Characterization of the Interaction of Indiplon, a Novel Pyrazolopyrimidine Sedative-Hypnotic, with the GABA$_A$ Receptor

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ABSTRACT

Clinically used benzodiazepine and nonbenzodiazepine sedative-hypnotic agents for the treatment of insomnia produce their therapeutic effects through allosteric enhancement of the effects of the inhibitory neurotransmitter GABA at the GABA$_A$ receptor. Indiplon is a novel pyrazolopyrimidine sedative-hypnotic agent, currently in development for insomnia. Using radioligand binding studies, indiplon inhibited the binding of [$^3$H]Ro 15-1788 (flumazenil) to rat cerebellar and cerebral cortex membranes with high affinity ($K_I$ values of 0.55 and 0.45 nM respectively). [$^3$H]IIndiplon binding to rat cerebellar and cerebral cortex membranes was reversible and of high affinity, with $K_D$ values of 1.01 and 0.45 nM, respectively, with a pharmacological specificity consistent with preferential labeling of GABA$_A$ receptors containing $\alpha_1$ subunits. In “GABA shift” experiments and in measurements of GABA-induced chloride conductance in rat cortical neurons in culture, indiplon behaved as an efficacious potentiator of GABA$_A$ receptor function. In both the radioligand binding and electrophysiological experiments, indiplon had a higher affinity than zolpidem or zaleplon. These in vitro properties are consistent with the in vivo properties of indiplon as an effective sedative-hypnotic acting through allosteric potentiation of the GABA$_A$ receptor.

The benzodiazepines produce their therapeutic effects through potentiation of the effects of the inhibitory neurotransmitter, GABA. This is achieved by binding to a specific site on the GABA$_A$ receptor to produce allosteric enhancement of chloride flux through this ligand-gated chloride channel (McKernan and Whiting, 1996; Möhler et al., 2002). Different benzodiazepines can enhance GABA-mediated chloride flux to varying maximal degrees, resulting in compounds that are full or partial agonists. Benzodiazepine “inverse” agonists (partial or full) reduce GABA-mediated chloride flux and neutral antagonists produce no change in GABA-mediated chloride flux by themselves, but they block the effects of benzodiazepine site agonists and inverse agonists. Benzodiazepine site agonists have found extensive clinical utility in the treatment of insomnia and anxiety. The “older” benzodiazepine hypnotics, such as triazolam, have been succeeded in recent years by the so-called “nonbenzodiazepines,” agents that act as full agonists at the benzodiazepine site but that are not benzodiazepine in structure (Mitler, 2000). These compounds, unlike the benzodiazepines, distinguish between the different GABA$_A$ receptor subtypes, a feature that is thought to contribute to their efficacy and pharmacological profile (Crestani et al., 2000).

The GABA$_A$ receptor is a pentameric structure made up of different transmembrane spanning subunits, termed $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, $\rho$, $\pi$, and $\theta$. In most native neuronal tissues, two $\alpha$ subunits, two $\beta$ subunits, and one $\gamma$ subunit form the typical GABA$_A$ receptor. The subunits identified as $\delta$, $\epsilon$, and $\rho$ have some reported selective functions but are not yet fully understood (Olsen and Tobin, 1990; Wilke et al., 1997; Bonnert et al., 1999). Theoretically, there are thousands of possible subunit combinations, but thus far, a limited number of subtype combinations have been found in native systems (Fritschy and Mohler, 1995; McKernan and Whiting, 1996) with expression localized to specific areas of the brain. Although the distribution, heterogeneity, and subunit composition of the receptor in rat brain has not been fully characterized, previous work has estimated that approximately 45% of the total GABA$_A$ receptor profile in the rat brain is composed of a receptor containing the $\alpha_1$, $\beta_2$, and $\gamma_2$ subunits. The $\alpha_1$ subunit is expressed in most brain regions,

ABBREVIATIONS: Indiplon, NBI 34060, N-methyl-N-[3-[3-(2-thienylcarbonyl)]-pyrazolo[1,5-a]pyrimidin-7-yl]phenylacetamide; Ro 15-1788, flumazenil; Ro 15-4513, ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate; DMSO, dimethyl sulfoxide.
with areas of highest receptor density localized to the cerebral cortex, cerebellum, and hippocampus (Duggan and Stephenson, 1990; Fritschi and Möhler, 1995; McKernan and Whiting, 1996; Gutierrez et al., 1997). The α2 and α3 subunits are found in the spinal cord, cerebral cortex and hippocampal pyramidal cells (Ruano et al., 1995; Bohhhalter et al., 1996); α4 expression has been localized to the cerebral cortex, thalamus, and dentate gyrus (Wisden et al., 1991; Khan et al., 1996b; Sur et al., 1999); α5 is found in the hippocampus and cerebral cortex (Ruano et al., 1995; Skolnick et al., 1997; Sur et al., 1998; Sanger et al., 1999), whereas α6 expression has been localized to the granular layer of the cerebellum (Luddens et al., 1990; Khan et al., 1996a; Gutierrez et al., 1997).

