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Research Article

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Synthesis and Pharmacological Evaluation of Analogs of Indole-Based Cannabimimetic Agents

Orazio Mazzoni[1,†], Maria V. Diurno[1,*], Antonio M. di Bosco[1], Ettore Novellino[1], Paolo Grieco[1], Giovanni Esposito[1], Alessia Bertamino[1], Antonio Calignano[2] and Roberto Russo[2]

[1]Dipartimento di Chimica Farmaceutica e Tossicologica, Via D. Montesano 49 Università di Napoli Federico II, Naples, Italy
[2]Dipartimento di Farmacologia Sperimentale, Via D. Montesano 49 Università di Napoli Federico II, Naples, Italy
**Corresponding author: Prof Maria V. Diurno, diurno@unina.it*
[†]This manuscript is dedicated to Orazio Mazzoni.

Aminoalkylindoles (AAIs), although structurally dissimilar from the classical cannabinoids, are known to be capable of binding to cannabinoid receptors and of evoking cannabinomimetic responses. With the aim of investigating the structure–activity relationships (SAR) for the binding of non-classical agonists to CB1 and CB2 cannabinoid receptors, we designed and synthesized a series of indole derivatives. The compounds were tested for their analgesic action by formalin test and compared to WIN 55212-2, an AAI acting to the cannabinoid receptors. In receptor binding assay, compound 5 showed affinity for the CB1 receptor comparable to WIN 55212-2.

Key words: aminoalkylindole analogs, analgesic agents, cannabimimetic agents, CB1 receptor, SAR study

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Cannabinoids interact with cannabinoid receptors to produce a variety of potential beneficial therapeutic effects including attenuation of nausea and vomiting in cancer chemotherapy, management of glaucoma, the suppression of muscle spasticity/spasm associated with multiple sclerosis (1), disorders associated with Alzheimer's disease (2,3), and therapeutic effects of analgesia (4–6). Furthermore, CB1 receptor antagonists/inverse agonists have therapeutic application as appetite suppressant (7,8); the rimonabant (SR141716A, Acomplia™, Sanofi-Aventis, Paris, France) (9) has been approved in some countries for the treatment of obesity (10–13) and in the management of schizophrenia (14,15), although recently this compound has been withdrawn from clinical development.

These therapeutic applications claimed for cannabinoid receptor agonists have triggered the synthesis of new compounds that are able to modulate the activity of cannabinoid receptors (16–20). One of the most common examples of non-classic type of cannabinoids is pravadoline that, together with WIN 55212-2 (Chart 1), belongs to the AAI class (21–23). These compounds show high affinity toward the cannabinoid receptors (24) and in addition, in several pharmacological and behavioral assays, they are more active with respect to the classical cannabinoid (CC) THC (Δ^9-tetrahydrocannabinol) acting as an agonist at cannabinoid receptors (25,26).

Originally, AAIs analogs were synthesized as cyclo-oxygenase inhibitors (27,28), but subsequently, they were also found to possess antinociceptive activity. Unlike the opioids, their inhibitory effects were not blocked by naloxone (29). More evidences have shown that AAIs analogs of pravadoline, lacking the cyclooxygenase inhibitory effect, endow cannabinomimetic response (27,30,31), by binding at cannabinoid receptors and represent an important class of cannabinoid receptor agonists.

The most potent AAIs analogs share some common features, such as an indole ring system, a tertiary amine moiety, and a naphthoyl or aroyl group. In contrast, the potent CCs are structures containing a phenolic moiety, a cyclohexyl ring and, a hydrophobic side chain (32,33). However, AAIs and CCs share a similar high lipophilicity.

Previously, have been reported two alternative binding conformations for WIN 55212-2, defined *aroyl-up1* and *aroyl-up2* (34), where the naphthyl ring of this ligand is oriented within the receptor upward with respect to the extracellular side. Aromatic–aromatic interactions are important not only for the binding of WIN but also for inducing receptor conformational change. It is possible that differences in the nature of the ligand binding could contribute to ligand-specific conformational changes in the receptor. In fact, it was observed that AAI structural analogs devoid of the carbonyl oxygen of the aroyl moiety showed an increased binding affinity compared with the corresponding AAI compound. This finding would suggest that the C3 carbonyl oxygen and its possible H-bonding would not be important for AAI binding to the CB1 receptor.

Starting from the structure of pravadoline and WIN 55212-2, we have synthesized a series of indole derivatives (General structures A and B of Chart 1) that show the common features of AAIs.

