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REVIEW

Biology and therapeutic potential of cannabinoid CB_2 receptor inverse agonists

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Evidence has emerged suggesting a role for the cannabinoid CB_2 receptor in immune cell motility. This provides a rationale for a novel and generalized immunoregulatory role for cannabinoid CB_2 receptor-specific compounds. In support of this possibility, we will review the biology of a class of cannabinoid CB_2 receptor—specific inverse agonist, the triaryl bis-sulfones. We will show that one candidate, Sch.414319, is potent and selective for the cannabinoid CB_2 receptor, based on profiling studies using biochemical assays for 45 enzymes and 80 G-protein coupled receptors and ion channels. We will describe initial mechanistic studies using this optimized triaryl bis-sulfone, showing that the compound exerts a broad effect on cellular protein phosphorylations in human monocytes. This profile includes the down regulation of a required phosphorylation of the monocyte-specific actin bundling protein L-plastin. We suggest that this observation may provide a mechanism for the observed activity of Sch.414319 *in vivo*. Our continued analysis of the *in vivo* efficacy of this compound in diverse disease models shows that Sch.414319 is a potent modulator of immune cell mobility *in vivo*, can modulate bone damage in antigen-induced mono-articular arthritis in the rat, and is uniquely potent at blocking experimental autoimmune encephalomyelitis in the rat.
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Abbreviations: EAE, experimental autoimmune encephalomyelitis; PMN, peripheral mononuclear cells

Introduction

Since its discovery in 1993, the cannabinoid CB_2 receptor has been an appealing therapeutic target for novel immunomodulators. Initial studies suggested that the receptor was not expressed in the central nervous system (Munro *et al*., 1993), suggesting that cannabinoid compounds specific for the CB_2 receptor would be free of the neural deficits seen in nonspecific cannabinoid compounds. The high-level expression within immune cells and the inducible expression of the receptor following inflammatory insult suggested that the receptor may serve to mediate the immune regulatory activities described for cannabinoids (Kaplan *et al*., 2003). Finally, studies showed that the primary active component in *Cannabis*, Δ9-tetrahydrocannabinol (THC), is a partial agonist at the cannabinoid receptor. This observation defined the pharmacology of a preferred therapeutic at the cannabinoid CB_2 receptor.

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However, this initial view of the cannabinoid system has proved to be simplistic. Detailed studies of the expression of the cannabinoid CB_2 receptor have shown that functional receptors can be found in neural tissue (Van Sickle *et al*., 2005; Beltramo *et al*., 2006; Onaivi *et al*., 2006). This may alter the utility of cannabinoid CB_2 receptor-specific compounds. In addition, selected animal models (Smith *et al*., 2000, 2001; Croci *et al*., 2003; Benamar *et al*., 2007) have suggested that cytokine levels can be mediated by manipulation of the cannabinoid CB_1 receptor. This suggests that the cannabinoid CB_2 receptor may not be the only cannabinoid-based mediator of the immune system, and may explain the fact that the cannabinoid CB_2 receptor-deficient mice fail to show significant immune deficit under standard conditions (Buckley *et al*., 2000; Ofek *et al*., 2006). Finally, other components of *Cannabis*, including cannabidiol, have proved to be potent immunomodulatory compounds (Malfait *et al*., 2001; Costa *et al*., 2004; Giudice *et al*., 2007). Recent evidence has shown that this compound behaves as an inverse agonist to the cannabinoid CB_2 receptor in standard biochemical assays (Thomas *et al*., 2007).

Taken together, these observations may suggest the value of adopting a broad view when considering the desired pharmacology of an immune therapeutic based on the cannabinoid CB_2 receptor.

Into this arena enters cannabinoid CB_2 receptor-specific triaryl bis-sulfones. In this review, we will discuss the biology of this novel class of cannabinoid CB_2 receptor-specific inverse agonists. We will further document the specificity of a member of this class, and describe a biology based on the ability of these compounds to modulate immune cell migration. We will provide initial data providing a potential mechanism for the activity of these compounds, based on controlling the phosphorylation required for activity of an actin bundling protein. Finally, we will describe the activity of an optimized triaryl bissulphone, Sch.414319, in arthritis and in experimental autoimmune encephalomyelitis (EAE). The ability of these compounds to modulate EAE mimics a diminished disease observed in animals lacking the cannabinoid CB_2 receptor. We conclude that cannabinoid CB_2 inverse agonists may play a part in the therapeutic pharmacopoeia that targets this class of receptors.

Identification and initial biochemistry of triaryl bis-sulphones

Triaryl bis-sulphones were identified from a compound library screening campaign, in which compounds were tested for a selective ability to block binding of [^{3}H]-CP55,940 to membrane preparations expressing recombinant human cannabinoid CB_2 receptor versus membrane preparations expressing recombinant human CB_1 receptor (Lunn *et al.*, 2006a).

The ability of the identified triaryl bis-sulphones to decrease basal binding of [^{35}S]-GTPγS to recombinant cell membranes expressing cannabinoid CB_2 (and CB_1) receptors, to bind with an increased potency to recombinant cell membranes in the presence of 100 μM GTPγS, and to decrease forskolin-stimulated cAMP levels in cells expressing the recombinant receptor are consistent with an inverse agonist pharmacology (Greasley and Clapham, 2006). This pharmacology has been maintained throughout an extensive medicinal chemistry effort designed to improve the pharmacological characteristics of triaryl bis-sulphones (Lavey *et al.*, 2005, 2007; Shankar *et al.*, 2005). An optimized compound, designated Sch.4141319, was engineered to contain a terminal trifluoromethane sulphonamide to permit salt formation and optimize solubility. This resulted in a compound that exhibited superior blood levels when dosed orally in rats (Table 1).

Our initial screening studies of Sch.414319 showed that the compound was specific for the CB_2 receptor over the CB_1 receptor. However, an optimized therapeutic candidate requires data demonstrating significant selectivity over additional enzymes and receptors separate from the target receptor. To this end, we carried out a generalized evaluation to investigate whether any activity identified for the optimized triaryl bissulphone Sch.414319 could result from a high-affinity interaction with some off-target effectors. Sch.414319 was submitted to MDS Pharma Services for testing its ability to modulate the activity of a broad collection of receptors and enzymes. The selected panel of proteins and receptors (Table 2) included 45 enzymes (including 11 kinases), 80 G-protein-coupled receptors and ion channels, and three recombinant cell-based adhesion inhibition assays mediated by fibronectin, VCAM-1 and ICAM-1. Eight of these biochemical screening assays were judged to be affected by the addition of 10 μM Sch.414319, showing greater than 50% inhibition (Table 2). These selected assays were retested using a four-point logarithmic dilution series of Sch.414319 to generate an estimation of compound potency in eight identified assay systems. As expected, Sch.414319 blocked binding of [^{3}H]-WIN55,212-2 to the human cannabinoid CB_2 receptor ($K_i \approx 70$ nM). The compound also blocked binding to the L-type calcium channels that measured using radiolabelled dihydropyridine ($K_i \approx 0.44$ μM), phenylalkylamine ($K_i \approx 2.43$ μM) and benzothiazepine ($K_i \approx 6.07$ μM). The compound also bound with modest affinity to several other receptor preparations, including the thromboxane A2 receptor ($K_i \approx 1.44$ μM), the non-selective sigma receptor ($K_i \approx 2.05$ μM) and the cholecystokinin B type (CCKB) receptor ($K_i \approx 5.7$ μM). Although this analysis is not exhaustive, it provides additional evidence that the biology of Sch.414319 is mediated primarily by the cannabinoid CB_2 receptor.

Table 1 Binding selectivity and pharmacokinetic profile of selected triaryl bissulphones

	CB_2 K_i	*CB_1 K_i*	*Ratio (CB_1/CB_2)*	*Rat AUC*
Sch.225336	0.4 nM	905 nM	2262	235 nM h@10 mg kg^{-1}
Sch.356036	1.3 nM	4387 nM	3375	3160 nM h@10 mg kg^{-1}
Sch.414319	2 nM	6785 nM	3393	27270 nM h@10 mg kg^{-1}

Competitive binding experiments were carried out versus [^{3}H] CP55,940, as described (Lunn *et al*, 2006a, b). The pharmacokinetic profiles of orally administered drug candidates were approximated by monitoring the plasma concentration versus time after dosing and calculating the area under the curve (AUC). For these determinations, rats were orally dosed at 10 mg kg^{-1} (in 0.4% w/v methylcellulose). Plasma levels were sampled (3 times per day, $n = 3$) and drug levels determined using standard quantitative HPLC methodologies (Cox *et al.*, 1999).

Cell biology of triaryl bis-sulphones

A number of biochemical and cellular activities associated with immune modulation by cannabinoids have been ascribed to the cannabinoid CB_2 receptor (Kaplan *et al.*, 2003). Some of the activities require high cannabinoid concentrations that are unexpected for cannabinoid receptor-mediated events. Indeed, several laboratories have reported cannabinoid effects that are mediated by such alternative mechanisms (Kraft *et al.*, 2004; Curran *et al.*, 2005; Kaplan *et al.*, 2005; McCollum *et al.*, 2007). We believe this set of observations speaks for the complexity of cannabinoid biology, and supports the value of considering novel pharmacological entities in targeting the cannabinoid CB_2 receptor.

