Synthesis and pharmacological evaluation of newly detected synthetic cannabinoid receptor agonists AB-4CN-BUTICA, MMB-4CN-BUTINACA, MDMB-4F-BUTICA, MDMB-4F-BUTINACA and their analogs

Eric Sparkes1,2, Rochelle Boyd1,3, Shuli Chen4, Jack W. Markham1,2,5, Jia Lin Luo1,3, Tahira Foyzun6, Humayra Zaman6, Charlotte Fletcher1,3, Ross Ellis7, Iain S. McGregor1,3, Marina J. Santiago6, Felicia Lai5, Roy R. Gerona7, Mark Connor6, David E. Hibbs5, Elizabeth A. Cairns1,3, Michelle Glass4, Adam Ametovski1,2,† and Samuel D. Banister1,2,†

1These authors have contributed equally to this work and share senior authorship

Synthetic cannabinoid receptor agonists (SCRAs) continue to make up a significant portion new psychoactive substances (NPS) detected and seized worldwide. Due to their often potent activation of central cannabinoid receptors in vivo, use of SCRAs can result in severe intoxication, in addition to other adverse health effects. Recent detections of AB-4CN-BUTICA, MMB-4CN-BUTINACA, MDMB-4F-BUTICA and MDMB-4F-BUTINACA mark a continuation in the appearance of SCRAs bearing novel tail substituents. The proactive characterization campaign described here has facilitated the detection of several new SCRAs in toxicological case work. Here we detail the synthesis, characterization, and pharmacological evaluation of recently detected SCRAs, as well as a systematic library of 32 compounds bearing head, tail, and core group combinations likely to appear in future. In vitro radioligand binding assays revealed most compounds showed moderate to high affinity at both CB1 (pK_i = 5.49 ± 0.03 to 9.92 ± 0.09 M) and CB2 (pK_i = 5.49 ± 0.03 to 9.92 ± 0.09 M) receptors. In vitro functional evaluation using a fluorescence-based membrane potential assay showed that most compounds were sub-micromolar to sub-nanomolar agonists at CB1 (pEC50 = 5.92 ± 0.16 to 8.64 ± 0.15 M)
Introduction

Synthetic cannabinoid receptor agonists (SCRAs) represent a large portion of detected new psychoactive substances (NPS) globally, accounting for 29% of the 1,047 NPS identified between 2009 and 2019 (1). Commonly sold as herbal blends, such as “Spice,” “K2,” and “Black Mamba” which are consumed by smoking, SCRAs are part of a conscious effort by manufacturers and retailers to mimic the effect of Δ9-tetrahydrocannabinol (THC, 1, Figure 1), the primary intoxicating compound in cannabis (2–6). Several mass intoxication events have been observed over the past decade, with clinically significant impact on patients including psychosis, seizure, respiratory failure, encephalopathy, necrotizing pancreatitis, acute kidney injury, and death (3,7–31).

SCRAs primarily target the endocannabinoid system, specifically as agonists of centrally expressed cannabinoid 1 receptors (CB1), to provide users with a high analogous to cannabis, although these compounds generally display equal or better affinity and potency at CB1 compared with THC. However, unlike THC, SCRAs such as JWH-018 (2), MDMB-FUBINACA (3), and ADB-BUTINACA (4) generally act as high efficacy agonists at CB1 resulting in significantly greater intoxication of users, and display differing profiles of tolerance, dependence, and withdrawal (32).

Modern SCRAs typically consist of amino acid-derived indole- and indazole-3-carboxamide type scaffolds, similar to compounds disclosed by Pfizer in a series of patents in 2009 (33–38). Appearance of NPS containing modification of these scaffolds has seemingly been a result of incoming legislation bringing detected compounds under national and international control, while attempting to retain CB1 activity. Recent approaches observed include scaffold hopping, fluorination, nitrogen walking, alkyl chain contraction, and homologation, as well as the coupling of different amino acid residues to the substituted heterocyclic cores. These modifications have led to a significant structural evolution of contemporary SCRAs from the late 2000s, with equivalent or increased potency at CB1 (39–41).

The utility of proactive synthesis programs to detect emerging NPS has recently facilitated the detection of two new SCRAs bearing the 4-cyanobutyl tail moiety. The indole and indazole derivatives AB-4CN-BUTICA (5) and MMB-4CN-BUTINACA (6, a.k.a. AMB-4CN-BUTINACA), respectively, were identified in Alabama, facilitated by proactive reference standard generation and toxicological screening by our laboratories (Figure 2) (42, 43). The 4-fluorobutyl compounds MDMB-4F-BUTICA (7) and MDMB-4F-BUTINACA (8) were also recently detected, with the latter involved in a suicide, as well as multiple fatal intoxications (7, 31, 44–49). With the appearance of these compounds, it is feasible that NPS manufacturers may introduce the 4-fluorobutyl or 4-cyanobutyl substituents to other amino acid-derived indole-, indazole- and 7-azaindole-3-carboxamide SCRAs in future. As such, this paper describes the synthesis, chemical analysis, and pharmacological characterization of the newly detected SCRAs, as well as a series of 32 systematic analogs (5–40, Figure 3). Due to the combinatorial design of the library, direct comparison of the contributions of substituents to the pharmacological profile of the compounds enabled identification of key structure-activity relationships (SARs). In vitro binding and functional data were obtained at both CB1 and CB2, and an in silico docking approach was utilized to explore observed SARs, especially concerning differences in binding between 4-cyanobutyl and 4-fluorobutyl tail moieties. Most compounds displayed high affinity, potency, and efficacy at both CB1 and CB2, suggesting these compounds should be included in NPS monitoring programs. Given the similarity in structure and pharmacological profile of the library evaluated in this work compared to existing SCRAs, these data provide important insights into the potential effect of these compounds in humans, pending evaluation in vivo.