These compounds, prepared by reaction of 1-morpholinoethyl-indole-2,3-dione or 1-morpholinoethyl-indole-3-aldheyde, with aryl or naphthyl amine (Schemes 1 and 2) display the spatial and electronic

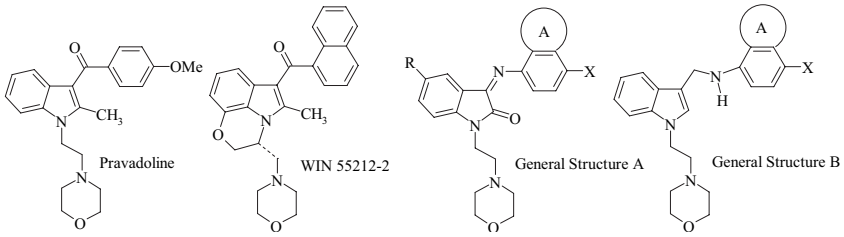


Chart 1: Design of Synthesized compounds in relation to Pravadoline and WIN55212-2.

requirements for binding to the cannabinoid receptors: two aromatic moieties, one of them represented by an indole nucleus which is connected to a cyclic lipophilic group and a hydrogen acceptor group, represented by an azomethinic or aminic nitrogen in our molecules.

We performed molecular modeling and molecular dynamic studies on compounds **1**–**10** and on pravadoline and WIN 55212-2, taken as reference compounds in this study. The Figure 1A–C shows the 3D structures of compound **5**, obtained by a simulated annealing method (see Experimental section) and the conformation of pravadoline and WIN 55212-2, (Cambridge database)[a]. As showed in Figure 1, compound **5**, pravadoline, and WIN 55212-2 are well overlapped (D–F).

Experimental Section

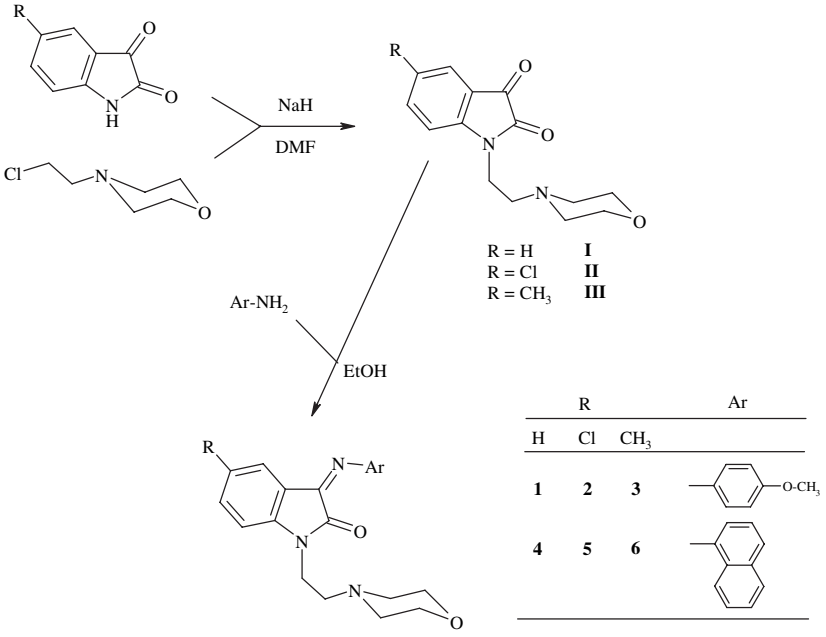
Chemistry

Melting points were determined by a Kofler apparatus and are uncorrected. The elemental analysis (C, H, and N) of reported compounds agrees with the calculated values and was within ±0.4% of theoretical values. Mass spectra (ESI) were obtained on an API 2000 spectrometer (Applied Biosystems, Foster City, CA, USA). The IR spectra were taken on a Perkin-Elmer 1760-X IFT spectrophotometer (Wellesley, MA, USA) in potassium bromide, and the amide carbonyl group ranges from 1690 to 1710/cm while the C=N stretching lies at 1640/cm. The purity of compounds was checked by ascending TLC on Merck's silica gel plates (0.25 mm) with fluorescent baking. NMR measurements (data reported in δ) were performed on a Varian 500 MHz spectrometer. The chemical shifts are referenced to $CDCl_3$ solvent signals at δ 7.26. Me_4Si was used as internal reference.

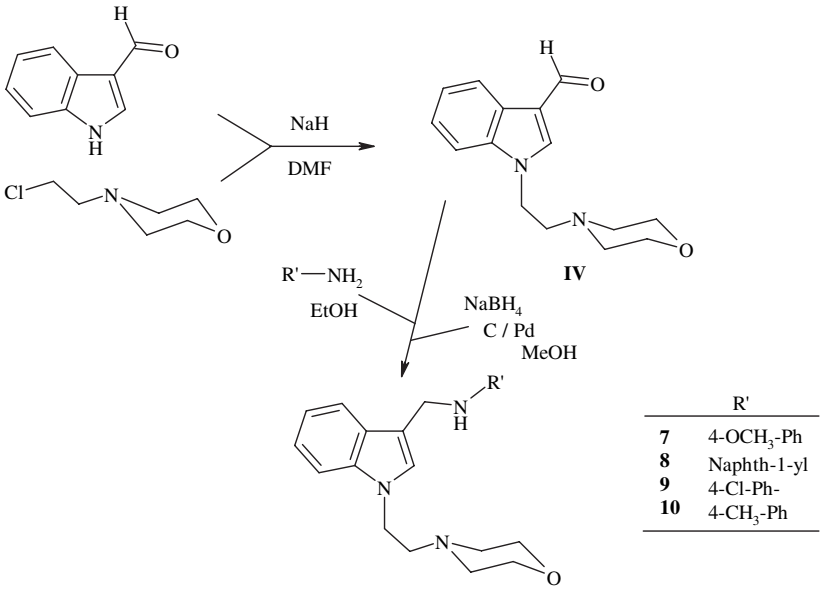
3-(Arylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one (1 and 4) and their 5-substituted-derivatives (2, 3, 5 and 6). General Procedure