An initial set of experiments were planned to test whether triaryl bis-sulphones could function as antagonists in several cannabinoid-induced *in vitro* and *in vivo* cytokine production

Table 2 Characterizing selectivity of Sch.414319 for the human cannabinoid CB_2 receptor

ID	*Name*	*Type*	*% Inhbt @ 10 μM*	*Protocol reference*
10730	Angiotensin-converting enzyme	Enz	8	Bunning P *et al. Biochemistry*. **22**:103, 1983
10800	Protease calpain	Enz	19	Murachi T *et al. Adv Enzyme Regul*. **19**: 407, 1980
11200	Carbonic anhydrase	Enz	3	Eveloff J *et al. Biochem Pharmacol*. **28**: 1434, 1979
11250	Protease cathepsin G	Enz	1	Mehdi S *et al. Biochem Biophys Res Commun*. **166**: 595, 1990
11300	Choline acetyltranferase	Enz	3	Maderdrut JL. *Neurochem Res*. **20**: 69, 1995
11501	Cyclooxygenase COX-1	Enz	−14	Warner TD *et al. Proc Natl Acad Sci USA*. **96**: 7563, 1999
11601	Cyclooxygenase COX-2	Enz	18	Warner TD *et al. Proc Natl Acad Sci USA*. **96**(13): 7563, 1999
12100	Myeloperoxidase	Enz	−7	Syensson BE. *Biochem J*. **242**: 673, 1987
12400	HMG-CoA reductase	Enz	−1	Kubo M and Strott CA. *Endocrinology*. **120**: 214, 1987
12800	Leukotriene A4 hydrolase	Enz	20	Iaumi T *et al. Biochem Biophys Res Commun*. **135**: 139, 1986
13200	Leukotriene C4 synthetase	Enz	−11	Bach MK *et al. Biochem Pharmacol*. **34**: 2695, 1985
13400	Lipid peroxidase	Enz	20	Mansuy D *et al. Biochem Biophys Res Commun*. **135**: 1015, 1986
13800	Lipoxygenase 15-LO	Enz	2	Averbach BJ *et al. Anal Biochem*. **201**: 375, 1992
14000	Monoamine oxidase MAOA	Enz	1	Medvedev AE *et al. Biochem Pharmacol*. **47**: 303, 1994
14010	Monoamine oxidase MAOB	Enz	−11	Egashira T *et al. Biochem Pharmacol*. **25**: 2583, 1976
14200	Nitric oxide sythase constitutive (cNOS)	Enz	3	Lowenstein CJ and Snyder SH. *Cell*. **70**: 705, 1992
14400	Nitric oxide sythase inducible (iNOS)	Enz	3	Nathan C. *FASEB J*. **6**: 3051, 1992
14600	Phosphodiesterase PDE1	Enz	65	Nicholsen CD *et al. Trends Pharmacol Sci*. **12**: 19, 1991
14800	Phosphodiesterase PDE2	Enz	25	Hidaka H and Asano T. *Biochem Biophys Acta*. **429**: 485, 1976
15200	Phosphodiesterase PDE3	Enz	12	Nicholsen CD *et al. Trends Pharmacol Sci*. **12**: 19, 1991
15400	Phosphodiesterase PDE4	Enz	27	Cortijo J *et al. Br J Pharmacol*. **108**: 562, 1993
15600	Phosphodiesterase PDE5	Enz	5	Hidaka H and Asano T. *Biochem Biophys Acta*. **429**: 485, 1976
16000	Phospholipase PLA2-1	Enz	19	Katsumata M *et al. Anal Biochem*. **154**: 676, 1986
16400	Protease neutral endopeptidase	Enz	−2	Erdos EG and Skidgel RA. *FASEB J*. **3**: 145, 1989
16600	Protease elastase	Enz	1	Baugh J and Travis J *Biochemistry*. **15**(4): 836, 1976
16800	Protein kinase II, Ca2+/calmodulin-dep.	Enz	18	Lai Y *et al. Proc Natl Acad Sci USA*. **83**: 4253, 1986.
17000	EGF receptor tyrosine kinase	Enz	−9	Geissler JF *et al. J Biol Chem*. **265**: 22255, 1990
17100	ERK1 serine/threonine kinase	Enz	10	Dudley DT *et al. Proc Natl Acad Sci USA*. **32**: 7686, 1995
17200	Fya (p59fya) tyrosine kinase	Enz	−6	Appleby MW *et al. Cell*. **70**: 751, 1992
17400	HER2 tyrosine kinase	Enz	12	Bargmann CI *et al. Cell*. **45**: 649, 1986
17600	Lck (p56lck) tyrosine	Enz	6	Caron L *et al. Mol Cell Biol*. **12**: 2720, 1992
17700	PKA non-selective	Enz	5	Quick J *et al. Biochem Biophys Res Commun*. **187**: 657, 1992
17800	Protein kinase C, non-selective	Enz	1	Jeng AY *et al. Cancer Res*. **46**: 1966, 1986
18001	Protein Kinase C alpha	Enz	1	Tamaki T *et al. Biochem Biophys Res Commun*. **135**: 397, 1986
18200	Protein kinase C- (I and II)	Enz	17	Woodgett JR and Hunter T. *J Biol Chem*. **262**: 4836, 1987
18400	Protein kinase C-	Enz	−14	Woodgett JR and Hunter T. *J Biol Chem*. **262**: 4836, 1987
18600	Calcineurin PP2B phosphatase	Enz	5	Klee CB *et al. Methods Enzymol*. **102**: 227, 1983
19000	CD45 tyrosine phosphatase	Enz	5	Zhang ZY *et al. Proc Natl Acad Sci USA*. **90**: 4446, 1993
19200	PTP1B tyrosine phosphatase	Enz	−2	Wiener JR *et al. J Natl Cancer last*. **86**: 372, 1994
19220	PTP1C tyrosine phosphatase	Enz	13	D'Ambrosio D *et al. Science*. **268**: 293, 1995
19300	T-cell tyrosine phosphatase	Enz	−2	Yakura H *Crit Rev Immunol*. **14**: 311, 1994
19350	Free radical scavenger, SOD mimetic	Enz	29	Sun Y *et al. Clin Chem*. **34**: 467, 1988
19400	Thromboxane synthetase	Enz	48	Fiddler GI and Lumley P. *Circulation*. **81**: 169, 1990
19500	Tyrosine hydroxylase	Enz	19	Roskoshi R Jr *et al. J Biochem*. **218**: 363, 1993
19800	Xanthine oxidase	Enz	−1	Hatano T *et al. Chem Pharm Bull*. **38**: 1224, 1990
20031	Adenosine A1	Bind	22	Libert F *et al. Biochem Biophys Res Commun*. **187**: 919, 1992
20061	Adenosine A2A	Bind	47	Jarvis MF *et al. J Pharmacol Exp Ther*. **251**: 888, 1989
20071	Adenosine A3	Bind	46	Olah ME *et al. Mol Pharmacol*. **45**: 978, 1994
20080	Adenosine A2B	Bind	38	Brackett LE and Daly JW. *Biochem Pharmacol*. **47**: 801, 1994
20340	Adrenergic-1D	Bind	27	Kenny BA *et al. Br. J Pharmacol*. **115**: 981, 1995
20362	Adrenergic-2A	Bind	3	Uhlen S *et al. J Pharmacol Exp Ther*. **271**: 1558, 1994
20380	Adrenergic-2C	Bind	13	Uhlen S *et al. J Pharmacol Exp Ther*. **271**: 1558, 1994
20401	Adrenergic 1	Bind	3	Feve B *et al. Proc Natl Acad Sci USA*. **91**: 5677, 1994
20411	Adrenergic-2	Bind	−5	McCrea KE and Hill SJ. *Br. J Pharmacol*. **110**: 619, 1993
20420	Adrenergic-3	Bind	36	Feve B *et al. Proc Natl Acad Sci USA*. **91**: 5677, 1994
20441	Adrenergic norepinephrine transporter	Bind	38	Galli A *et al. J Exp Biol*. **198**: 2197, 1995
21001	Angiotensin AT1	Bind	22	Dudley DT *et al. Mol Pharmacol*. **38**: 370, 1990
21011	Angiotensin AT2	Bind	7	Whitebread SE *et al. Biochem Biophys Res Commun*. **181**: 1365, 1991
21261	Bradykinin B2	Bind	10	Eggerickx D *et al. Biochem Biophys Res Commun*. **187**(3): 1306, 1992
21361	Calcitonin	Bind	9	Findlay DM *et al. Cancer Res*. **40**: 1311, 1980
21401	Calcitonin gene-related peptide (CGRP)	Bind	−5	Zimmermann U *et al. Peptide*. **16**(3): 421, 1995
21450	Calcium channel type l, benzothiazepine	Bind	69	Schoemaker H and Langer SZ. *Eur J Pharmacol*. **111**: 273, 1985
21460	Calcium channel type l, dihydropyridine	Bind	92	Gould RJ *et al. Proc Natl Acad Sci USA*. **79**: 3656, 1982
21500	Calcium channel type l, phenylalkylamine	Bind	72	Reynolds IJ *et al. J Pharmacol Exp Ther*. **237**: 731, 1986
21600	Calcium channel type N	Bind	−1	Moresco RM *et al. Neurobiol Aging*. **11**(4): 433, 1990
21701	Cannabinoid CB1	Bind	10	Felder CC *et al. Mol Pharmacol*. **48**: 443, 1995
21710	Cannabinoid CB2	Bind	85	Munro S *et al. Nature*. **365**: 61, 1993
21801	Cholecystokinin CCKA	Bind	10	Jensen RT *et al. Ann N Y Acad Sci*. **713**: 88, 1994
21811	Cholecystokinin CCKB	Bind	55	Jensen RT *et al. Ann N Y Acad Sci*. **713**: 88, 1994

