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Novel indole-based compounds that differentiate alkylindole-sensitive receptors from cannabinoid receptors and microtubules: Characterization of their activity on glioma cell migration

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A B S T R A C T

Indole-based compounds, such as the alkyl-indole (AI) compound WIN55212-2, activate the cannabinoid receptors, CB_1 and CB_2, two well-characterized G protein-coupled receptors (GPCR). Reports indicate that several indole-based cannabinoid agonists, including WIN55212-2, lack selectivity and interact with at least two additional targets: AI-sensitive GPCRs and microtubules. Studying how indole-based compounds modulate the activity of these 4 targets has been difficult as selective chemical tools were not available. Here we report the pharmacological characterization of six newly-developed indole-based compounds (ST-11, ST-23, ST-25, ST-29, ST-47 and ST-48) that exhibit distinct binding affinities at AI-sensitive receptors, cannabinoid CB_1 and CB_2 receptors and the colchicine site of tubulin. Several compounds exhibit some level of selectivity for AI-sensitive receptors, including ST-11 that binds AI-sensitive receptors with a K_d of 52 nM and appears to have a weaker affinity for the colchicine site of tubulin (K_d = 3.2 μM) and does not bind CB_1/CB_2 receptors. Leveraging these characteristics, we show that activation of AI-sensitive receptors with ST-11 inhibits both the basal and stimulated migration of the Delayed Brain Tumor (DBT) mouse glioma cell line. Our study describes a new series of indole-based compounds that enable the pharmacological and functional differentiation of alkylindole-sensitive receptors from cannabinoid receptors and microtubules.

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1. Introduction

G protein-coupled receptors (GPCRs) represent the largest family of proteins targeted by current medicines. Recently, small molecules that target GPCRs were shown to exhibit promising therapeutic efficacy for the treatment of various types of cancers, including gliomas (reviewed in [1,2]). Thus, developing compounds that selectively target GPCR is of prime importance to better understand the involvement of these signaling proteins in pathological processes such as the development and growth of cancer, and advance novel therapeutic modalities to treat such devastating diseases.

Cannabinoids activate two well-characterized GPCRs, CB_1 and CB_2, expressed by many healthy cell types and malignant cells, including glioblastoma multiform (GBM, a devastating subtype of brain cancer) [3,4]. Selective activation of either CB_1 or CB_2 receptors expressed by GBM promotes their cell death by apoptosis although with limited efficacy [5,6]. Over 40 years of medicinal chemistry has led to the development of distinct classes of

Abbreviations: 2-AG, arachidonoylglycerol; AI, alkylindole; CB, cannabinoid; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CSA4, combretastatin A4; DBT, delayed Brain Tumor; HA, hemagglutinin; LPA, lysophosphatidic acid; GBM, glioblastoma multiforme; SAR, structure activity relationship; THC, tetrahydrocannabinol; WIN-2, WIN55212-2.

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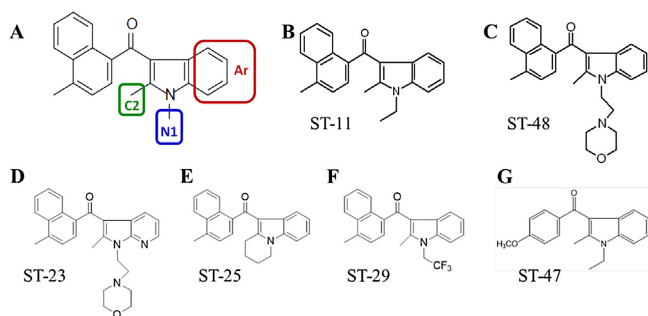

Fig. 1. Chemical structures of ST compounds.
Chemical scaffold shows the 3 moieties – the phenyl portion of the indole (Aroyl, Ar) and the C-2 and N-1 substituents – that were modified in B. ST-11, C. ST-48, D. ST-23, E. ST-25, F. ST-29 and G. ST-47.

synthetic cannabinoids that exhibit nanomolar potencies at CB_1 and CB_2, including HU-210 and JWH-133 (analogs of the plant-derived cannabinoid tetrahydrocannabinol), and WIN55,212-2 (WIN-2) and JWH-015 (synthetic indole-based cannabinoids) [7]. Results from both pharmacological and genetic experiments indicate that many indole-based cannabinoids interact with additional undefined protein targets that are independent of cannabinoid receptors [8]. For example, they interact with alkyl-indole sensitive GPCRs that regulate multiple cellular functions, including the release of neurotransmitters by neurons and the directed migration of microglia [9–11]. The ability of indole-based cannabinoids to curb the growth of cancer, including GBM, is also in part mediated through undefined protein targets. Specifically, WIN-2 and JWH-015 reduce the growth of cancer cells when applied at micromolar concentrations (2–3 orders of magnitude above its described potency and activity at CB receptors), and this anti-neoplastic response is only partially blocked by CB receptor antagonists [12–15]. Our laboratory recently showed that a significant portion of the anti-proliferative activity of indole-based cannabinoids is likely mediated through their direct interaction with tubulin and microtubules [16]. Thus, several indole-based cannabinoids are likely to interact with at least four protein targets (AI-sensitive GPCRs, cannabinoid CB_1 and CB_2 receptors and microtubules). Identifying compounds that differentiate these targets will help both better understand the participation of each protein in various cellular processes, including cancer pathogenesis, and help develop new therapies.