To a mixture of indole-2,3-dione (or 5-substituted-indole-2,3-dione) (10 mmol) and sodium hydride (15 mmol), in anhydrous Dimethylformamide (DMF) (20 mL) at room temperature, was added a solution of *N*-(chloroethyl)morpholine hydrochloride (10 mmol) in anhydrous acetonitrile. The reaction mixture, stirred for 8 h at reflux temperature, kept at room temperature for 48 h and evaporated in vacuum yielded the corresponding intermediate 1-(2-morpholin-4-yl-ethyl)-1*H*-indole-2,3-dione (**I**) (or its 5-substituted derivatives (**II** and **III**)) as a brown oil. Chromatography on silica gel using ethyl acetate: carbon tetrachloride (3:2, v:v) as eluent afforded compounds as colored powder.

I (or its 5-substituted derivatives **II** and **III**) was dissolved in absolute ethanol, the appropriate aromatic amine was added to the solution, and the mixture was stirred for 4 h at refluxing tempera-



Scheme 1: Synthesis of compound 1–6.



	R'
7	4-OCH_3-Ph
8	Naphth-1-yl
9	4-Cl-Ph-
10	4-CH_3-Ph

Scheme 2: Synthesis of compounds 7–10.

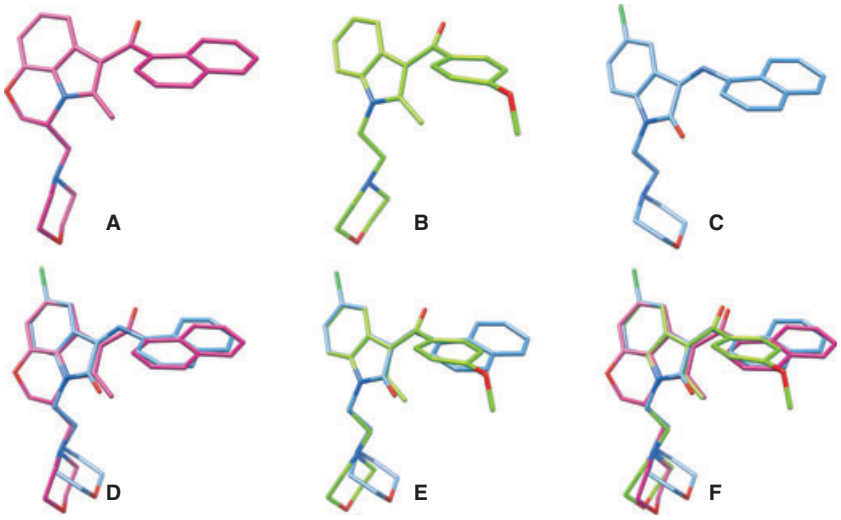


Figure 1: The 3D structures of WIN 55212-2 (A), pravadoline (B) obtained by Cambridge database, and compound **5** (C) obtained by molecular dynamics. The superimposition of WIN 55212-2 and compound **5** (D), pravadoline and compound **5** (E), and WIN 55212-2, pravadoline, and compound **5** (F).

ture. After cooling, the mixture was evaporated in vacuo yielding the crude product that was recrystallized from a mixture of ethyl acetate–ethanol (2:1, v:v).

(I) 1-(2-Morpholin-4-yl-ethyl)-1*H*-indole-2,3-dione

Orange powder. Yield: 45%. Mp 172–4 °C; ^{1}H NMR 7.83 (1H, d; J = 8.0 Hz), 7.79 (1H, d; J = 8.0 Hz), 7.52 (1H, t; J = 8.0 Hz), 7.19 (1H, t; J = 8.0 Hz), 3.65 (4H, dt; J = 10.0 Hz), 3.09 (2H, t; J = 7.1 Hz), 2.60 (2H, t; J = 7.1 Hz), 2.36 (4H, dt; J = 10.0 Hz); anal $C_{14}H_{16}N_2O_3$ C, H, N; Mass calcd. 260.29; MS (ESI+) *m/z* found 261.31 (M + H^+; 100%).