Table 2 *Continued*

ID	*Name*	*Type*	*% Inhbt @ 10 μM*	*Protocol reference*
21850	Dopamine D1	Bind	−1	Dearry A *et al. Nature*. **347**: 72, 1990
21860	Dopamine D2L	Bind	26	Hayes G *et al. Mol Endocrinol*. **6**: 920, 1992
21870	Dopamine D2S	Bind	18	Grandy DK *et al. Proc Natl Acad Sci USA*. **86**: 9762, 1989
21880	Dopamine D3	Bind	30	Sokoloff P *et al. Nature*. **347**: 146, 1990
21890	Dopamine D4-2	Bind	11	Van Tol HHM *et al. Nature*. **358**: 149, 1992
22000	Dopamine D4-4	Bind	−6	Van Tol HHM *et al. Nature*. **350**: 610, 1991
22010	Dopamine D4-7	Bind	−8	Van Tol HHM *et al. Nature*. **358**: 149, 1992
22020	Dopamine D5	Bind	20	Sunahara RK *et al. Nature*. **350**: 614, 1991
22032	Dopamine transporter	Bind	36	Gu H *et al. J. Biol Chem*. **269**: 7124, 1994
22410	Endothelin ETB	Bind	−14	Mihara S *et al. J Pharmacol Exp Ther*. **268**: 1122, 1994
22550	Epidermal growth factor (EGF)	Bind	6	Dittadi R *et al. Clin Chem*. **36**: 849, 1990
22601	Estrogen ER	Bind	8	Obourn JD *et al. Biochem*. **32**: 6229, 1993
23131	Galanin	Bind	−4	Heullet E *et al. Eur J Pharmacol*. **269**: 139, 1994
23170	Glucagon-like peptide-1 (GLP-1)	Bind	14	Dillon JS *et al. Endocrinology*. **133**: 1907, 1993
23201	Glucocorticoid	Bind	14	Cidlowski JA and Cidlowski NB. *Endocrinology*. **109**: 1975, 1981
23350	Histamine H1, central	Bind	4	Hill SJ *et al. J Neurochem*. **31**: 997, 1978
23360	Histamine H1, peripheral	Bind	−14	Dini S *et al. Agents Actions*. **33**: 181, 1991
23370	Histamine H2	Bind	23	Traiffort E *et al. Eur J Pharmacol*. **207**: 143, 1991
23380	Histamine H3	Bind	11	West RE *et al. Mol Pharmacol*. **38**: 610, 1990
24410	Laterleukin IL-6	Bind	9	Cornfield LJ and sills MA. *Eur J Pharmacol*. **202**: 113, 1991
24430	Chemokine CXCR1/2 (IL-8, non-selective)	Bind	−17	Moser B *et al. J Biol Chem*. **266**: 10666, 1991
24440	Chemokine CXCR1	Bind	3	Ahuja SK *et al. J Biol Chem*. **271**: 20545, 1996
24450	Chemokine CXCR2	Bind	8	Ahuja SK *et al. J Biol Chem*. **271**: 20545, 1996
25051	Leukotriene B4	Bind	−10	Winkler JD *et al. J Pharmacol Exp Ther*. **246**: 204, 1988
25060	Leukotriene D4	Bind	6	Mong S *et al. Eur J Pharmacol*. **102**: 1, 1984
25260	Muscarinic M1	Bind	−23	Buckley NJ *et al. Mol Pharmacol*. **35**: 469, 1989
25270	Muscarinic M2	Bind	2	Buckley NJ *et al. Mol Pharmacol*. **35**: 469, 1989
25280	Muscarinic M3	Bind	−7	Buckley NJ *et al. Mol Pharmacol*. **35**: 469, 1989
25290	Muscarinic M4	Bind	−5	Buckley NJ *et al. Mol Pharmacol*. **35**: 469, 1989
25300	Muscarinic M5	Bind	−18	Buckley NJ *et al. Mol Pharmacol*. **35**: 469, 1989
25551	Tachykinin NK1	Bind	14	Patacchini R and Maggi CA. *Arch Int Pharmacodyn Ther*. **329**: 161, 1995
25560	Tachykinin NK2	Bind	1	Patacchini R and Maggi CA. *Arch Int Pharmacodyn Ther*. **329**: 161, 1995
25570	Tachykinin NK3	Bind	0	Patacchini R and Maggi CA. *Arch Int Pharmacodyn Ther*. **329**: 161, 1995
25700	Neuropeptide Y1	Bind	22	Fuhlendorff J *et al. Proc Natl Acad Sci USA*. **87**: 182, 1990
25711	Neuropeptide Y2	Bind	−11	Rose PM *et al. J Biol Chem*. **270**(39): 22661, 1995
26011	Opiate delta	Bind	9	Simonin F *et al. Mol Pharmacol*. **46**: 1015, 1994
26021	Opiate-	Bind	8	Simonin F *et al. Proc Natl Acad Sci USA*. **92**(15): 7006, 1995
26041	Opiate-	Bind	23	Wang JB *et al. FEBS Lett*. **338**: 217, 1994
26060	Orphanin (ORL1)	Bind	7	Ardati A *et al. Mol Pharmacol*. **51**: 816, 1997
26330	Potassium channel [KA]	Bind	23	Rehm H and Laadunski M. *Proc Natl Acad Sci USA*. **85**: 4919, 1988
26370	Potassium channel [KV]	Bind	−5	Vazquez J *et al. J Biol Chem*. **264**: 20902, 1989
26560	Potassium channel [SKCA]	Bind	2	Mourre C *et al. Brain Res*. **382**: 239, 1986
26670	Purinergic P2X	Bind	7	Ziganshin AU *et al. Br J Pharmacol*. **110**: 1431, 1993
27111	Serotonin 5-HT1A	Bind	2	Martin GR and Humphrey PPA. *Neuropharmacol*. **33**: 261, 1994
27130	Serotonin 5-HT1D	Bind	−2	Domenech T *et al. Naunyn Schmiedebergs Arch Pharmacol*. **356**: 328, 1997
27165	Serotonin 5-HT2A	Bind	31	Saucier C and Albert PR. *J Neurochem*. **68**: 1998, 1997
27210	Serotonin 5-HT5A	Bind	21	Rees S *et al. FEBS Lett*. **355**: 242, 1994
27220	Serotonin 5-HT6	Bind	54	Monsma FJ Jr *et al. Mol Pharmacol*. **43**: 320, 1993
27230	Serotonin 5-HT7	Bind	28	Roth BL *et al. J Pharmacol Exp Ther*. **268**: 1403, 1994
27402	Serotonin transporter	Bind	−5	Gu H *et al. J Biol Chem*. **269**: 7124, 1994
27830	Sigma, non-selective	Bind	70	Weber E *et al. Proc Natl Acad Sci USA*. **83**: 8784, 1986
28550	Thromboxane A2 [TXA2]	Bind	59	Hedberg A *et al. J Pharmacol Exp Ther*. **245**: 786, 1988
28631	Tumor hecrosis factor, non-selective	Bind	11	Baglioni C *et al. J Biol Chem*. **260**: 13395, 1985
28680	Vascular endothelinal growth factor	Bind	13	Gitay-Goren H *et al. J Biol Chem*. **271**: 5519, 1996
28701	Vasoactive intestinal peptide VIP1	Bind	−12	Couvineau A *et al. Biochem J*. **231**: 139, 1985
28751	Vasopressia VIA	Bind	18	Thibonnier M *et al. J Biol Chem*. **269**: 3304, 1994
30500	Adhesion, fibronectin-mediated	Cell	5.9	Nowlin D *et al. J Biol Chem*. **268**: 20351, 1993
30510	Adhesion, ICAM-1 mediated	Cell	−4.3	Cobb RR *et al. Biochem Biophys Res Commun*. **185**: 1022, 1992
30750	Adhesion, VCAM-1-mediated antagonist	Cell	26.3	Stoltenborg JK *et al. J Immunol Methods*. **175**: 59, 1994.