KEYWORDS

synthetic cannabinoid, cannabinoid receptor 1 agonists, pharmacology, cannabinoids, SCRAs, docking, in vitro evaluation, synthesis
Experimental

General chemical synthesis details

All reactions were performed under an atmosphere of nitrogen unless otherwise specified. Methyl 1H-indazole-3-carboxylate (42) and methyl 1H-pyrrrolo[2,3-b]pyridine-3-carboxylate (43) were purchased from Fluorochem LTD (Derbyshire, UK) and used as received. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Oakwood Chemical (Estill, SC, USA). Deuterated solvents (CD$_3$OD, CDCl$_3$, and DMSO-d$_6$) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Unless otherwise stated, all other reagents and solvents used for this manuscript were obtained from Sigma-Aldrich/Merck (Castle Hill, NSW, Australia) and used as purchased. Analytical thin-layer chromatography was performed using Merck aluminum-backed silica gel 60 F254 (0.2 mm) plates (Merck, Darmstadt, Germany), which were visualized using shortwave (254 nm) UV fluorescence. Flash chromatography was performed using a Biotage Isolera Spektra One and Biotage SNAP KP-Sil silica cartridges (Uppsala, Sweden), with gradient elution terminating at the solvent combination indicated for each compound (vide infra). One and Biotage SNAP KP-Sil silica cartridges (Uppsala, Sweden), with gradient elution terminating at the solvent combination indicated for each compound (vide infra).

In vitro binding evaluation

Human embryonic kidney (HEK) cells expressing either human CB$_1$ receptors N-terminally tagged with pplss (preprolactin signal sequence) and 3HA (3x haemagglutinin) epitopes or human CB$_2$ receptor N-terminally tagged with 3HA were harvested in 5 mM EDTA in PBS, and “P2” membranes were prepared in sucrose buffer as previously described (52, 53). Protein content was estimated using a BioRad (Hercules, CA) DC protein assay (modified Lowry assay). For binding assays, radioligand ([3H]-CP55,940, PerkinElmer, Waltham, MA, USA), non-radiolabeled drugs, and P2 membrane preparations were diluted in binding buffer (50 mM HEPES pH 7.4, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 2 mg/mL NZ-origin BSA, MP Biomedicals, Santa Ana, CA, USA) and dispensed into 96-well, polypropylene V-well plates (Hangzhou Gene Era Biotech Co Ltd, Zhejiang, China) in a final reaction volume of 200 µL (membranes were dispensed last). Final radioligand concentration was 1 nM for all assays. Protein content was 3 µg/point for pplss-3HA-hCB$_1$ HEK membranes, and 2 µg/point for 3HA-hCB$_2$ HEK membranes for the assays using type A harvest plate (PerkinElmer, GF/C filters, 1.2 µm pores), these plates were discontinued during the course of this study, and replaced with type B harvest plates (Merk Millipore, GF/C filters, 1.2 µm pores). As these plates appear to retain a smaller proportion of the protein, membrane content was increased to 8 µg/point for CB$_1$ membranes, and 3 µg/point for CB$_2$ membranes. When all components had been dispensed, the V well plate was sealed and incubated for 1 h at 30°C. During the incubation, the 96 well harvest plate was treated with 0.1% w/v branched polyethyleneimine (PEI, Sigma Aldrich) in H$_2$O. Immediately prior to washing, PEI was washed through the filters using a vacuum manifold (Pall Corporation, Port Washington, NY) and all wells were washed once with ice cold...
washes of wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 1 mg/mL BSA). Equilibrated binding mixture was then transferred to the harvest plate under vacuum, and samples washed through. For the assays using type A harvest plate, binding wells were rinsed once with wash buffer and transferred to the harvest plate, and then wells were washed three more times with 200 µL of wash buffer. The plate was then removed, and filters allowed to dry overnight. The next day, the plate bottom was sealed, and 50 µL of Ultima Gold XR scintillation fluid (PerkinElmer) was dispensed to each well and the plate loaded into the 96 well “rigid” cassette. For the assays using type B harvest plate, samples were rapidly washed 4 times with 100 µL of wash buffer. The bottom of the plate was then removed, and filters allowed to dry overnight. The next day, the plate was inserted into a 96 well “rigid” cassette. The base of the cassette was sealed and 50 µL of scintillation fluid was added to each well. The top of the plate was sealed prior to loading into a Wallac MicroBeta2⃝ TriLux Liquid Scintillation Counter (PerkinElmer). Scintillation was detected after a 30 min delay, for 2 min per well. Counts were corrected for detector efficiency. Data were then exported and analyzed in GraphPad Prism v9 (GraphPad Software Inc., La Jolla, CA, USA). Kd was determined through fit of “Competition binding- One site fit Kd” in GraphPad prism using a Kd of 3.50 nM for the radioligand for the assays using type A harvest plate; while for the assays using harvest plate B, Kd was 3.58 nM for binding at CB1 and 1.162 nM for binding at CB2 (Kd was determined empirically using homologous competition assay under matching conditions). log Kd were determined for at least three independent experiments (maximum 6) and combined to determine mean pKd ± SEM reported in Tables 1, 2. Assays for compounds 5, 6, 10–12, 14, 15, 17, 18, 20–22, 24, 29, 34 used type A harvest plate. Assays for compounds 7–9, 13, 16, 19, 23, 25–28, 30–33, 35–40 used type B harvest plates.