(II) 5-Chloro-1-(2-morpholin-4-yl-ethyl)-1*H*-indole-2,3-dione

Dark orange powder. Yield: 38%. Mp 120–1 °C; ^{1}H NMR 7.80 (1H, s), 7.77 (1H, d; J = 8.1 Hz), 7.53 (1H, d; J = 8.1 Hz), 3.67 (4H, dt; J = 10.0 Hz), 3.06 (2H, t; J = 7.1 Hz), 2.62 (2H, t; J = 7.1 Hz), 2.37 (4H, dt; J = 10.0 Hz); anal $C_{14}H_{15}ClN_2O_3$ C, H, N; Mass calcd. 294.73; MS (ESI+) *m/z* 295.70 (M + H^+; 100%).

(III) 5-Methyl-1-(2-morpholin-4-yl-ethyl)-1*H*-indole-2,3-dione

Brown powder. Yield: 56%. Mp 130–2 °C; ^{1}H NMR 7.71 (1H, d; J = 8.2 Hz), 7.59 (1H, s), 7.32 (1H, d; J = 8.2 Hz), 3.67 (4H, dt; J = 10.0 Hz), 3.07 (2H, t; J = 7.1 Hz), 2.61 (2H, t; J = 7.1 Hz), 2.37 (4H, dt; J = 10.0 Hz), 2.35 (3H, s); anal $C_{15}H_{18}N_2O_3$ C, H, N; Mass calcd. 274.32; MS (ESI+) *m/z* 275.31 (M + H^+; 100%).

(1) 3-(4-Methoxy-phenylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Yellow powder. Yield: 65%. Mp 174–75 °C; ^{1}H NMR 7.67 (1H, d; J = 8.2 Hz), 7.60 (1H, d; J = 8.2 Hz), 7.27 (1H, t; J = 8.1 Hz), 7.20

(2H, d; J = 8.3 Hz), 7.03 (1H, t; J = 8.2 Hz), 6.80 (2H, d; J = 8.3 Hz), 3.73 (3H, s), 3.67 (4H, dt; J = 11.0 Hz), 3.06 (2H, t; J = 7.2 Hz), 2.62 (2H, t; J = 7.2 Hz), 2.37 (4H, dt; J = 11.0 Hz); anal $C_{21}H_{23}N_3O_3$ C, H, N; Mass calcd. 365.43; MS (ESI+) *m/z* 366.42 (M + H^+; 100%).

(2) 5-Chloro-3-(4-methoxy-phenylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Orange powder. Yield: 62%. Mp 205–6 °C; ^{1}H NMR 7.61 (2H, d+s; J = 8.2 Hz), 7.28 (1H, d; J = 8.1 Hz), 7.22 (2H, d; J = 8.3 Hz), 6.78 (2H, d; J = 8.3 Hz), 3.75 (3H, s), 3.68 (4H, dt; J = 11.0 Hz), 3.05 (2H, t; J = 7.2 Hz), 2.60 (2H, t; J = 7.2 Hz), 2.37 (4H, dt; J = 11.0 Hz); anal $C_{21}H_{21}ClN_3O_3$ C, H, N; Mass calcd. 398.86; MS (ESI+) *m/z* 399.78 (M + H^+; 100%).

(3) 5-Methyl-3-(4-methoxy-phenylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Dark orange powder. Yield: 58%. Mp 140–1 °C; ^{1}H NMR 7.55 (1H, d; J = 8.1 Hz), 7.40 (1H, s), 7.19 (2H, d; J = 8.3 Hz), 7.08 (1H, d; J = 8.1 Hz), 6.81 (2H, d; J = 8.2 Hz), 3.70 (3H, s), 3.65 (4H, dt; J = 11.0 Hz), 3.08 (2H, t; J = 7.0 Hz), 2.61 (2H, t; J = 7.0 Hz), 2.39 (4H, dt; J = 11.0 Hz); anal $C_{22}H_{24}N_3O_3$ C, H, N; Mass calcd. 378.44; MS (ESI+) *m/z* 379.46 (M + H^+; 100%).

(4) 3-(Naphthalen-1-ylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Orange powder. Yield: 71%. Mp 178–80 °C; ^{1}H NMR 7.70 (3H, m), 7.67 (1H, d; J = 8.5 Hz), 7.60 (1H, d; J = 8.4 Hz), 7.30 (4H, m), 7.27 (1H, t; J = 8.4 Hz), 7.03 (1H, t; J = 8.5 Hz), 3.67 (4H, dt; J = 11.0 Hz), 3.07 (2H, t; J = 7.2 Hz), 2.62 (2H, t; J = 7.2 Hz), 2.37 (4H, dt; J = 11.0 Hz); anal $C_{24}H_{22}N_3O_2$ C, H, N; Mass calcd. 384.45; MS (ESI+) *m/z* 385.46 (M + H^+; 100%).

