{50} of 0.1 mg/kg), an effect blocked by administration of the CB2 antagonist AM630 [70]. The compound was also active in the CCI model [71]. Quite interestingly subchronic administration (4-5 days) in both the CFA and CCI models did not induce tolerance to the analgesic effect [70, 71]. In addition no side effects, such as catalepsy and hyperthermia, were observed at a dose 100-fold higher than an antihyperalgesic dose in the CFA [70]. Thus GW842166X looks as a very promising compound being active on pain of both inflammatory and neuropathic origin at low p.o. doses. On the other hand its quite puzzling *in vitro* pharmacology remains to be further explored.

THIAZOLE

A-836339

Very recently another CB_2 agonist, A-836339, was disclosed by Abbott Laboratories (**8**, Fig. **1**). The compound has

Table 5. Analgesic Effect of L768242 in Pre-Clinical Pain Models

Pain Model	Behavioral Readout	Administration Route	Active Doses	Reference
SNL (spinal nerve ligation)	Tactile allodynia (Von Frey hairs)	i.p.	30 mg/kg	[58]
Partial sciatic nerve ligation	Tactile allodynia (Von Frey hairs)	i.p.	30 mg/kg	[68]
	Mechanical hyperalgesia (Randall-Selitto)	i.p.	0.1-0.3-1-10-30 mg/kg	[12]
Formalin	Nocifensive response (2^{nd} phase)	i.v.	6-10 mg/kg	[58]
		i.p.	30 mg/kg	[58]
Freund's complete adjuvant	Mechanical hyperalgesia (Randall-Selitto)	i.p.	0.3-1-3-10-30 mg/kg	[12]
	Tactile allodynia (Von Frey hairs)	i.p.	10-30 mg/kg	[68] [12]
Carrageenan (2%)	Mechanical hypersensitivity (Weight bearing)	i.p.	3-10 mg/kg	[67]
Tail flick	Withdrawal latency	i.p.	100 mg/kg	[12]
		i.p.	100 mg/kg	[68]
Hot plate	Nocifensive response	i.p.	100 mg/kg	[12]
		i.p.	100 mg/kg	[68]
Incision model of postoperative pain	Mechanical hyperalgesia (Randall-Selitto)	i.p.	3-10-30 mg/kg	[12]
	Tactile allodynia (Von Frey hairs)	i.p.	30 mg/kg	[44]

a subnanomolar affinity for CB2 and, compared to A-796260, shows an improved selectivity over rCB1 (Tables **1** and **2**). In CFA model of chronic pain and in an acute post-operative pain model A-836339 resulted more potent compared to A-796260 (ED_{50} in CFA model 0.45 mg/kg *vs* 2.8 mg/kg, and in acute post-operative pain model 2.5 mg/kg versus 18 mg/kg respectively) (Table **3**). Analgesia induced by A-836339 was reversed by SR144258 but not by SR141716A [72, 73]. All these studies were performed using the i.p. route of administration, whereas no information are available on its efficacy after oral administration.

Carboxiamide

Taisho Compounds

Recently Taisho Pharmaceutical published a series of reports disclosing the identification of a novel group of CB2 agonists derived from carboxiamide [74-76]. This class of compounds shows good affinity for hCB2, selectivity over CB1 (Table **1**) and a good pharmacokinetic profile. The structure of the best compound is reported in Fig. **1** (**9**). Available information on its activity *in vivo* is extremely limited (Table **3**), however an analgesic effect was reported using a yeast induced inflammatory pain model [76].

THIAZINE

Shionogi Compounds

Kai and colleagues [77] have disclosed a new class of CB2 agonists that are thiazine derivatives. The best compound identified so far (**10**, Fig. **1**) showed a high affinity for CB2 and was claimed to have full efficacy in the cAMP functional assay, even though no numerical data was reported. Its analgesic activity (Table **3**) was assessed in the formalin test where, after s.c. administration into the paw, it was able to reduce the nociceptive behaviour in both the early and late phase of the test [77]. The analgesic effect observed in the second phase was fully reverted by SR144528, whereas the one observed in the first phase was only partially reverted. Conversely the CB1 antagonist SR141716A had no effect on the analgesia triggered by this compound.

UNKNOWN STRUCTURE

GRC10622

The structure of this compound, discovered by Glenmark researchers, has not been disclosed and the available information on GRC10622 profile has been presented only at

scientific meetings [78]. Nevertheless GRC10622 looks very interesting and, apart from GW842166X, it is the only CB2 selective agonist that so far has been claimed to be analgesic after single oral dose. The compound was tested in the CFA, CCI and partial spinal nerve ligation showing efficacy in all models (Table **3**) reaching a full reversal (80-90%) at 10 mg/kg. GRC10622 displays a good pharmacokinetic profile with a plasma half life of more than 6 hours and absolute bioavailability of 51%. Available data indicate that the compound is more potent in the inflammatory pain model where it has been claimed to be active at 0.1 mg/kg after oral administration.