In vitro functional evaluation

Mouse AtT20 FlpIn adenocarcinoma cells stably transfected with human CB1 or CB2 were cultured in DMEM containing 10% FBS, 100 U penicillin/streptomycin, and 80 µg/mL of hygromycin, as previously described (54). Cells were passaged at 80% confluency, cells for assays were grown in 75 cm² flasks and used at 90% confluency. Between the assay cells were detached from the flask with trypsin/EDTA (Sigma Aldrich) and resuspended in 10 mL of Leibovitz’s L-15 media (L-15) supplemented with 1% FBS, 100 Units penicillin/streptomycin, and 15 µM glucose. The cells were plated in volume of 90 µL in black walled, clear bottomed 96-well microplates (Corning, Corning, NY, USA) and incubated overnight at 37°C in ambient CO₂. Membrane potential was measured using a Membrane Potential Assay Kit (blue) from Molecular Devices (San Jose, CA, USA), as described previously (55, 56). The dye was reconstituted with assay buffer [145 mM NaCl, 22 mM HEPES, 0.338 mM Na₂HPO₄, 4.17 mM NaHCO₃, 0.441 mM KH₂PO₄, 0.407 mM MgSO₄, 0.493 mM MgCl₂, 1.26 mM CaCl₂, 5.56 mM glucose (pH 7.4)]. Prior to the assay, cells were loaded with 90 µL/well of the dye solution without removal of the L-15. Plates were then incubated at 37°C in ambient CO₂ for 60 min. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 s for at least 2 min, at which time either drug or vehicle was added in a volume of 20 µL. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle (DMSO, final concentration was no more than 0.1%) addition. Concentration-response data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA), using a four-parameter non-linear regression to fit concentration-response curves on data from 3 to 6 independent experiments.

Molecular modeling

Protein preparation

The cryo-EM structures of the CB1 receptor (PDB: 6N4B) and the CB2 receptor (PDB: 6KPF) were retrieved from RCSB PDB (57–59). The structures were prepared with Maestro’s Protein Preparation Wizard as follows (60). The G proteins and cholesterol were removed, leaving only the CB1 and CB2 receptor and their cognate ligands. The preparation process consisted of assigning bond orders, adding hydrogens, generation of disulfide bonds, generation of missing side chains using Prime, generating het states using Epik at pH 7.4 ± 1.0, and deleting water molecules beyond 3 Å from het groups (61, 62). The hydrogen bonding network was optimized, the pKₐ values of the protein were predicted using PROPKA, and target pH value was set at 7.4 (63). Lastly, the protein structure was minimized using the OPLS4 force field where RMSD of the atom displacement for terminating the minimization was set as 0.3 Å (64).

Ligand preparation

Ligands were prepared using LigPrep to generate energy minimized 3D structures (65). OPLS4 force field was used for minimization. Epik was used to generate all possible ionized states at pH 7.4 ± 1.0.

Ligand docking

A receptor grid was generated using Glide, with a van der Waals radius scaling factor of 1.0 and a partial charge cutoff at 0.25 (66). The binding site was defined by the centroid
of the cognate ligand for each structure. The Van der Waals scaling factor for the ligands was set to 0.80 with a partial charge cut-off at 0.15. The precision was set to Extra Precision (XP) with flexible ligand sampling. Nitrogen inversions and ring conformations were sampled and Epik state penalties were added to the docking scores. The ligand cores were restricted to the position of the cognate ligand by their maximum common substructure and post docking minimization was performed. Poses that had the lowest RMSD between the 4-fluorobutyl and 4-cyanobutyl analogs were taken for direct comparison.

Strain calculations

The internal strain of ligands in their bound and unbound states was calculated using MacroModel (65). The 4RDDD solvation forcefield was used, a constant 4.00 kcal/mol energy offset selected, a penalty scale factor of 0.25 and Cartesian restraints with a bound state half-width of 0.3 Å and a 120 kcal/mol/Å² force constant.

Binding site mapping

The binding site regions for both the CB₁ and CB₂ structures were evaluated using SiteMap (67). SiteMap was run in evaluate mode using the structures cognate ligand and a 5 Å buffer. A minimum of 15 site points per binding site was set, using the “more restrictive” definition of hydrophobicity and a standard grid. Site maps were cropped at 4 Å from the nearest site point.