(5) 5-Chloro-3-(naphthalen-1-ylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Orange powder. Yield: 68%. Mp 130–31 °C; ^{1}H NMR 7.72 (3H, m), 7.63 (2H, d+s; J = 8.3 Hz), 7.28 (4H, m), 7.28 (1H, d; J = 8.3 Hz), 3.65 (4H, dt; J = 10.0 Hz), 3.09 (2H, t; J = 7.1 Hz), 2.60 (2H, t; J = 7.1 Hz), 2.37 (4H, dt; J = 10.0 Hz); anal $C_{24}H_{21}ClN_3O_2$ C, H, N; Mass calcd. 418.9; MS (ESI+) *m/z* 419.91 (M + H^+; 100%).

(6) 5-Methyl-3-(naphthalen-1-ylimino)-1-(2-morpholin-4-yl-ethyl)-1,3-dihydro-indole-2-one

Dark orange powder. Yield: 55%. Mp 188–89 °C; ^{1}H NMR 7.70 (3H, m), 7.55 (1H, d; J = 8.3 Hz), 7.40 (1H, s), 7.31 (4H, m), 7.07 (1H, d; J = 8.3 Hz), 3.67 (4H, dt; J = 10.0 Hz), 3.07 (2H, t; J = 7.1 Hz), 2.61 (2H, t; J = 7.1 Hz), 2.37 (4H, dt; J = 10.0 Hz); anal $C_{25}H_{24}N_3O_2$ C, H, N; Mass calcd. 398.48; MS (ESI+) *m/z* 399.51 (M + H^+; 100%).

(4-Aryl)-[1-(2-morpholin-4-yl-ethyl)-1H-indole-3-ylmethyl]-amine (7–10). General Procedure

To a mixture of indole-3-aldehyde (10 mmol) and sodium hydride (30 mmol), in anhydrous DMF (20 mL) at room temperature, was added a solution of *N*-(chloroethyl)morpholine hydrochloride (10 mmol). The reaction mixture, stirred for 12 h at reflux temperature, kept at room temperature 24 h and evaporated in vacuum, yielded the corresponding 1-(2-morpholin-4-yl-ethyl)-1*H*-indole-3-carbaldheyde (**IV**) as a brown oil. Chromatography on silica gel using ethyl acetate: chloroform 2:1 v:v, as eluent afforded pure **IV** as an orange powder.

IV was dissolved in absolute ethanol, and appropriate arylamine was added to the solution. The mixture was stirred for 4 h at refluxing temperature. After cooling, the mixture was evaporated in vacuum yielding the crude product. Owing to the instability of the indole immine derivative, it was submitted to the reduction without purification.

To a suspension of $NaBH_4$ (10 mmol) and C/Pd (15 mmol) in ethanol/water (1:1 v:v) (30 mL), crude immine was added, and the mixture was stirred for 3 h at room temperature. The resulting mixture was treated with HCl 2N (20 mL), filtered, and evaporated in vacuum. The resulting residue was collected, dissolved in chloroform and washed with water (3 × 50 mL). Organic layer was evaporated and residue purified by chromatography on silica gel using ethyl acetate: *n*-hexane 1:1 as eluent yielded products a colored oils.

(IV) 1-(2-Morpholin-4-yl-ethyl)-1*H*-indole-3-carbaldheyde

Dark orange oil. Yield: 52%. ^{1}H NMR 9.95 (1H, s), 8,27 (1H, d; J = 7.8Hz), 7.79 (1H, s), 7.25 (3H, d+t+t), 4.24 (2H, t; J = 7.5 Hz), 3.65 (4H, m), 2.74 (2H, t; J = 7.5 Hz), 2.42 (4H, m); anal $C_{15}H_{18}N_2O_2$ C, H, N; Mass calcd. 258.32; MS (ESI+) *m/z* 259.31 (M + H^+; 100%).

(7) (4-Methoxy-phenyl)-[1-(2-morpholin-4-yl-ethyl)-1*H*-indole-3-ylmethyl]-amine

Orange oil. Yield: 52%. ^{1}H NMR 7.66 (1H, d; *J* = 7.6 Hz); 7.35 (1H, d; J = 7.6Hz); 7.25 (1H, t; J = 7.6 Hz); 7.13 (1H, t; J = 7.6 Hz); 6.81 (2H, d; J = 8.4 Hz); 6.76 (2H, d; J = 8.8 Hz), 6.71 (1H, s), 4.42 (2H, s), 4.22 (2H, t; J = 7.8 Hz), 3.76 (3H, s), 3.69 (4H, m), 2.74 (2H, t; J = 7.8 Hz), 2.49 (4H, m); anal $C_{22}H_{27}N_3O_2$ C, H, N; Mass calcd. 365.47; MS (ESI+) *m/z* 366.42 (M + H^+; 100%).