Abbreviations: Bind, specific binding; EGF, epidermal growth factor; Enz, enzymatic activity; ERK1, extracellular signal-regulated kinase 1; HMG, 3-hydroxy-3-methyl-glutaryl-CoA reductase; PKA, protein kinase A; ICAM, intercellular adhesion molecule; SOD, superoxide dismutase; VCAM, vascular cell adhesion molecule. The ability of Sch.414319 to affect the biochemical activity of selected assays is presented. Assay results are presented as the percent inhibition of specific binding (Bind), enzymatic activity (Enz) or cellular activity (Cell) according to experimental procedures listed in the references provided.

systems. We were unable to demonstrate an effect of the compounds on human peripheral blood mononuclear cell and murine T-cell proliferation, T-cell and monocyte cytokine production, and surface-marker upregulation—the compounds were either inactive or demonstrated limited activity at concentrations $>5\,\mu M$, deemed inconsistent with a CB_2 receptor-mediated response (data not shown; Lunn *et al*., 2006a, b).

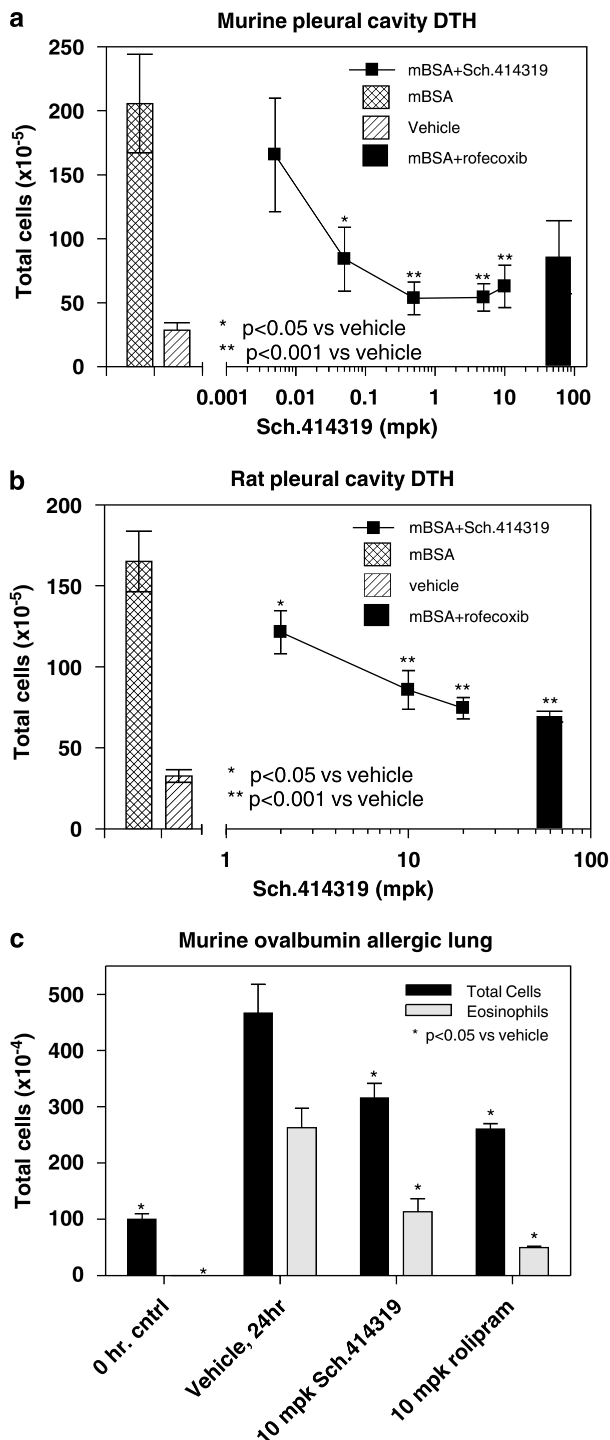
As part of this effort, we evaluated the effect of WIN 55212-2 and HU-210 on lipopolysaccharide-induced cytokines in *Corynebacterium parvum*-primed and unprimed mice. As hoped, the compounds modified inflammatory cytokine levels, decreasing serum tumour necrosis factor- and interleukin-12, and increasing interleukin-10 (Smith *et al*., 2000). However, the compounds appeared to function through the cannabinoid CB_1 receptor. Consistent with this conclusion, the cannabinoids showed similar cytokine responses at significantly lower doses when administered intracerebroventricularly, (Smith *et al*., 2001).

We next examined the ability of triaryl bis-sulphones to control immune cell movement. Modulating immune cells' migration to the site of inflammatory insult has been a powerful driving force behind campaigns to develop chemokine antagonists (De Clercq, 2003; Horuk, 2003; Chen *et al*., 2004). Such chemokine receptor antagonists have shown promise for the treatment of asthma (Varnes *et al*., 2004) and chronic obstructive pulmonary disease, arthritis and reperfusion injury (Widdowson *et al*., 2004). Other laboratories have suggested a link between the cannabinoid CB_2 receptor and cellular movement. Cannabinoids have been demonstrated to mediate cell migration in a variety of cells, including myeloid leukaemic cell line 32D/G-CSF-R, isolated mouse splenocytes (Jorda *et al*., 2002), 1,25-$(OH)_2$ vitamin D_3-treated HL-60 cells (Kishimoto *et al*., 2006), EoL-1 human eosinophilic leukaemia cells and human peripheral blood eosinophils (Oka *et al*., 2004). However, the mechanism by which cannabinoids mediate this effect is complex. In many of the above papers, cannabinoids are shown to attract cells expressing the receptor. This suggests that cannabinoid agonists may be the therapeutically relevant class of compounds. However, cannabinoids upregulated chemokine expression in several cell types (Jbilo *et al*., 1999; Derocq *et al*., 2000). More recently, Coopman *et al*. (2007) have demonstrated an upregulation of chemokine receptors following T-lymphocyte activation. Adding to this complication, the behaviour of cells exposed to cannabinoid agonists did not mimic the behaviour of chemokines. Lopez-Cepero *et al*. (1986) showed that THC suppressed macrophage spreading. Sacerdote *et al*. (2000) argued that the nonspecific cannabinoid agonist CP55,940 blocked both chemokinesis (non-directed cell movement) and N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced cellular chemotaxis of rat peritoneal macrophages, with an $EC_{50}\approx 10$–$30\,nM$. Jorda *et al*. (2002) also argued that chemokinesis played a significant role in this biology—administration of the agonist 2-arachidonoyl glycerol on both sides of the membrane in a transwell chemotaxis chamber generated significant cellular motion to the distal surface. Gokoh (Gokoh *et al*., 2005) used differentiated HL-60 cells to show a time- and dose-dependent increase in F-actin staining (therefore actin polymerization) following 2-arachidonoyl glycerol treatment. This phenotype, abolished when cells were treated with SR144528, was accompanied by the extension of non-directed pseudopods. The investigators suggested the potential involvement of phosphoinositide 3-kinase, Rho-family small G-proteins and a tyrosine kinase in this process. Kurihara *et al*. (2006) observed a repeating random extension F-actin-containing pseudopods in HL60 cells treated with the cannabinoid CB_2 receptor agonist JWH015 and 2-arachidonoyl glycerol. The activity of Rho-GTPase RhoA decreased, and Rac1, Rac2 and Cdc42 activity increased with cannabinoid treatment. These CB_2 receptor-mediated changes appear to be similar to those observed using the Rho-dependent protein kinase (p160-ROCK) inhibitor Y27632. The authors concluded that the CB_2 receptor might exert an immunomodulatory role by controlling the RhoA–ROCK pathway.

Our initial studies (Lunn et al., 2006) showed that the cannabinoid CB_2 receptor inverse agonist Sch.225336 blocked recombinant cells chemotaxis to 2-arachidonoyl glycerol *in vitro*, and blocked migration of immune cells to a CC chemokine ligand (CCL)2-soaked gel foam sponge implanted intraperitoneally. We also showed that oral doses of Sch.225336 blocked cell migration into the peritoneal cavity following antigen challenge, and blocked cell migration to the lungs of sensitized mice following an aerosol antigen challenge. Further studies showed that oral administration of a cannabinoid CB_2 receptor-specific triaryl bis-sulphone blocked cannabinoid-induced migration mediated by HU210, the protein immunogen methylated bovine serum albumin (mBSA), thioglycolate, zymosan and lipopolysaccharide (CA Lunn *et al*., in press). These results have extended to Sch.414319. Figure 1 shows that oral administration of Sch.414319 blocks accumulation of immune cells into the pleural cavity of sensitized CF1 outbred mice (panel A) and sensitized Sprague–Dawley female rats (panel B) challenged with $200\,\mu g$ mBSA. In these models, the triaryl bis-sulphone is almost as effective as $10\,mg\,kg^{-1}$ rofecoxib. Likewise, the figure (panel C) shows that oral administration of Sch.414319 blocks lung eosinophilia following aerosol administration of an ovalbumin antigen to ovalbumin/alum-sensitized male B6D2F1/J mice to an extent comparable to $10\,mg\,kg^{-1}$ rolipram. In all cases, Sch.414319 and the control compound was dosed orally in 0.4% methylcellulose at days -2, -1, 1 h before challenge and 3 h post-challenge. Twenty-four hours after challenge, cells were collected from the pleural cavity or from a bronchial alveolar lavage and quantitated. We conclude from these studies and from the results in the literature that immune cell mobility can be modulated through the cannabinoid CB_2 receptor.