Results and discussion

Synthesis

Indole-based SCRA*s were derived from indole (41), with a convenient one-pot alkylation and trifluoroacetylation procedure employed to give intermediate N-alkyl-5-(3-(2,2,2-trifluoroacetyl)-1H-indoles before subsequent hydrolysis gave N-alkyl-1H-indole-3-carboxylic acids 44 and 45 in good yield (Scheme 1, eq. 1). This was then subjected to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/1-hydroxybenzotriazole (HOBt)-mediated amide coupling with the appropriate amino acid derivatives to furnish the final indole derived compounds (5, 7, 9, 10, 17–19, 25–27, 34, 35). The indazole and 7-azaindole derivatives were synthesized using a similar procedure from methyl indazole- or methyl-7-azaindole-3-carboxylates 42 and 43 (Scheme 1, eq. 2). Thus, alkylation with the appropriate alkylbromide and sodium hydride provided methyl N-alkyl-indazole- or methyl N-alkyl-7-azaindole-3-carboxylates 46–49 before hydrolysis to give the corresponding carboxylic acids 50–53. In the same manner as described previously, EDC/HOBt-mediated amide coupling furnished N-alkyl-indazole- or -alkyl-7-azaindole-3-carboxylamides 6, 8, 11–16, 20–24, 28–33, 36–40 (6, 50, 51, 54, 68, 69). Synthetic procedures and characterization data (¹H and 13C NMR, melting point, Rₜ, FTIR, HRMS, and LCMS) for all novel compounds can be found in the supporting information.

In vitro binding affinity trends for 5–40

Following our previous efforts in the characterization of existing SCRA*s, in vitro binding data were obtained via a competitive radio binding assay in HEK293 cells stably transfected with human cannabinoid receptor 1 (hCB₁) or human cannabinoid receptor 2 (hCB₂) (6, 50, 54, 68, 69). Ligand affinity (pKᵢ) was determined based on extent to which compounds displaced the tritiated standard [³H]CP55,940 (Tables 1, 2). Except for terminal amide bearing 7-azaindoles SCRA*s 38, 33 and 16, all compounds displayed micromolar to sub-nanomolar affinity for CB₁ receptors (pKᵢ = 8.89 ± 0.09–5.48 ± 0.11). Similar or increased CB₂ affinity was observed in almost all cases (pKᵢ = 9.92 ± 0.09–5.49 ± 0.03), with several examples exhibiting sub-nanomolar affinities for this receptor subtype (20, pKᵢ = 9.53 ± 0.07; 29, pKᵢ = 9.16 ± 0.04; 7, pKᵢ = 9.30 ± 0.12; 8, 9.92 ± 0.09; 37, pKᵢ = 9.34 ± 0.05), which represent the highest affinities in the series. Across the 4-cyanobutyl and 4-fluorobutyl tail substituents, no clear trend was apparent for CB₁ except within methyl tert-leucinate (18, 20, 23 > 7, 8, 39) and methyl phenylalaninate (35, 37, 40 > 19, 21, 24) subgroups, whereby 4-cyanobutyl and 4-fluorobutyl substitution, respectively, conveyed increased affinity, albeit slightly. Only 4-cyanobutyl bearing 15 (CB₁ pKᵢ = 6.81 ± 0.09) showed affinity an order of magnitude greater than its 4-fluorobutyl analog 32 (CB₁ pKᵢ = 5.90 ± 0.06). Conversely, 4-fluorobutyl derivatives were in general equal or better ligands at CB₂. Variation of the headgroup within the indazole series (AR: 28 > 11; ADB: 29 > 12; APP: 30 > 13; MMB: 36 > 4; MDDB: 8 ≈ 20; MMP: 37 ≈ 21) highlights this trend. A clear structure-affinity trend within the core scaffold was observed, with indazoles (CB₁, pKᵢ = 8.89 ± 0.09–5.48 ± 0.11; CB₂, pKᵢ = 9.53 ± 0.07–6.78 ± 0.03) providing the best affinity to both CB₁ and CB₂, followed closely by indoles (CB₁, pKᵢ = 8.18 ± 0.11–5.50 ± 0.10; CB₂, pKᵢ = 9.30 ± 0.12–6.22 ± 0.13). Finally, 7-azaindoles (CB₁, pKᵢ = 8.03 ± 0.13–< 5; CB₂, pKᵢ = 8.67 ± 0.07–5.49 ± 0.03) exhibited reduced affinity compared with the other groups, often by an order of magnitude. This is in keeping with previous studies detailing related indole, indazole and 7-azaindole carboxamides (36, 51, 54). Notably, the indazole core was required to achieve sub-nanomolar affinity for CB₂ (i.e., 20, 29, 8, 37) except in the case of 7, whereby high affinity was maintained with combination of an indole core, and importantly, the methyl tert-leucinate head group. In each core and tail sub-group, the tert-leucine
derivatives (MDMB > ADB) were consistently the best ligands for both CB$_1$ and CB$_2$, followed by phenylalanine methyl esters (MPP), which were better than both valine derivatives (MMB > AB), and finally, phenylalaninamides (APP). For example, within the indole set, a tert-leucinate conferred the highest CB$_1$ affinity ($18$, $pK_i = 8.18 \pm 0.11$), which was reduced by an order of magnitude for the corresponding phenylalaninate ($19$, $pK_i = 7.07 \pm 0.09$), and further halved for valine derivatives ($17$, $pK_i = 6.80 \pm 0.14$). An alternate order was observed for the corresponding terminal amides of tert-leucine ($9$, $pK_i = 8.17 \pm 0.12$), valine ($5$, $pK_i = 6.26 \pm 0.09$) and phenylalanine ($10$, $pK_i = 5.48 \pm 0.11$), which conferred one of the lowest affinities in the set. Affinities for CB$_2$ followed the same general trend, although phenylalaninamides exhibited affinities up to an order of magnitude greater compared to CB$_1$ (e.g., $13$, CB$_1$ $pK_i = 5.48 \pm 0.11$; CB$_2$ $pK_i = 6.78 \pm 0.03$). Indeed, APP derivatives were not well-tolerated at CB$_1$ and thus modest selectivity for CB$_2$ (4- to 50-fold) was observed in this class (i.e., $10$, $33$, $16$, $27$, $30$).