(8) [1-(2-Morpholin-4-yl-ethyl)-1*H*-indole-3-ylmethyl]-naphthalen-1-yl-amine

Pale yellow oil. Yield: 64%. ^{1}H NMR 7.81 (1H, d; J = 7.6 Hz), 7.74 (2H, m), 7.44 (3H, m), 7.30 (3H, m), 7.22 (1H, s), 7.15 (1H, t; J = 7.6 Hz), 6.79 (1H, d; J = 8.0 Hz), 4.63 (2H, s), 4.25 (2H, t; J = 7.8 Hz), 3.71 (4H, m), 2.77 (2H, t; J = 7.8 Hz), 2.51 (4H, m); anal $C_{25}H_{27}N_3O$ C, H, N; Mass calcd. 385.50; MS (ESI+) *m/z* 386.51 (M + H^+; 100%).

(9) (4-Chloro-phenyl)-[1-(2-morpholin-4-yl-ethyl)-1*H*-indole-3-ylmethyl]-amine

Pale yellow oil. Yield: 48%. ^{1}HNMR 7.62 (1H, d; J = 8.8 Hz); 7.39 (1H, d; J = 8,8 Hz); 7.23 (1H, t; J = 8.8 Hz); 7.12 (3H, m); 7.02 (1H,

s; 6.49 (2H, d; J = 8.8 Hz); 4.41 (2H, s); 4.21 (2H, t; J = 7.8 Hz); 3.64 (4H, m); 2.75 (2H, t; J = 7.8 Hz); 2.44 (4H, m); anal $C_{21}H_{24}ClN_3O$ C, H, N; Mass calcd. 369.89; MS (ESI+) *m/z* 370.89 ($M + H^+$; 100%).

(10) [1-(2-morpholin-4-yl-ethyl)-1*H*-indole-3-ylmethyl]-p-tolyl-amine

Orange oil. Yield: 64%. ^{1}H NMR 7.62 (1H, d; J = 8.0 Hz) 7.35 (1H, d; J = 8.0 Hz), 7.20 (1H, s), 7.1 (2H, t; J = 7.6 Hz), 7.01 (2H, d; J = 8.0 Hz), 6.62 (2H, d; J = 8.0 Hz), 4.40 (2H, s), 4.18 (2H, t J = 7.8 Hz), 3.65 (4H, m), 2.71 (2H, t; J = 7.8 Hz), 2.46 (4H, m), 2.22 (3H, s); anal $C_{22}H_{27}N_3O$ C, H, N; Mass calcd. 349.47; MS (ESI+) *m/z* 350.48 ($M + H^+$; 100%).

Pharmacology

Paw formalin assay

The formalin test was performed in mice that had been individually exposed to the observation chamber for 45 min before experiments. For the formalin injection, 10 μL 5% formalin was administered into the plantar surface of the right hind paw using a 30-gauge needle. The animals were then placed in a clear Plexiglas cylinder (20 × 30 cm) for observation. The pain behavior was quantified by determining the amount of time (s) spent by mouse licking the injected paw, over 40–60 min using 5-min bins. Two phases of spontaneous licking-behavior were observed after the formalin injection. The interval from 0 to 15 min has been defined as Phase I, and the interval from 15 to 40 min has been defined as Phase II (35).

Criteria for exclusion from the study included incomplete formalin injection or excessive bleeding from the injection site. Time–response data were presented as the mean ± SEM of 5-min bins over 40 min.

All the compounds, pravadoline, WIN 55212-2, and the antagonist SR141716A were dissolved in dimethylsulfoxide (DMSO) and diluted in saline containing DMSO 10% final concentrations. Ten microliters of these solutions (containing 100 μg of compounds or controls) was administered by intraplantar injection.

All tested compounds and references were given 30 min before formalin administration and were compared to saline injections (control group). For the response analysis for the paw formalin assay, data from Phase I and Phase II observations were considered separately. In each case, the licking response was calculated for each mouse, and the response expressed as percentage of control. Response values and their 95% confidence intervals were calculated using linear regression analysis (38). Displacement test of compounds by CB1 and CB2 antagonists was performed by intravenous administration of SR 141716A and SR144528, 30 min before test compounds.

Animals

All the experiments were performed on male *Swiss* mice (20–25 g; Harlan Italy), maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Mice were housed in groups of five until tested. The experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Statistical analysis

All data were expressed as a mean ± SEM. In each case, the cumulative licking-response was calculated for each mouse, and the dose–response curves were expressed as the licking activity (seconds) for the first 15 min and last 15–40 min. Response values and its 95% confidence intervals were calculated using linear regression analysis. The data were examined by analysis of variance (ANOVA) for multiple comparisons with a single control group. When the analysis was restricted to two means, Student's test (two-tailed) was used. Level of significance was set to 5% (p 0.05).