The mechanism by which the cannabinoid CB_2 receptor is linked to cell motility, and the explanation for the effects of both cannabinoid agonists and inverse agonists on this process remain complex. Chemotaxis involves an interaction between both signalling elements (for example, receptors) and structural elements (linked to maintaining cell polarity), with numerous pharmacological strategies available for modulating the process. A number of neuronal receptor systems have been shown to desensitize chemokine receptors (Zhang and Oppenheim, 2005). For example,

heterologous desensitization of chemokine receptors via opioid receptor signalling has also been documented (for review see Steele *et al.*, 2002), a desensitization mediated by phosphorylation of the chemokine receptor via calcium-independent protein kinase C isotypes (Ali *et al.*, 1999; Zhang *et al.*, 2003). Zhang and coworkers have also shown that activation of the A2a adenosine receptors suppresses chemokine receptor function, based on protein kinase A-mediated heterologous desensitization (Zhang *et al.*, 2006).



This is believed responsible, in part, for the anti-inflammatory activity of adenosine.

With these thoughts in mind, and the observation that the first cannabinoid CB_2 receptor inverse agonist, the biarylpyrazole SR144528, was shown to alter p42/p44 MAPK signalling mediated by receptors to insulin and LPA (Bouaboula *et al.*, 1999), we sought to investigate this problem by studying the effect of Sch.414319 on global pattern of protein phosphorylation in isolated immune cells. This decision was stimulated by kinetic experiments showing that initial inhibitory effects we observed on cellular chemotaxis appeared within 30 min of treatment with the cannabinoid receptor inverse agonist (L Bober, data not shown). We also were unable to detect significant changes in proteins synthesized by Jurkat cells treated with 10 nM Sch.414319, as measured by microarray expression profiling (D Lundell, personal communication). Based on these observations, we ruled out a role for new protein synthesis in the biology of Sch.414319 and speculated that the cannabinoid effect was the result of post-translational modification of existing proteins. As an initial test of this hypothesis, we determined the ability of Sch.414319 to modulate cellular protein phosphorylation in peripheral blood mononuclear cells. Peripheral blood mononuclear cells (1×10^7 cells ml^{-1}) from normal human volunteers were resuspended in phosphate-free RPMI medium (Invitrogen Corporation, Carlsbad, CA, USA) containing 1% dialyzed foetal bovine serum (Invitrogen, Corporation, Carlsbad, CA, USA), and then treated with 0.5 mCi ml^{-1} [^{32}P]orthophosphoric acid in the presence or absence of 10 nM Sch.414319 or 10 nM HU210. After incubation at 37 °C for 60 min, cell-free extracts were prepared for two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (O'Farrell, 1975), autoradiography (7-day exposure at −70 °C on Kodak XAR film with intensifier screen) and comparative digital imaging (Kendrick Laboratories Inc., Madison, WI, USA).

Figure 2 shows that we successfully visualized a number of phosphorylated proteins from the radiolabelled cell extracts. Comparative digital imaging of the gels detected 21 polypeptides that showed a difference in phosphorylation following cannabinoid treatment (Table 3). The cannabinoid agonist HU210 altered phosphorylation of five proteins—three of these proteins were also affected by the cannabinoid inverse agonist. However, there was no reciprocal relation

Figure 1 Sch.414319 modulates cell migration *in vivo*. Murine delayed type hypersensitivity reaction was carried out using female CF1 mice as described (Fine *et al.*, 2003). (**a**) Sensitized female CF1 outbred mice were challenged with vehicle or with mBSA (200 μg). Sch.414319 or rofecoxib (10 mg kg^{-1}) was dosed orally in 0.4% methylcellulose at days −2, −1 and 1 h before challenge and 3 h post-challenge. Twenty-four hours after challenge, cells were collected from the pleural cavity and quantitated. (**b**) Sensitized Sprague–Dawley female rats were challenged with vehicle or with mBSA (200 μg), and then dosed as above. (**c**) B6D2F1/J mice sensitized with ovalbumin/alum were challenged with aerosolized ovalbumin after dosing with vehicle, Sch.414319 or rolipram, as described above. Twenty-four hours after challenge, total cells and eosinophils (using differential Wright–Giemsa staining) recovered from a broncheoalveolar lavage were quantitated. mBSA, methylated bovine serum albumin.

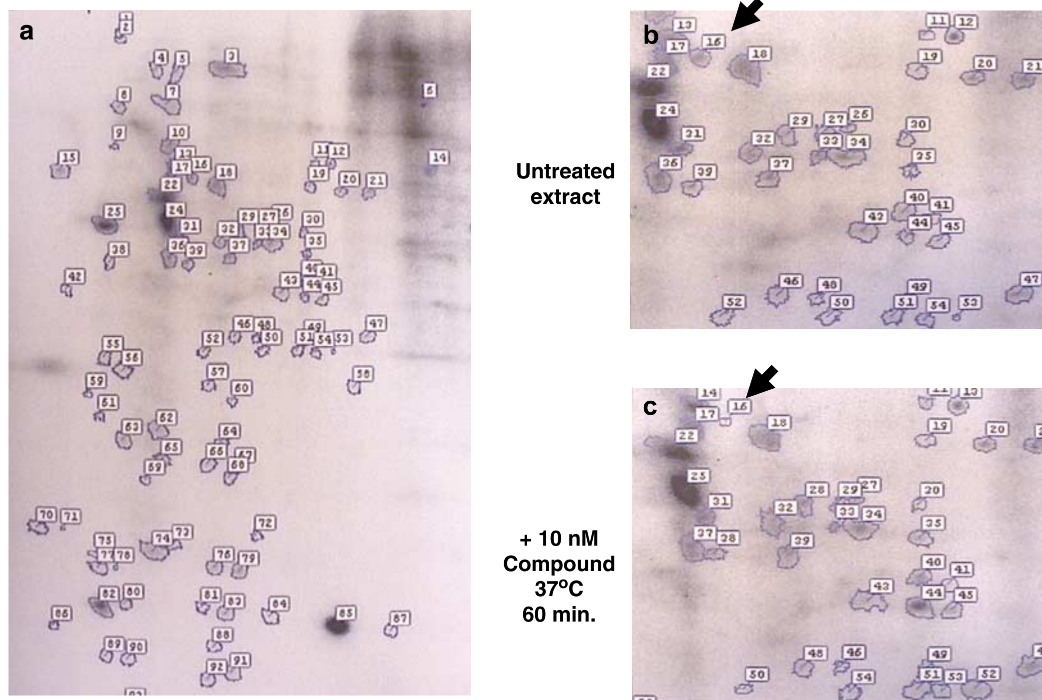


Figure 2 The effect of Sch.414319 on phosphoproteins detected in human mononuclear cell preparations. Isolated human peripheral blood mononuclear cells were treated for 60 min in low phosphate medium containing 0.5 mCi ml^{-1} [^{32}P]orthophosphoric acid in the absence or presence of 10 nM Sch.414319 or 10 nM HU210. Solubilized protein fractions from treated cells were submitted to Kendrick Laboratories for two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (O'Farrell, 1975) autoradiography and comparative digital imaging. (**a**) Autoradiogram of untreated extract. (**b**) Portion of **a** showing protein no. 16 (arrow). (**c**) Corresponding autoradiogram of extract from cells treated with 10 nM Sch.414319 at 37 °C for 60 min. Protein no. 16 is designated by an arrow.

Table 3 Quantitating the effect of Sch.414319 on phosphoproteins detected in human mononuclear cell preparations

Protein reference no.	*Protein pI*	*Protein MWt*	*Sch.414319 Difference*	*HU210 Difference*	
23	8.34	64 541	2616	—	
35	6.62	56 000	301	—	
44	6.6	47 000	554	—	
53	6.81	40 833	6559	—	
71	4.9	25 814	4879	1423	
78	5.24	23 173	2138	—	
1	ND	ND	−91	−97	
2	5.28	194 711	−88	—	
5	5.67	156 000	−89	—	
16	5.77	71 000	−98	—	L-plastin
52	5.84	41 000	−78	—	
57	5.87	37 616	−97	—	
61	5.15	35 000	−69	—	14-3-3-ε
69	5.46	28 647	—	−91	
70	4.74	26 000	−99	—	
72	6.24	25 057	−72	—	
76	5.94	23 000	−70	—	
77	5.13	23 424	−96	—	
86	4.83	20 000	−78	—	
96	ND	ND	—	−91	
7	5.61	119 000	−52	137	

Abbreviations: MWt, molecualr weight; ND, not done.