In vitro CB$_1$ and CB$_2$ functional characterization of 5–40

SARs for compounds 5–40 were also investigated via a fluorescence-based functional assay using AtT20 cells transfected with hCB$_1$ and hCB$_2$. This method measures change in membrane potential resulting from $G_{\beta\gamma}$-coupled activation of inwardly rectifying potassium channels (GIRKs). The full concentration-response curves of 5–40 are shown in Figure 4, with potencies and efficacies referenced to $1\mu$M CP55,940 in Tables 1, 2. Most compounds, except for selected phenylalaninamide and/or 7-azaindole derivatives ($10$, $13$, $16$, $27$, $33$, and $38$), had maximal efficacy ($E_{\text{max}} = 102−122\%$), and exhibited nanomolar to sub-nanomolar potencies ($pEC_{50} = 9.48 \pm 0.14−6.30 \pm 0.05$) at CB$_1$. In general, this group activated CB$_2$ with equal or increased potency ($pEC_{50} = 8.67 \pm 0.17−6.41 \pm 0.10$) and efficacy, albeit, with no ligands active at sub-nanomolar levels. As with other SCRAs, these compounds are generally similar or greater potency, but more efficacious than.
Structures of amino acid-derived 4-cyanobutyl and 4-fluorobutyl-substituted indole, indazole and 7-aza-indole SCRAs explored in this study. AB, [(S)-2-amino-3-methylbutanamide]; ADB, [(S)-2-amino-3,3-dimethylbutanamide]; APP, [(S)-2-amino-3-phenylpropanamide]; MMB, [methyl (S)-2-amino-3-methylbutanoate]; MDMB, [methyl (S)-2-amino-3,3-dimethylbutanoate]; MPP, [methyl (S)-2-amino-3-phenylpropanoate]; 4-CN-BUT, 4-cyanobutyl; 4F-BUT, 4-fluorobutyl; ICA, indole-3-carboxamide; INACA, indazole-3-carboxamide; 7AICA, 7-aza-indole-3-carboxamide.
THC (pEC$_{50}$ = 6.76 ± 0.09 and $E_{\text{max}} = 58\% \pm 3\%$ at CB$_1$, and 32% ± 1% at 30 μM at CB$_2$) (36). Potency compared with THC is also similar (pK$_i$ = 8.09 ± 0.02 and 7.50 ± 0.07 at CB$_1$ and CB$_2$, respectively) (70).

Effect of 4-cyanobutyl and 4-fluorobutyl substituents on CB$_1$ and CB$_2$ activation

Of the 3 chemical modifications investigated in the present study, variation of the tail substituent had the least significant impact on potency and efficacy. The 4-cyanobutyl and 4-fluorobutyl derivatives commonly displayed similar potency at both CB$_1$ and CB$_2$, for example in the tert-leucinate subgroup (18, 20, 23 ≈ 7, 8, 39, respectively), or conversely, exhibited differing potency with no discernable preference for either tail group. Indeed, both tail substituents produced active ligands with sub-nanomolar potency for CB$_1$ (20: pEC$_{50}$ = 9.14 ± 0.14; 8: pEC$_{50}$ = 9.39 ± 0.17) and low nanomolar potency for CB$_2$ (20: pEC$_{50}$ = 8.45 ± 0.07; 8: pEC$_{50}$ = 8.48 ± 0.14). These data are consistent with the corresponding structure-affinity trends; however, binding is not predictive of relative potency in some cases. For example, MDMB-4F-BUTINACA (8) exhibits activation (pEC$_{50}$ = 9.39 ± 0.17) an order of magnitude greater than its affinity (pK$_i$ = 8.21 ± 0.13) for CB$_1$, whilst for CB$_2$, the opposite is true (pEC$_{50}$ = 8.48 ± 0.14; pK$_i$ = 9.92 ± 0.09).

Effect of indole, indazole and 7-azaindole core on CB$_1$ and CB$_2$ activation

In the present study, the nature of the heterocyclic core greatly impacts potency, giving rise to the same trends as previously documented (51, 54, 71). Rank order potency of activity at CB$_1$ closely mirrored the observed binding affinities, whereby indoles (pEC$_{50}$ = 9.39 ± 0.17–6.31 ± 0.05) demonstrated equal or greater potency to the corresponding indazoles (pEC$_{50}$ = 8.57 ± 0.13–<5), which were, again, more potent than the corresponding 7-azaindoles (pEC$_{50}$ = 8.05 ± 0.21–<5). In all cases, the indazole core was required to produce sub-nanomolar potency (pEC$_{50}$ = 9.39 ± 0.17, 9.48 ± 0.14, 9.14 ± 0.14) observed for compounds 8, 12, and 20, respectively. While the same general trend is realized for activation of CB$_2$ (indazoles: pEC$_{50}$ = 8.75 ± 0.03–7.00 ± 0.36; indoles: pEC$_{50}$ = 8.54 ± 0.05–6.49 ± 0.2; 7-azaindoles: pEC$_{50}$ = 8.41 ± 0.08–5.92 ± 0.16), differentiation of heterocyclic cores is less pronounced.
for this receptor, with the MPP subgroup (21 > 19 > 24) highlighting this effect. Notably, this is not apparent for MDMB derivatives (7 ≈ 8 ≈ 39), whereby all core motifs conferred similar potency.