Binding assays

CB1 competitive binding assays were performed in rat cerebellar membranes using [^{3}H]WIN 55212-2 (Perkin Elmer) as a radioligand. Binding studies were performed at 25 °C with 20 μg of receptor protein in a 20 mM HEPES/1 mM $MgCl_2$ (pH = 7.4) buffer along with [^{3}H]WIN 55212-2 at a concentration of 100 nM. Final assay volume was 500 μL, and the incubation time was 90 min. Receptor-bound [^{3}H]WIN 55212-2 was separated from free [^{3}H]WIN 55212-2 by filtration. The filter paper was washed twice with 200 μL of cold assay buffer and counted by liquid scintillation (46). The *K*i values were calculated based on the Cheng–Prusoff equation: $Ki = IC_{50}/(1 + L/Kd)$.

Molecular modeling

Molecular modeling and graphic manipulations were performed using the InsightII software package (Accelrys, San Diego, CA, USA) running on Silicon Graphics Octane2 workstation and on Dell Precision 780, a dual-core Pentium D 3800 GHz, machine running Linux Fedora 4 as operating system.

The 3D structures of analyzed compounds were constructed using the module Builder of Insight program and then optimized using a molecular dynamic simulation, calculations were performed by a simulated annealing method (46–48), *in vacuo*, using the consistent-valence force field (49,50) in InsightII/Discover software packages.

Results and Discussion

The antinociceptive and cannabinomimetic activities of compounds **1**–**10** were evaluated at peripheral level by formalin-induced pain in the mouse (paw-licking test) (35,36). Paw-licking test is the widely employed test to identify pharmacological properties of new molecules.

The pain behavior is quantified by determining the amount of time (in seconds) that the mouse spent licking the injected paw, over 40–60 min using 5-min bins. Two phases of spontaneous licking behavior are observed after the formalin injection. The interval from

0 to 15 min has been defined as Phase I, and it is related to the direct interaction with cannabinoid receptors, while the interval from 15 to 40 min has been defined as Phase II, and it is related to the production of inflammatory mediators (37).

Compounds **1**–**10**, WIN 55212-2, and pravadoline, used as references, the CB1 antagonist SR141716A and the CB2 antagonist SR144528 were given 30 min before formalin administration and were compared to saline injections (control group). For the response analysis of the formalin assay, data from Phase I and Phase II observations were considered separately. In each case, the licking response was calculated for each mouse. The responses reported in Table 1 are expressed as licking time and in parenthesis are reported the percentage of inhibition of pain with respect to untreated control (39). Compounds that gave more interesting results were subsequently screened to evaluate the analgesic reversion by contemporary administration of CB1 and CB2 receptor antagonists.

The maximal nociceptive response was observed during the first 15 min, with a subsequent drop of licking activity for the next 15–40 min. Chloroisatin derivatives **2**, **5**, and the indole derivative **10** showed a licking time reduction values ranging between 35% and 45%, while the other compounds showed only a slight antinociceptive effect in the Phase I. No activity (except for the compound **5**) was detected in the Phase II. The values of reduction of nociception in the Phase I were comparable to pravadoline but less active when compared to WIN 55212-2.

Differently to other compounds reported in literature (2,39–44) among the indole 2,3-dione derivatives **1**–**6,** the activity seems to be affected by the electron-withdrawing nature of the substituent on the indole moiety. In fact, the 5-chloro compounds **2** and **5** were the most active in this study. Regarding the derivatives **7**–**10**, sharing the indole moiety, the activity seems to be affected by the arylamino methylene moiety in position 3. The activity decreases in the order **10** > **8**>**7** > **9** and seems to be related to the electron-donating nature of the aminic aryl substituent. The activity of compound **10** could be explained by an increase in the electronic density on aminic nitrogen and consequently to generate a hydrogen bond with the target.

Compounds **2**, **5**, **10**, and the slightly active compound **6** were subsequently screened in the presence of CB1 and CB2 antagonists to evaluate receptorial displacement. The results seem to confirm a biologic activity similar to AAIs (Table 2). The reversion of the antinociceptive activity in the presence of antagonist suggests that the analgesia could be related to the interaction with cannabinoid receptors. It is interesting to note that the reversion of the activity is obtained only by CB1 antagonist suggesting selectivity for the tested compounds.

Binding affinity at rat native CB1 receptor was performed in rat cerebellar membranes, according to the procedure reported in experimental section, using [^{3}H]WIN 55212-2 (Perkin Elmer) as ligand. The results reported in Table 3 show that indole derivatives exhibited good affinities toward CB1 cannabinoid receptor. Compound **5** showed an affinity comparable to that of reference WIN 55212-2 (8.4 and 7.3 nM, respectively).

The compounds **7**–**10** displaying the substitution of the imino group with a methyleneamino group showed a consistent reduction in the analgesic activity *in vitro*. In particular, the compounds **7** and **9** resulted to be almost inactive, while compounds **8** and **10** showed to endow a moderate analgesic activity (Table 1). The difference between binding and *in vivo* values seems to suggest a partial agonism or, more importantly, the interference of pharmacokinetic factors for the compound **10**. Further investigation is currently in progress.