Cell-free extracts from [^{32}P] orthophosphoric acid-labelled human peripheral blood mononuclear cells plus/minus drug treatment (10 nM, 37 °C, 60 min) were analysed by two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by autoradiography. Resulting films were digitally analysed and quantitated by Kendrick Laboratories (Madison, WI, USA). Differences in film darkening were quantitated according to the following formula:

Difference = (1−spot% sample × /spot % sample ref.)(−100).

Proteins in grey were selected for peptide sequencing by MALDI-MS (Protein Chemistry Core Facility, Columbia University).

between cannabinoid agonist treatment and the cannabinoid inverse agonist treatment (excepting protein no. 7). Two species (protein no. 71 and protein no. 1) showed equivalent changes with treatment by the agonist and inverse agonist. Two species (protein no. 69 and no. 96) were only affected by the agonist. The majority of the detected differences were seen only with Sch.414319 treatment. Because the cannabinoid inverse agonist does not reverse the phosphorylation pattern of the agonist, we question whether we should expect Sch.414319 to function as a classic inverse agonist—reversing the effects of an agonist. Similarly, the biarylpyrazole cannabinoid CB$_2$ receptor inverse agonist SR144528 alone failed to induce significant pain hypersensitivity in model systems where cannabinoid CB$_2$ receptor agonists clearly demonstrate decreased pain sensitivity (see Nackley *et al*., 2003; Elmes *et al*., 2004; Hohmann *et al*., 2004; Beltramo *et al*., 2006; La Rana *et al*., 2006; Gutierrez *et al*., 2007).

Proteins present in sufficient quantities and showing significant changes in phosphorylation following drug treatment were excised from stained daughter gel sheet, digested with endoproteinase Lys-C and trypsin and the resulting peptides sequenced by matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-MS; Protein Chemistry Core Facility, Columbia University). The majority of the proteins tested could not be resolved from background noise. However, two proteins were identified. Figure 3 shows that analysis of protein no. 61 generated seven peptide sequences consistent with human 14-3-3-ε isoform protein (accession number: AAD00026.1) and analysis of protein no. 16 generated eight peptide sequences, consistent with human L-plastin (accession number: P13796; also known as Lymphocyte cytosolic protein 1, LCP-1, LC64P). Examining the biology of these proteins may offer insight into the pathway by which triaryl bis-sulphones regulate cell motility. The first protein, 14-3-3-ε is a member of a conserved acidic protein family known to bind a number of signalling proteins, including kinases, phosphatases and transmembrane receptors. With such a broad distribution of activities, the reported association of 14-3-3 protein with the regulation of cell spreading (Rodriguez and Guan, 2005) may reflect the broad regulatory role of this protein rather than a specific role in cannabinoid CB$_2$ receptor biology.

Of more interest is the modulation of L-plastin phosphorylation by Sch.414319. Plastins are a set of actin-bundling proteins involved in the regulation of the actin cytoskeleton (Delanote *et al*., 2005). Three isoforms have been identified. T-plastin is constitutively expressed in epithelial and mesenchymal cells, I-plastin expression is restricted to absorptive intestinal and kidney cells and finally, L-plastin is expressed in hematopoetic cells, and is overexpressed in many solid tumours. Localization studies find L-plastin associated with structures involved in locomotion, adhesion and immune defense, including filopodia and immune complexes (Jones and Brown, 1996; Babb *et al*., 1997; Samstag *et al*., 2003). Recent studies have shown that phosphorylation of Ser5 increases F-actin-binding activity to the periphery of membrane protrusions in Vero cells (Janji *et al*., 2006). Boldt *et al*. (2006) showed that L-plastin is phosphorylated in polymorphonuclear neutrophils upon

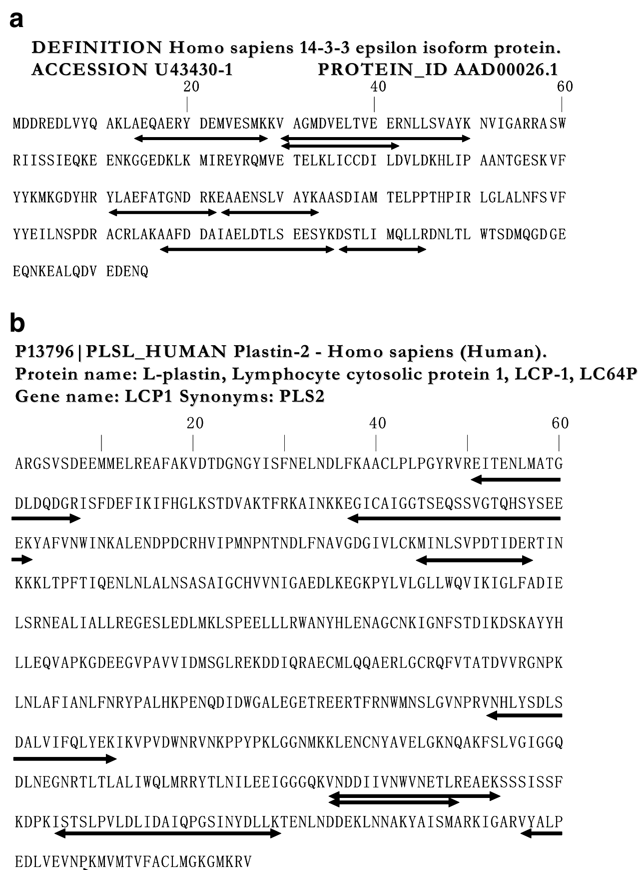


Figure 3 Identification of phosphoproteins selectively modulated by treatment with Sch.414319. Phosphoproteins modulated by treatment of human mononuclear cell preparations with Sch.414319 were detected by two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by autoradiography and quantitative digital imaging of the resulting autoradiogram. Proteins present in sufficient quantities and showing significant changes in phosphorylation following drug treatment were isolated from stained daughter gels, digested with endoproteinase Lys-C and trypsin and submitted for sequencing (Protein Chemistry Core Facility, Columbia University). Sequences and sequence alignments derived from the analysis of protein spot no. 61 (**a**) and protein spot no. 16 (**b**) are presented as lines under specific protein sequences.

FPRL-1 activation with 0.1 μM W-peptide (Trp-Lys-Tyr-Met-Val-Met-NH2) or 1 μM sCKβ1-8 (amino acids 46–137 of the β-chemokine CKβ8, whereas other proteins associated with the cytoskeleton (moesin, cofilin and stathmin) are dephosphorylated. Finally, cells expressing a non-phosphorylatable ser5ala mutant of L-plastin showed decreased expressin of CD25 and CD69 at the cell surface (Wabnitz *et al*., 2007), suggesting phosphorylation of L-plastin may control receptor transport to the cell surface.

All of these studies suggest that control of L-plastin phosphorylation may represent a unique mechanism independent of chemokine receptor desensitization by which triaryl bis-sulphones could control of immune cell mobility and immune modulation. This model suggests that the cannabinoid CB$_2$ receptor-specific triaryl bis-sulphones decreases L-plastin phosphorylation, which decreases the ability to the L-plastin to organize the actin fibrils involved

in cellular polarization, required for controlled cellular chemotaxis. We would argue that this biology is not the direct result of inhibition of the kinases involved in L-plastin phosphorylation. Both protein kinase A (Wang and Brown, 1999) and protein kinase C (Paclet *et al*., 2004) have been implicated in the phosphorylation of L-plastin. We have shown that Sch.414319 does not inhibit the biochemical activity of several protein kinases, including a Ca^{2+}/calmodulin-dependent protein kinase II, epidermal growth factor receptor tyrosine kinase, extracellular signal-regulated kinase 1 serine/threonine kinase, the fyn, HER2 and lck tyrosine kinases, non-selective protein kinase A, a non-selective protein kinase C and selective assays for protein kinase C-, - (I and II) and - (Table 2). This suggests that the decreased phosphorylation of L-plastin following triaryl bis-sulphone treatment is not due to inhibition of these protein kinases independent of cannabinoid CB_2 signalling.

The ability of cannabinoid CB_2 receptor-specific triaryl bis-sulphones to modulate plastin phosphorylation may provide a rationale for linking this class of compounds with cancer cell motility (invasiveness). A number of neoplastic cells from solid tumours are shown to express L-plastin, including 68% from carcinoma cell lines and 53% from mesenchymal cell lines (Lin *et al*., 1993; Park *et al*., 1994). The L-plastin promoter has been used to specifically target gene expression to these cells (Chung *et al*., 1999; Peng *et al*., 2001; Akbulut *et al*., 2004), and in experiments seeking to control invasiveness using antisense RNA (Zheng *et al*., 1999). Klemke *et al*. (2007) showed that L-plastin expression *per se* did not correlate to the penetration depth or the stage of melanoma in human tissue biopsies. However, in the same paper, Klemke and co-workers did show a correlation between L-plastin phosphorylation and tumour cell invasiveness *in vitro* and in a mouse B16 melanoma model.