Effect of amino acid derived head groups on CB$_1$ and CB$_2$ activation

As described previously, tert-leucine derived compounds (MDMB/ADB) are commonly more potent than the corresponding valine (AB/AV) species at CB$_1$, despite differing only by a single methyl group (36, 51, 54, 69, 71, 72). In line with this is the greater potencies of MDMB/ADB vs. MMB/AB analogs for indole (18, 9 > 5, 17), indazole (20, 12 > 6, 11) and 7-azaindole (23, 15 > 22, 14) sub-groups observed here. Further, MDMB- and ADB-4F-BUTINACA (8 and 29) represent two of only three substrates with sub-nanomolar potency, and indeed the highest potencies observed for CB$_1$. While the methyl esters of phenylalanine (MPP) were commonly equipotent or greater than valine (AB and MMB) analogs, unlike with tert-leucine derivatives, switching to the terminal amide (APP) was detrimental to potency at CB$_1$. Specifically, when combined with an indole (10 and 27) or 7-azaindole core (16 and 33), the APP substituent produced submaximal agonist activity at the highest concentration tested (10 μM), whereas full efficacy was maintained with the indazole core (i.e., 13: E$_{\text{max}}$ = 104%). In terms of CB$_2$, structure-activity trends align closely with CB$_1$; however, with all phenylalaninamides (APP) except APP-4CN-BUT7AICA (16: E$_{\text{max}}$ = 73%, pEC$_{50}$ = 6.63 ± 0.11) having full agonist effect with moderate potency. As with binding, these findings indicate a CB$_2$ receptor subtype
TABLE 2  Affinities and functional activities of 4-fluorobutyl derived SCRAs at hCB1 and hCB2.

| Compound         | pKi ± SEM (K1, nM) | pEC50 ± SEM (EC50, nM) | Emax ± SEM (% CP55,940) | pKi ± SEM (K1, nM) | pEC50 ± SEM (EC50, nM) | Emax ± SEM (% CP55,940) |
|------------------|-------------------|------------------------|--------------------------|-------------------|------------------------|--------------------------|
| AB-4F-BUTICA (25) | 6.00 ± 0.11 (1,000) | 7.02 ± 0.05 (96.5) | 108 ± 3 | 7.09 ± 0.08 (82) | 7.63 ± 0.05 (23.5) | 97 ± 2 |
| ADB-4F-BUTICA (26) | 7.46 ± 0.08 (35) | 7.39 ± 0.09 (40.4) | 110 ± 4 | 8.10 ± 0.11 (8.0) | 7.36 ± 0.12 (4.4) | 105 ± 4 |
| APP-4F-BUTICA (27) | 5.50 ± 0.10 (3,200) | DNCb | 82 ± 4d | 6.76 ± 0.01 (170) | 6.51 ± 0.16 (360) | 108 ± 8 |
| AB-4F-BUTINACA (28) | 6.88 ± 0.13 (130) | 8.22 ± 0.06 (5.98) | 107 ± 2 | 8.07 ± 0.07 (8.5) | 8.31 ± 0.04 (4.85) | 96 ± 1 |
| ADB-4F-BUTINACA (29) | 8.39 ± 0.08 (4.07) | 8.79 ± 0.06 (1.61) | 116 ± 2 | 9.16 ± 0.04 (0.69) | 8.28 ± 0.07 (5.28) | 103 ± 2 |
| APP-4F-BUTINACA (30) | 5.86 ± 0.13 (1,380) | 6.30 ± 0.03 (504) | 103 ± 3 | 7.55 ± 0.15 (28.2) | 8.08 ± 0.13 (8.35) | 101 ± 12 |
| AB-4F-BUT7AICA (31) | <5 | DNCb | 82 ± 4d | 5.86 ± 0.07 (1.400) | 8.66 ± 0.05 (137) | 98 ± 4 |
| ADB-4F-BUT7AICA (32) | 5.90 ± 0.06 (1,300) | 7.34 ± 0.13 (46.0) | 112 ± 6 | 7.34 ± 0.06 (46) | 8.30 ± 0.04 (4.99) | 108 ± 2 |
| APP-4F-BUT7AICA (33) | <5 | NDd | 25 ± 14d | 5.62 ± 0.04 (2,400) | 5.93 ± 0.16 (1,170) | 99 ± 12 |
| MMB-4F-BUTICA (34) | 7.01 ± 0.10 (97.7) | 7.11 ± 0.04 (76.9) | 116 ± 3 | 7.57 ± 0.05 (26.9) | 7.95 ± 0.05 (11.4) | 102 ± 2 |
| MDMB-4F-BUTICA (7) | 7.88 ± 0.12 (13.2) | 8.47 ± 0.08 (3.43) | 114 ± 3 | 9.30 ± 0.12 (0.35) | 8.54 ± 0.05 (2.87) | 110 ± 3 |
| MPP-4F-BUTICA (35) | 6.57 ± 0.10 (270) | 7.44 ± 0.06 (36.6) | 105 ± 2 | 8.22 ± 0.06 (6.1) | 8.00 ± 0.05 (10.1) | 100 ± 2 |
| MMB-4F-BUTINACA (36) | 7.50 ± 0.15 (32) | 8.41 ± 0.08 (3.93) | 103 ± 2 | 8.81 ± 0.09 (1.6) | 8.42 ± 0.08 (3.76) | 100 ± 2 |
| MDMB-4F-BUTINACA (8) | 8.21 ± 0.13 (6.2) | 9.39 ± 0.17 (0.41) | 106 ± 3 | 9.92 ± 0.09 (0.10) | 8.48 ± 0.14 (3.28) | 103 ± 5 |
| MPP-4F-BUTINACA (37) | 7.47 ± 0.13 (34) | 8.25 ± 0.12 (5.62) | 106 ± 3 | 9.34 ± 0.05 (0.50) | 8.33 ± 0.10 (4.65) | 101 ± 3 |
| MMB-4F-BUT7AICA (38) | 6.65 ± 0.05 (220) | 5.86 ± 0.18 (1,390) | 115 ± 16 | 7.22 ± 0.02 (61) | 7.30 ± 0.13 (49.6) | 114 ± 6 |
| MDMB-4F-BUT7AICA (39) | 6.97 ± 0.08 (107) | 7.65 ± 0.08 (22.2) | 109 ± 3 | 8.67 ± 0.07 (2.14) | 8.41 ± 0.08 (3.88) | 95 ± 2 |
| MPP-4F-BUT7AICA (40) | 6.06 ± 0.13 (880) | 6.20 ± 0.14 (637) | 122 ± 10 | 7.54 ± 0.08 (29) | 7.57 ± 0.17 (26.9) | 110 ± 6 |