In order to summarize various CB2 agonists have been discovered belonging to several different chemical classes. These agonists show a wide range of oral bioavailability from poor (AM1241) to satisfactory (GRC10622). Based on binding data these compounds in general show good selectivity over hCB1 whereas high selectivity over rCB1 seems more difficult to achieve, indicating a clear species-specific difference in the SAR. A difference could be noticed in compounds potency and efficacy at h and rCB2. In this case, however, the difference could be due a bias introduced by the use of recombinant systems where a different constitutive activity of the cell lines expressing h and rCB2 may influence the functional efficacy of the tested compounds.

Even though with various degrees of efficacy, these compounds are able to reduce nociception in a variety of preclinical pain models. For most of these compounds, however, available information are limited to a few articles or even a single one, the only exceptions being AM1241 and L768242. Therefore general speculation should be taken with caution. Nevertheless, the emerging picture would imply a very broad spectrum of analgesic activity of CB2 agonists. Thus they could be useful for the treatment of both chronic inflammatory and neuropathic pain, even though potency and efficacy in preclinical models seem to indicate a stronger effect on inflammatory pain.

On the other hand it should be mentioned that other groups [79,80], using either mixed (CP55940 and WIN55212-2) or selective (L768242 and GW842166X) CB2 agonists in both wild type and KO mice, have not found evidence in support of a CB2 agonist-mediated analgesia. Several reasons could account for these discordant data. For example in the same species different groups have reported for the same compound significant differences in selectivity and efficacy, and several compounds showed species-specific difference (Tables **1** and **2**). In addition data on the pharmacology of compounds on native cellular systems are almost completely missing and this is an important piece of information that could help to reconcile these puzzling data.

Another interesting aspect to underline is that when probed on subchronic dosing CB2 agonist seems not to induce tolerance. If further confirmed also in clinical trials this would surely be a definite asset for CB2-based drugs.

Finally an extremely intriguing question remains still open that is: which between full agonist, partial agonists and protean agonist would result in the best analgesic profile.

MECHANISMS OF CB2 AGONIST-INDUCED ANALGESIA

Experimental observations supporting a role for CB2 agonists in cannabinoid-induced analgesia originated the question about the mechanisms contributing to this behavioral effect. Data on expression and distribution of CB2 indicate a predominant localization in immune system cells [4, 81-83] whereas its localization on DRG and spinal cord is more controversial [36, 53, 58, 84-89].

The capacity of cannabinoids to influence the inflammatory response is well known even though both pro and anti-inflammatory effect have been described [90]. Inflammatory pain is the result of inflammatory cell recruitment to the site of injury where these cells release several mediators (cytokine, prostanoids, NGF, histamine, etc.) that act as pronociceptive agents. In this context the activation of CB2 present in these cell types could be an important factor in reducing nociception [91].

Albeit the action on immune and inflammatory cells could help to explain the CB2 agonists- induced analgesia observed in inflammatory pain, nevertheless it would not assist to explain analgesia in acute pain and in chronic neuropathic pain. To solve this riddle it was suggested that CB2 activation could induce analgesia through the reduction of basal level of the proalgesic molecule NGF [91]. Actually hyperalgesia induced by NGF administration is reduced by palmitoylethanolamide through a mechanism that is blocked by the CB2 antagonist SR144528 [92,93]. However, palmitoylethanolamide is not a CB2 ligand and the reversal of its effect by SR144528 could be due to an action on a CB2-like receptor [92]. On the other hand, studies on the direct effect of cannabinoid on the NGF system are inconclusive, showing either an activation of the mechanism involved in NGF induction [30], or a reduction of the level of NGF Trk receptor [94]. Thus, a clear experimental evidence to support this hypothesis is still missing.

Another interesting theory on the mechanism of action underpinning CB2 agonist analgesic effect is coming from the study of AM1241. When applied on cultured keratinocytes AM1241 stimulated the release of β-endorphin [65]. The released β-endorphin would act locally by activating the μ-opioid receptor present on the nerve terminal and thus inducing analgesia. Interestingly in μ-opioid receptor deficient mice the analgesic effect of AM1241 was lost [65]. However, more recent studies showed that this mechanism is probably compound specific. These studies demonstrated that for other CB2 agonists, namely A-796260 and L768242, the μ-opioid receptor was not necessary to display their analgesic effect because the μ-opioid receptor antagonists naltrexone or naloxone did not influence their analgesic efficacy [14, 68].

A fourth hypothesis to explain the antinociception elicited by CB2 agonists is a direct action on CB2 located in the neural part of the pain pathway. Actually the literature on the subject is controversial. Historically, based on *in situ* hybridization, PCR and binding studies [84-86], CB2 has been considered absent from the CNS. However, more recently several studies were published claiming the presence of CB2