While these models are interesting, they are complicated by the biology of L-plastin knockout ($LPL^{-/-}$) mice. Chen *et al*. (2003) showed that $LPL^{-/-}$ mice were unable to control *Staphylococcus aureus* infection in subcutaneous abscess model. This effect was not the result of altered immune cell migration—$LPL^{-/-}$ peripheral mononuclear cell (PMN) migration to infection site appeared normal. $LPL^{-/-}$ PMNs bound and ingested opsinized *S. aureus* normally. The authors concluded that the immune deficit in this model resulted from an attenuated respiratory burst in the $LPL^{-/-}$ PMNs. Further studies are needed for comparing this biology with that using small-molecule inhibitors to determine the ultimate mechanism by which cannabinoid inverse agonists function.

Cannabinoid CB_2 receptor and disease: arthritis

The demonstrated ability of triaryl bis-sulphones to modulate immune cell motility provides a generalized, well-validated mechanism for generalized immunoregulation. With the identification of Sch.414319 as an optimized member of this compound class, we have confirmed the ability of this compound to modulate cell migration in a number of animal models described previously. Sch.414319 inhibits leukocyte migration into the pleural cavity of mice and rats sensitized to mBSA antigen, and blocks eosinophilia in fluid recovered from the lungs of mice and guinea pigs sensitized to ovalbumin antigen (data not shown). However, we were most interested in determining the ability of this compound to impact a more complex disease model—antigen-induced arthritis. Studies with a congener of Sch.414319, designated Sch.356036, showed that the compound can ameliorate bone damage in a rat model of relapsing–remitting arthritis, a particularly harmful property of this inflammatory joint disease. In leukocyte recruitment models, this CB_2 receptor-selective compound shows efficacy when added in concert with suboptimal doses of selected anti-inflammatory agents, consistent with its unique function and indicative of its potential therapeutic utility (CA Lunn, *et al*., in press).

The recruitment of inflammatory cells into the joint space has been implicated in the initiation and maintenance of the pannus, a defining characteristic of rheumatoid arthritis. Both T cells (Kraan *et al*., 2004; Desmetz *et al*., 2007) and macrophages (Haringman *et al*., 2005; Szekanecz and Koch, 2007) have been suggested as playing a significant role in the pathology of this disease. The value of chemokine inhibition (Tak, 2006) as well as the ability of current therapeutics to modulate cellular infiltration into the joint (Taylor *et al*., 2000; Jenkins *et al*., 2002; Bukhari *et al*., 2003; Sesin and Bingham, 2005) add to the significance of this therapeutic approach. Based on these data, we sought to evaluate Sch.414319 in a rat model of relapsing–remitting arthritis. For studies using triaryl bis-sulphones, monoarticular arthritis was induced according to described procedures (Buchner *et al*., 1995). Male inbred Lewis rats were first sensitized with mBSA in complete Freund's adjuvant injected near the inguinal lymph node. Fourteen days later, arthritis was induced by injecting 500 μg of a sterile mBSA solution in a volume of 5 μl into the left talar–navicular joint space of animals anesthetized with isofurane vapors. The right paw was similarly injected with sterile saline to serve as an internal control. All animals are acclimated to treatment regime by dosing for two days before disease induction. Animals were handled in accordance with protocols and guidelines established by our institution's Animal Care and Use Committee, and showed no weight loss during progression of the monoarticular arthritis. Figure 4 shows that oral administration of $10\,mg\,kg^{-1}$ Sch.414319 2 days before through 5 days after antigen challenge showed improved bone damage score when measured 2 weeks after the last drug dose. The effect was similar to that seen with $3\,mg\,kg^{-1}$ rofecoxib. This result suggests that treatment with Sch.414319 slowed the development of the antigen-induced monoarticular arthritis, perhaps by slowing the movement of immune cells to the talar–navicular joint space. This fact and the ability of triaryl bis-sulphones to synergize with other anti-inflammatory agents including the cyclooxygenase inhibitor piroxicam, the PDE4 inhibitor rolipram and the steroid dexamethasone, provide a potential use for the class of compound (CA Lunn, *et al*., in press).

The ability to modulate the effects of antigen insult on bone structure using two cannabinoid CB_2 receptor inverse agonists (Sch.414319 and Sch.356036) suggests a link between this receptor and bone physiology. However,

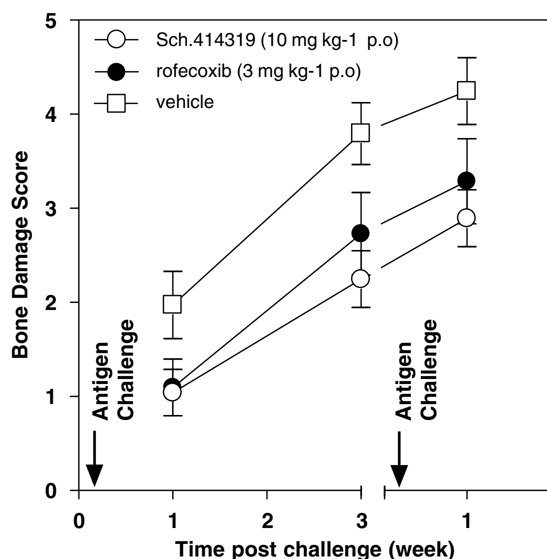


Figure 4 Effect Sch.414319 on bone damage in antigen-induced mono-articular arthritis. Sensitized male inbred Lewis rats (nine per group) were challenged by injection of 5 μl (500 μg) mBSA antigen into the left talar–navicular joint space. The animals were treated 2 days before through 5 days after antigen challenges with vehicle, rofecoxib ($3\,mg\,kg^{-1}$ p.o.) or Sch.414319 ($10\,mg\,kg^{-1}$ p.o.)—animals received no treatment between first and second challenges. Radiologic evaluation was performed on the hind paws using Polaroid type 55 films and a microradiographic X-ray unit (Faxitron Hewlett Packard, McMinnville, OR, USA). Each paw was scored for soft tissue swelling (scale 0–3), erosions (scale 0–3), cartilage space changes (scale 0–3) and periostitis (scale 0–3), making for a total possible score of 12. Animals were used in accordance with protocols and guidelines established by our institution's Animal Care and Use Committee, and showed no weight loss during progression of the monoarticular arthritis. mBSA, methylated bovine serum albumin.

mimicking the complex role of cannabinoid CB_2 receptor pharmacology in cell migration, the role of the cannabinoid CB_2 receptor in bone physiology is complex. Idris *et al.* (2005) have reported that CB_2 (and CB_1) receptor antagonists/inverse agonists AM630 and SR144528 inhibited osteoclast formation in C57BL/6 cell cultures stimulated with RANKL and M-CSF—agonists stimulated osteoclast formation. This result is consistent with the benefit of inverse agonists in maintaining bone density. On the other hand, a study of 388 postmenopausal women showed a significant association of polymorphisms and haplotypes containing the CB_2 receptor gene CNR2 on human chromosome 1p36 with osteoporosis (Karsak *et al.*, 2005). Assuming that the mutations detected in this study would decrease expression or activity of the CB_2 receptor, Karsak suggested the data are consistent with the value of a cannabinoid CB_2 receptor agonist as a valuable therapeutic. Ofek *et al.* (2006) also reported that, while young mice appeared normal, aged (1 year old), CB_2 receptor-deficient mice exhibited an accelerated trabecular bone loss and cortical expansion. He also showed that the cannabinoid CB_2 receptor-specific agonist HU308 stimulated proliferation of osteoblastogenic cultures and restricted osteoclastogenic cultures.

Further investigations are required to rationalize the therapeutic benefit seen using our cannabinoid CB_2 receptor-specific inverse agonists with that observed with cannabinoid CB_2 receptor-specific agonists. We argue that the beneficial therapeutic effect of cannabinoid CB_2 receptor inverse agonists on bone damage is mediated by limiting cell accumulation at the site of insult. Additional mechanisms could be involved in this complex disease, including precursor cell differentiation/proliferation (see above). Other laboratories have described effects of cannabinoid compounds on bone density based on other mechanisms (Sumariwalla *et al.*, 2004; Burstein, 2005; Mbvundula *et al.*, 2005; see Burstein and Zurier, 2004).