*Data represent mean values ± standard error of the mean (SEM) from 3 to 6 independent experiments; 
*Not determined (ND); <50% change in fluorescence at 10 µM; 
*Maximal response at 10 µM.

AB, [S]-2-amino-3-methylbutanamide; ADB, [S]-2-amino-3,3-dimethylbutanamide; APP, [(S)-2-amino-3-phenylpropanamide]; MMB, [methyl (S)-2-amino-3-methylbutanoate]; MDMB, [methyl (S)-2-amino-3,3-dimethylbutanoate]; MPP, methyl [(S)-2-amino-3-phenylpropanate]; 4-CN-BUT, 4-cyanobutyl; 4F-BUT, 4-fluorobutyl; ICA, indole-3-carboxamide; INACA, indazole-3-carboxamide; 7AICA, 7-aza-indole-3-carboxamide.

selectivity for the phenylalaninamide group. In general, the SARs described here corroborate the observed structure-affinity trends for the compounds in the present study, as well as those previously found (54).

**In silico molecular docking study**

To rationalize the novel SARs observed in vitro at CB1 and CB2, we performed docking for each of the screened ligands using the cryo-EM structures of the CB1 (PDB ID: 6N4B) and the CB2 (PDB ID: 6PTO) receptors retrieved from the Research Collaboratory for Structural Bioinformatics Protein Databank (RCSB PDB) (57–59). These agonist-bound structures were chosen for the similarity between our compounds and the cognate ligands MDMB-FUBINACA (6N4B) and WIN 55,212-2 (6PTO).

When docking at CB1 there were π-π interactions between each of the ligands and residues PHE200 and PHE268. The flexible 4-fluorobutyl and 4-cyanobutyl tail groups occupied a hydrophobic pocket comprised of MET363, LEU226, TYR275, LEU193, and ILE271. There is also a common polar interaction between the amide linker carbonyl and SER383, as well as the terminal carbonyl and HIE178.

Docking at CB2 was dictated by hydrophobic interactions between the core and PHE117, and PHE183 residues. The flexible tail occupied a pocket comprised of TRP194, ILE186, TYR190, and SER165. The amide and ester head groups occupied a pocket comprised of PHE91, PHE94, HIS95, and PRO184. Ligands bearing a terminal amide had the potential for H-bonding with the backbone carbonyl of PHE183. Pairs of docking poses for ligands that differed by tail substitution were selected for strain rescoring (see SI, figures S105–116 for all calculated docking poses). This allowed us to investigate the SAR described in the in vitro section, wherein many 4-fluorobutyl ligands showed higher affinity for the CB2 receptor than their 4-cyanobutyl counterparts. Given the lack of formal intermolecular interactions possible for this substituent, the
effect is likely due to the size-constraints of the binding pocket for CB$_2$ as compared with CB$_1$. Figure 5 shows an overlay of compounds 9 and 26 at both CB$_1$ and CB$_2$, as well as the computed hydrophobic regions of the binding site. Relative to CB$_1$, the CB$_2$ binding site region occupied by the 4-fluorobutyl or 4-cyanobutyl tail is significantly constrained. This was observed by Sitemap calculation on the active site of CB$_1$ and CB$_2$ receptors as illustrated in Figure 5. This may force the longer and more rigid 4-cyanobutyl tail into a higher strain conformation to adopt the predicted binding pose. The strain energy of the ligands in their bound state relative to their unbound state was calculated, and the values compared for analogous ligands (see SI Table T1). The mean difference and standard error between the docked 4-cyanobutyl and 4-fluorobutyl ligands were 0.220 ± 0.400 kcal/mol at CB$_1$ and 2.552 ± 0.468 kcal/mol at CB$_2$.