Figure 1 shows a graphical representation of the preferred conformer of the molecule **5**, as representative of the series, with the three key pharmacophores, the aryl, morpholino, and the azomethinic nitrogen lone pair, aligned with the corresponding groups of the references pravadoline and WIN 55212-2. The molecule of compound **5** fits in with the reference compounds with a 0.4 Å average deviation of atoms, suggesting the possibility of interactions with CB1 through the exploration of the same receptorial spaces. In fact, although the formal substitution of the AAI carbonyl with an azomethinic group, the napthyl residues of **5**, and WIN well overlap each other, such as the indole and the alkylaminic moieties. The capability of **5** to interact with CB1 is consistent with the hypothesis that the potential interactions of the AAI carbonyl group with CB1 are unnecessary for both the binding and the activation (34).

Besides, the presence of the chlorine atom in position 5 of the indole-2-one residue also seems to be important (compare **5** with **4** and **6**, and **2** with **1** and **3**). This could be related to the extension of

Table 1: Paw-licking time and, in parenthesis, the percentage of inhibition of pain induced by formalin with respect to untreated control

	Compound												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	Pravadoline	WIN 55212-2	Formalin
Phase I	101 ± 9	71 ± 7	96 ± 8	104 ± 9	60 ± 5	80 ± 4	111 ± 12	88 ± 6	110 ± 1	67 ± 5	58 ± 3	44 ± 4	110 ± 18
% inhib.	(8)	(35)	(12)	(5)	(45)	(26.5)	(0)	(19.8)	(0)	(39)	(48)	(60)	–
Phase II	162 ± 10	160 ± 12	165 ± 2	163 ± 11	122 ± 9	164 ± 13	163 ± 16	164 ± 12	141 ± 2	165 ± 14	120 ± 9	59 ± 6	165 ± 16
% inhib.	(0)	(0)	(0)	(0)	(25.6)	(0)	(0)	(0)	(15)	(0)	(27)	(65)	–

Groups of mice received formalin injection into the paw (5%–10 μL, 10 mice/group). The licking response was observed for each mouse and response expressed as paw-licking time in second, and in parenthesis was reported percentage of reduction of pain with respect to control. Results were determined using linear regression analysis as described in the methods.

Table 2: Pain reversion in paw-licking test obtained by contemporary administration of antagonist at CB1 and CB2 receptors

			SR 141716A		SR 144528	
Comp	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
2	71 ± 1 (35)	160 ± 2 (3)	107 ± 2 (2)	(0)	71 ± 2 (33)	(0)
5	60 ± 1 (45)	122 ± 3 (25.6)	104 ± 1 (5)	(0)	75 ± 2 (41)	123 ± 2 (25)
6	80 ± 1 (26.5)	164 ± 1 (0)	107 ± 2 (2)	(0)	83 ± 2 (24)	(0)
10	67 ± 2 (39)	165 ± 1 (0)	106 ± 1 (3)	(0)	66 ± 2 (40)	(0)
Pravadoline	58 ± 1 (48)	120 ± 2 (27)	108 ± 2 (2)	(0)	59 ± 2 (46)	112 ± 2 (28)
WIN 55212-2	44 ± 2 (60)	59 ± 3 (65)	107 ± 2 (2)	75 ± 2 (50)	49 ± 1 (45)	60 ± 2 (27)
Formalin	110 ± 18	165 ± 16				

Groups of mice received formalin into either paw. The mice were treated intravenously with antagonists (2 mg/kg) 30 min before compounds administration. The licking response was observed for each mouse and expressed also as % of reduction of pain with respect to control. The reported results were determined using linear regression analysis as described in the methods.

Table 3: Binding affinity values at rat native CB1 receptor

	Ki (nM)		Ki (nM)
Comp	rCB1	Comp	rCB1
1	311 ± 14.8	**6**	69.6 ± 3.3
2	74.6 ± 3.8	**7**	42.6 ± 2.9
3	390 ± 20.4	**8**	49.4 ± 3.4
4	553 ± 36.1	**9**	24.1 ± 2.2
5	8.4 ± 2.0	**10**	15.7 ± 1.9
WIN 55212-2	7.3 ± 1.3		

The reported results are expressed as the mean ± SEM of at least three independent experiments.

the electron-withdrawing effect until the arylamino moiety through the electronic conjugate system, and consequently, to the formation of the necessary π-stacking interactions with CB1 receptor.

Conclusion

Although the activity of some compounds reported here are comparable to pravadoline but less active when compared to WIN, these indole derivatives represent an interesting starting point for further studies in the field of cannabimimetic agents.

In particular, the synthetic method developed to produce these compounds could be used to perform libraries by combinatorial approach to optimize the activity of these indole derivatives. In fact, the results obtained indicate that it is possible to design analogs that could be more effective on nociceptive response by introducing appropriate structural modifications into indole moiety.

In conclusion, the first results confirm the validity of our synthetic method providing practical access to indole-based derivatives of intense current interest in antinociceptive effect.

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Note

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