Cannabinoid CB_2 receptor and disease: EAE

Another important therapeutic target for cannabinoid CB_2 receptor-specific agents is inflammation of the central nervous system. Like arthritis, this disease involves migration of inflammatory cells to a restricted site, and then action of the cells at that site (Izikson *et al.*, 2002). Early studies showed that the nonspecific cannabinoid agonist WIN55,212-2 could control spasticity and tremors in a chronic relapsing EAE (Baker *et al.*, 2000), although this effect is believed to have been mediated by the cannabinoid CB_1 receptor (Pryce and Baker, 2007). Although initially believed to be uniquely associated with immune cells, functional cannabinoid CB_2 receptors have been found associated with central nervous system tissue (Van Sickle *et al.*, 2005; Beltramo *et al.*, 2006), and the detectible receptor mRNA increases in activated microglia cells with development of transgenic mice expressing a T-cell receptor transgene specific for the acetylated NH_2-terminal peptide of myelin basic protein (Ac1-11) bound to I-A^u (MBP-TCR) (CD4+ T-cell-induced EAE model (Maresz *et al.*, 2005). This effect is believed due to the cannabinoid system within the central nervous system directly suppressing T-cell effector function via the CB_2 receptor (Maresz *et al.*, 2007). Ni *et al.* (2004) have shown that the increased leukocyte/endothelium interactions seen in mice following induction of EAE using myelin oligodendrocyte glycoprotein, can be alleviated with the cannabinoid agonist WIN55,212-2 via the CB_2 receptor. Sipe *et al.* (2005) have extended these studies to human disease, showing the potential linkage of a cannabinoid CB_2 receptor polymorphism associated with autoimmune disorders, including multiple sclerosis.

We have carried out preliminary investigations to investigate the role of the cannabinoid CB_2 receptor in inflammatory diseases of the central nervous system, using the cannabinoid CB_2 receptor-deficient mice. We have shown that the ability of a peptide derived from myelin oligodendrocyte glycoprotein (MOG_{35-55}) to elicit EAE (Reich *et al.*, 2005) is diminished in a cannabinoid CB_2 receptor-deficient mouse strain (Lunn *et al.*, 2006b). The CB_2 receptor-deficient strain showed a significant delay in disease onset, attenuation in clinical disease score and improved survivability (with six of 15 mice dying in the control, but only one of 15 dying in the receptor-deficient group). We have now extended these initial studies to probe the ability of the cannabinoid CB_2 receptor inverse agonist Sch.414319 to modulate EAE in the rat. For these experiments, male Lewis rats were injected with 30 mg of guinea pig spinal cord homogenate in complete Freund's adjuvant into the footpad

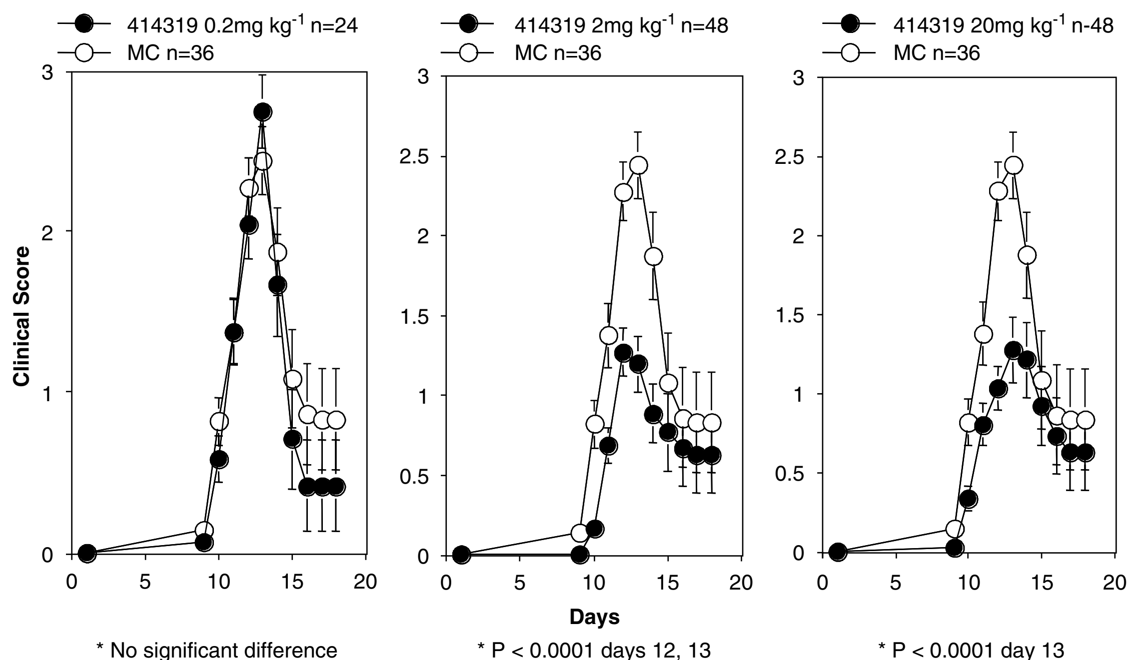


Figure 5 Sch.414319 protects against development of EAE. Male Lewis rats challenged by injection of 50 μl (30 mg) of a guinea pig spinal cord homogenate in complete Freund's adjuvant into one footpad. The animals were treated starting at day 0 and oral dosing continued throughout the 3-week disease course, with varying amounts of Sch.414319 in 0.4% methylcellulose (MC) p.o. Animals were scored for disease severity: 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness; 3, complete hind limb paralysis; 4, complete hind limb paralysis, forelimb weakness or paralysis; 5, death. Data presented represent the sum of two independent experiments. All animals were used in accordance with protocols and guidelines established by our institution's Animal Care and Use Committee. EAE, experimental autoimmune encephalomyelitis.

(Smith *et al.*, 1993). The animals were treated orally with Sch.414319 in 0.4% methylcellulose starting at day 0 then continuing throughout the 3-week disease course. Animals were evaluated daily for clinical disease score. Figure 5 shows a summation of the results of two independent experiments using at least 24 rats per treatment group. The figure shows that oral administration of 20 and $2\,mg\,kg^{-1}$ Sch.414319 significantly modulates the clinical signs of EAE, converting complete hind-limb paralysis to a flaccid tail phenotype. Viewed in another way, we observed that roughly 30% of the vehicle-treated animals showed total limb paralysis, whereas no animals showed total limb paralysis at day 13, when treated with $2\,mg\,kg^{-1}$ Sch.414319. The effect appears dose dependent—treatment with $0.2\,mg\,kg^{-1}$ Sch.414319 is ineffective.

Conclusions

An ability to control the migration of inflammatory cells to the site of insult is a powerful strategy for the development of immunomodulators. Our work on triaryl bis-sulphones suggest that the cannabinoid CB_2 receptor-specific inverse agonists may serve as such immune modulators. We have demonstrated that this class of compound behaves as inverse agonists in selected biochemical assays, including ligand-mediated GTPγS binding, forskolin-stimulated cAMP and in competition binding assays with known cannabinoid agonists/inverse agonists. Recent studies (Gonsiorek *et al.*, 2006) have demonstrated that competition binding in the presence of a radiolabelled triaryl bis-sulphone significantly shifts the observed K_i relative to values obtained using an radiolabelled agonist ligand, consistent with the inverse agonist label. However, cell-based bioassays probing the effect of these compounds on the monocyte kinome (Table 3) suggest that the pharmacology of this class of compounds may be more complex. In this review, we have demonstrated that an optimized triaryl bis-sulphone, Sch.414319, modulates cell migration *in vivo* (Figure 1), can modulate bone damage in antigen-induced mono-articular arthritis (Figure 4) and can modulate the clinical signs of EAE in the Lewis rat strain (Figure 5). We have argued that these effects can all result from a control of inflammatory cell migration, and have presented preliminary evidence for a mechanism involving L-plastin phosphorylation could be involved.

We have not been the only investigators to identify immunomodulatory activities associated with cannabinoid CB_2 receptor inverse agonists. Iwamura *et al.* (2001) showed that oral administration of the inverse agonists JTE-907 and SR144528 inhibited carrageenin-induced paw oedema in mice. Ueda *et al.* (2005) reported that orally administered JTE-907 ($0.1–10\,mg\,kg^{-1}$) and SR144528 ($1\,mg\,kg^{-1}$) significantly inhibited dinitrofluorobenzene-induced ear swelling, with increased cannabinoid CB_2 receptor mRNA expression observed in the inflamed ear. Oka *et al.* (2005) showed that SR144528 treatment blocked 12-*O*-tetradecanoylphorbol-13-acetate-induced ear swelling, consistent with an observed decrease in leukotriene B4 production and decrease in

neutrophil infiltration into the treated mouse ear. Oka *et al.* (2006) also observed that ear swelling was suppressed by administration of SR144528 immediately after sensitization (sensitization phase), or upon challenge (elicitation phase) of oxazolone-induced contact dermatitis. This result correlated with suppressed proinflammatory cytokine mRNA expression and attenuated eosinophil recruitment into the treated ear. Further studies, using these and other CB_2 receptor-specific compounds, will be required to resolve the complex pharmacology of cannabinoids and the cannabinoid CB_2 receptor, and to determine the most effective pharmacology to exploit this therapeutic target.

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Conflict of interest

Work described has been carried out by members of the Schering-Plough Research Institute.

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