Conclusions

The detected SCRs AB-4CN-BUTICA, MMB-4CN-BUTINACA, MDMB-4F-BUTICA, MDMB-4F-BUTINACA, as well as a series of 32 analogs, were synthesized, characterized, and evaluated in vitro using a radioligand binding assay and a functional membrane potential assay at both human CB$_1$ and CB$_2$. These data confirm that AB-4CN-BUTICA, MMB-4CN-BUTINACA, MDMB-4F-BUTICA, MDMB-4F-BUTINACA are potent and efficacious cannabinoid receptor ligands. Most analogs in the present study, barring the APP (phenylalaninamide) derivatives, were also potent and efficacious cannabinoid ligands. SAR trends observed here were consistent with those previously described for with respect to head group and core group contributions to ligand activity (30, 36, 50, 54, 69, 72). The increased affinity for
Computationally predicted binding modes of 9 (orange) and 26 (green) overlayed at CB$_2$ (A), PDB:6N4B and CB$_2$ (B), PDB:6PT0 (57, 58). The hydrophobic regions of the binding site (grey) were evaluated with SiteMap (67). Glide docking was performed using the Schrodinger computational chemistry suite (66).

4-fluorobutyl derivatives at CB$_2$ is likely to arise from increased strain of the 4-cyanobutyl tail in the hydrophobic tail pocket. Given these pharmacological data, availability of precursory chemical building blocks, ease of synthesis, and structural similarity to previous SCRAs, the compounds evaluated in this study should be monitored as potential emerging NPS in the marketplace.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

ES and JL synthesized and characterized compounds under the supervision of AA and SB. In vitro binding data were obtained by SC under the supervision of MG. In vitro functional characterization was performed by RB and CF under the supervision of EC and by TF and HZ under the supervision of MS and MC. In silico docking experiments were performed by JM under the supervision of FL and DH. HRMS data were obtained by RE under the supervision of RG. ES, JM, IM, EC, MC, MG, AA, and SB conceived the experiments. ES, JM, EC, AA, and SB wrote the manuscript. All authors reviewed drafts of the manuscript and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.1010501/full#supplementary-material
BUTINACA agonist (SCRA) new psychoactive substances (NPS) 5F-PY-PIC A, 5F-PY-PINACA, et al. The chemistry and pharmacology of putative synthetic cannabinoid receptor agonists AB-PINACA, 5F-AB-PINACA and 5F-ADB-PINACA in mice. Tolerance, dependence and withdrawal. Drug Alcohol Depend. (2022) 236:109468. doi: 10.1016/j.drugalcdep.2022.109468

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Glossary

[^3]H]CP55,940, Tritiated 2-((1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)-5-(2-methyloctan-2-yl)phenol
AB-4CN-BUTICA, (S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-cyanobutyl)-1H-indole-3-carboxamide
ADB-BUTINACA, (S)-N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-butyl-1H-indazole-3-carboxamide
AtT20, Mouse AtT20 FlpIn adenocarcinoma cells
BSA, Bovine serum albumin
CB1, Cannabinoid receptor 1
CB2, Cannabinoid receptor 2
CD3OD, Deuterated methanol (d4)
CDCl3, Deuterated chloroform
CH2Cl2, dichloromethane
CP55,940, 2-((1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)-5-(2-methyloctan-2-yl)phenol
cryo-EM, Cryogenic electron microscopy
DMEM, Dulbecco’s modified eagle medium
DMF, Dimethylformamide
DMSO, Dimethyl sulfoxide
DMSO-d6, Deuterated dimethyl sulfoxide (d6)
DNC, Did not converge
EC50, Half maximal effective concentration
EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA, Ethylenediaminetetraacetic acid
E_max, Maximum effect
Et3N, Triethylamine
EtOAc, Ethyl acetate
FBS, Fetal bovine serum
FTIR, Fourier-transform infrared spectroscopy
Gβγ, G beta-gamma subunit
GIRK, G protein-coupled inwardly rectifying potassium channel
HA, Haemagglutinin
hCB1, Human cannabinoid receptor 1
hCB2, Human cannabinoid receptor 2
HEK, Human embryonic kidney cells
HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid
HOBr, 1-hydroxybenzotriazole
HRMS, High resolution mass spectrometry
JWH-018, Naphthalen-1-yl(1-pentyl-1H-indol-3-yl)methanone
K_d, Equilibrium dissociation constant
K_i, Inhibition constant
LC-UV, Liquid chromatography- Ultraviolet
m.p., Melting point
MDMB-4F-BUTICA, Methyl (S)-2-(1-(4-fluorobutyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate
MDMB-4F-BUTINACA, Methyl (S)-2-(1-(4-fluorobutyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate
MDMB-FUBINACA, Methyl (S)-2-(1-(4-fluorobenzyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate
Methanol
MgSO4, Magnesium sulfate
MMB-4CN-BUTINACA, a.k.a. AMB-4CN-BUTINACA, methyl (1-(4-cyanobutyl)-1H-indazole-3-carboxamido)-L-valinate
MS, Mass spectrometry
Na2SO4, Sodium sulfate
NaH, Sodium hydride
ND, Not determined
NMR, Nuclear magnetic resonance
NPS, New psychoactive substance
PBS, Phosphate buffered saline
PEI, Branched polyethyleneimine
pplss, Preprolactin signal sequence
RCSB PDB, Research Collaboratory for Structural Bioinformatics Protein Databank
RMSD, Root-mean-square deviation
SARs, Structure activity relationships
SCRA, Synthetic cannabinoid receptor agonist
SEM, Standard error of the mean
THC, Δ[^9]-tetrahydrocannabinol
XP, Extra Precision