Here we characterized the binding affinity of six newly-developed indole-based compounds (ST compounds, Fig. 1) [9,17,18] at four targets: AI-sensitive receptors endogenously expressed by DBT cells, CB_1 and CB_2 receptors heterologously-expressed by HEK293 cells and purified tubulin. We then took advantage of these findings to study how AI-sensitive receptors regulate DBT cell migration.

2. Materials and Methods

2.1. Materials

ST-11 was synthesized by John W. Huffman at Clemson University, and ST-23, ST-25, ST-29, ST-47 and ST-48 were synthesized by Medchem Source (Seattle, WA). CP55940, SR141617 and SR144528 were provided by the NIDA drug supply program (RTI International, Research Triangle Park, NC). WIN55212-2, 2-arachidonoylglycerol (2-AG) and AM630 were purchased from Tocris (Minneapolis, MN). C18:1 LPA (857230P) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). $[^3H]$WIN55212-2 was purchased from PerkinElmer (Boston, MA). All drug dilutions and experiments were performed using silanized glass test tubes (Alltech, Deerfield, IL) and silanized pipette tips (VWR Scientific, Brisbane, CA) to minimize loss of hydrophobic compounds.

2.2. Radioligand binding to alkylindole-sensitive, CB_1 and CB_2 receptors

P2 membrane fractions were generated and binding performed as previously described, with modifications [9]. Briefly, DBT cells, mCB_1-HEK293 cells and mCB_2-HEK293 cell were expanded in 10 cm dishes until confluent, then rinsed with PBS and collected using homogenization buffer (50 mM Tris, 1 mM EDTA, 3 mM $MgCl_2$). Homogenates were centrifuged at $200 \times g$ for 10 min at 4 °C and the resulting supernatant (P1 fraction) was collected and centrifuged at $125{,}000 \times g$ for 60 min at 4 °C and stored as P2 fractions at −80 °C until further use. P2 membrane fractions were incubated for 1 h with radioligand and compounds in a 30 °C water bath with mild agitation. Reactions were terminated using a Brandel harvester (Brandel, Gaithersburg, MD), collecting radioactive protein on Whatman GFB filter strips (Brandel, Gaithersburg MD). Filters were transferred to 7 ml glass scintillation vials (VWR Scientific, Brisbane, CA), and 4 ml scintillation fluid (National Diagnostics, Atlanta GA; Ecoscint XR) was added to each vial. Samples were vortexed for 10 s and incubated for 18 h at room temperature prior to quantifying radioactivity with a scintillation counter (PerkinElmer, Boston, MA).

2.3. Cell culture and migration

DBT cells were grown in DMEM supplemented with 10% FBS, 5 mM HEPES, 5 mM $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were used in an A series, 96-well format Boyden chamber (Neuroprobe, Gaithersburg, MD) for migration assays as previously described [9,19]. Briefly, 70,000 cells labeled with DRAQ5 (BioStatus, Shepshed, Leicestershire, UK) were loaded to the upper portion of each well of the Boyden chamber fitted with a filter with 10 μm pores. Cells were allowed to migrate for 3 h at 37 °C, after which migrated cells were quantified using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

2.4. Cannabinoid receptor internalization

This assay measures the integrated intensity of the fluorescent signal across the well of a plate. HA-mCB_1 or HA-mCB_2 expressing human embryonic kidney (HEK) cells were grown to 95% confluence in DMEM + 10% FBS + 0.5% Penicillin/Streptomycin [20,21]. Cells were washed once with HEPES buffered saline (HBS)/bovine serum albumen (BSA) (BSA, 0.08 mg/ml) 200 μl per well. Drugs in HBS/BSA were applied at the indicated concentrations to cells and were incubated for the indicated time at 37 °C. As SR144528 is an inverse agonist of CB_2 receptors, we used AM630 as the antagonist in the internalization assay [22]. Cells were then fixed with 4% PFA for 20 min and washed 4 times (200 μl per well) with Tris-buffered saline (TBS). Blocking buffer (Odyssey Blocking buffer, LI-COR, Inc. Lincoln, NE) was applied at 100 μl per well for 1 h at room temperature. Anti-HA antibody, (1:500,Cat#901503, Lot#B207272, BioLegend, San Diego, CA) diluted in 50:50 Odyssey Blocking Buffer and PBS, was then applied for 1 h at room temperature. Following this, the plate was washed 4 times (200 μl per well) with TBS. Secondary antibody diluted (anti-mouse 680 antibody 1:800, LI-COR, Inc.) in 50:50 blocking buffer and PBS, was then applied for one hour at room temperature. The plate was then washed 4 times (200 μl per well) with TBS. The plate was imaged using an Odyssey scanner (700 nm channel, 5.0 intensity, LI-COR, Inc.).

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