in the DRG and spinal cord. Evidence demonstrating the localization and functional activity of CB2 in DRG and spinal cord were obtained using a set of different techniques ranging from *in situ* hybridization [87] to immunocytochemistry [88], from real time quantitative PCR [58, 89] to electrophysiology [36, 53] and finally by analgesia evaluation after i.t. administration of CB2 agonist [49]. In addition it has been also reported an increase of CB2 expression in the spinal cord following various types of nerve injury/damage [58, 87, 88] that has been tentatively linked to upregulation of CB2 in microglia. Because of spinal cord cell population heterogeneity it is difficult to extrapolate which cell population expresses CB2. However, the presence of CB2 in microglia was shown performing QRT-PCR on mRNA extract from spinal cord microglia cell culture [58]. A result that is in agreement with previously data obtained using *in situ* hybridization and suggesting a microglia localization for CB2 [87]. Conversely the presence of mRNA messengers in the DRG could be more easily linked to an expression in the nociceptors. The existence of functional CB2 on nociceptor is also supported by the blockade of capsaicin-induced CGRP release from dissociated DRG neurons and spinal cord slices following CB2 agonist administration [36, 37, 58]. Additional evidence of DRG involvement on CB2-mediated analgesia were produced by performing i.t. or intra-DRG injection of A-836339 that resulted in an analgesic effect [95].

Based on this information, and on the known lipophilicity of cannabinoids, CB2 agonist-induced analgesia could be explained by a mechanism of action that involves both peripheral and central nervous system components. The CB2 located on both peripheral and central terminals of nociceptors could diminish nociceptors excitability and could reduce the release of pronociceptive mediators from primary afferents. On the other hand CB2 present on microglia could dampen microglia activation elicited by nociceptors overactivation, and consequently could reduce or block pro-algesic substances release (e.g. IL-1b, IL-6, TNFα and excitatory amino acids) from microglia. This second part of the hypothesis, however, has still to be supported by clear experimental evidence.

It is important to point out that the different mechanisms evoked to explain CB2 analgesia are not mutually exclusive. Even though some of them may better explain the CB2-mediated analgesia observed in a specific type of pain and not in others, nevertheless it should not be ruled out that in certain cases they could be acting simultaneously to achieve the observed analgesic effects.

CLINICAL TRIALS

Since interest on CB2 as a therapeutic target for chronic pain is quite recent there is a very limited number of clinical trials assessing CB2 agonists analgesic value.

Pharmos Corporation's drug Cannabinor was evaluated in two separate phase 2a trails: single-center, double blinded, placebo controlled single dose, 2-way cross over study in experimentally-induced capsaicin pain model and single-center, double blinded, placebo controlled single dose, parallel treatment in post-operative pain (third molar extraction). In the first trial were compared 48mg of cannabinor delivered intravenously versus placebo on capsaicin-evoked allodynia and hyperalgesia. The drug candidate did not meet the primary endpoint defined by analgesic effects compared to placebo, but confirmed safety and tolerability observed in previous studies. In the other trial, that is third molar extraction, results were puzzling, indicating analgesic efficacy of the dose of 12 mg/kg at 2 and 8 hours post administration, whereas higher doses, 24 and 48 mg/kg were not active. Thus in both trails the effect of Cannabinor felt short to show convincing analgesic effect.

GSK compound GW842166X is at present in clinical trials. Information about the trial phase is, however, conflicting. GSK official website reports the compound to be in Phase II for inflammatory pain but no detailed information is given. Whereas database search reports GW842166 in Phase I with the aim to assess the dose response and efficacy in healthy volunteers with the primary endpoint of measuring heat pain threshold. Recruitment for this trial was concluded in November 2007 and results are expected to be released in the first half of 2008.

Finally Glenmark is planning to start a Phase I trail for neuropathic pain, osteoarthritis, reumathoid arthritis and other inflammatory pain in the third quarter of 2008 using GRC10693, a coumpound on which there is no published information.

CONCLUSIONS

Several lines of evidence support the analgesic potential of CB2 agonists in preclinical model of chronic inflammatory and neuropathic pain. Nevertheless there is still a series of open questions that would be important to address in order to fully understand the true value of this target.

Most compounds are less selective in rat than in human and functional assays indicate that compounds efficacy could be quite different at rat and human CB2. In addition the best characterized compounds (AM1241 and L768242) have a complex *in vitro* pharmacology behaving as a protean agonist, making it difficult to draw clear-cut conclusions on their activity. This makes *in vitro* data not always particularly useful to predict analgesic effect in pain models. A possible way to overcome this problem could be to develop functional assays in native receptor systems which, in principle, should match more closely the *in vivo* situation.

Another potential issue is that at present only two compounds have shown significant analgesic properties after oral administration. Thus the development of highly selective and potent orally available CB2 agonist is feasible, but seems to represent a challenging task.

A potential advantage of targeting CB2 is that agonists were shown to be active in a broad range of preclinical pain models mimicking various pathological conditions. None of the commercially available treatments has so far shown such a broad spectrum of activity, thus the potential market for such a type of medicine would be extremely important.

The development of an agonist drug for chronic treatment faces the problem of tolerance, an effect well known for opioid. However, available data suggest that this actually seems not to be the case for CB2 agonists; if confirmed, this

would be a distinct advantage from a drug discovery perspective.

Finally, but most important, it should be mentioned that a clear proof of concept of CB2 selective agonist analgesic properties in man has not yet been produced. The outcome of ongoing and planned clinical trials would be essential to verify if this target holds its promises.

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