Benzodiazepines have long been widely used as tranquilizers, sedatives, anxiolytics, anticonvulsants, muscle relaxants, and hypnotics. The benzodiazepine binding site on the GABA<sub>A</sub> receptor lies at the interface between the α and γ subunits (Rabow et al., 1995; Sieghart, 1995; McKernan and Whiting, 1996; Siggel and Buhr, 1997). The diverse pharmacological properties of benzodiazepines, such as sedation, muscle relaxation, anxiolyis, anticonvulsant, and memory impairment, have been attributed to interaction with these different receptor subtypes (McKernan and Whiting, 1996; Möhler et al., 2002). This work has shown that certain pharmacological effects of benzodiazepines are a result of an interaction with particular α subunits (Rudolph et al., 1999; Low et al., 2000; McKernan et al., 2000; Crestani et al., 2002). In general, the benzodiazepine drugs do not distinguish between the α1, α2, α3, or α5 subunit-containing GABA<sub>A</sub> receptor subtypes, but the “nombenzodiazepine” drugs have preferential affinity for GABA<sub>A</sub> receptors containing certain α subunits, and in the case of agents such as zolpidem, particularly the α1-containing GABA<sub>A</sub> receptors (Crestani et al., 2000).

Indiplon, (NBI 34060; N-methyl-N-[3-[3-(2-thienylcarbonyl)pyrazolo[1,5-a]pyrimidin-7-yl]phenyl]acetamide) is a novel pyrazolopyrimidine (Fig. 1) with sedative and hypnotic properties (Crestani et al., 2000). The actual tritiation was performed under contract by American Radiolabeled Chemical (St. Louis, MO). Typically, each batch of the acquired radiolabel yielded a specific activity of 35 Ci/mmol, with a compound purity greater than 98% as determined by both thin layer chromatography and preparative high-performance liquid chromatography. The product (ART 1023) was stored at −20°C until use.

Membrane Preparation

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) rats weighing approximately 200 to 250 g were sacrificed by decapitation. The brains were quickly removed and dissected on ice, and the cerebral cortex and cerebellum were rapidly frozen by immersion in liquid nitrogen and stored at −80°C until ready for use. On the day of assay, membranes were prepared from frozen tissue by homogenization in ice-cold 0.32 M sucrose in 50 mM Tris HCl, pH 7.4, using a Dounce Teflon homogenizer. The homogenate was spun at 400g for 10 min at 4°C, and the supernatant was transferred to a separate tube and centrifuged at 20,000g for 20 min at 4°C. The resulting pellets were washed once more in ice-cold buffer without sucrose (50 mM Tris HCl, pH 7.4) and centrifuged at 20,000g for 20 min at 4°C. Protein concentrations were determined with a Coomassie Plus Protein Reagent kit (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. Titration analysis using a wide range of protein concentrations determined the optimal protein concentration to be 50 μg/well final concentration. This concentration was used in all subsequent binding studies.

Radioligand Binding Assays

Association studies were conducted to determine the optimal time of equilibrium of the <sup>3</sup>H radioligand. Membranes were prepared as described above and added to a 96-well plate containing either 50 μl of 5 nM <sup>3</sup>H indiplon, 1 nM <sup>3</sup>H Ro 15-1788, or 10 nM <sup>3</sup>H Ro 15-4513 (final concentrations) and 50 μl of buffer (total binding), or 50 μl of 10 μM triazolam (final concentration) to define the nonspecific binding. Membranes were incubated in a total volume of 200 μl for the various times indicated and filtered to determine the specific association of the ligand. Bound from free radioligand was determined by rapid vacuum filtration as outlined below.

For dissociation studies, 50 μg of membrane protein was incubated with 50 μl of <sup>3</sup>H indiplon or <sup>3</sup>H Ro 15-1788 and 50 μl of buffer, or 50 μl of 10 μM triazolam (to define nonspecific binding)
until equilibrium was reached. Dissociation was initiated by the addition of 10 μl of 10 μM triazolam (final concentration) to all tubes and filtered at various times. Bound from free radioligand was determined again by rapid vacuum filtration as outlined below.

For homologous and heterologous competition assays, membranes (50 μg of protein) were incubated with 50 μl of the 3H ligand and 50 μl of varying concentrations of unlabeled competitors triazolam, zolpidem (Sigma-Aldrich), indipen (as the free base), and zaleplon (synthesized in house) from 1 μM to 100 μM for a total volume in each well of 200 μl. Incubations were carried out for 60 min, as predetermined by the association binding experiments described above, at 4°C, and terminated by rapid vacuum filtration onto GF/B filter plates (Whatman, Clifton, NJ) using a Filtermate 96 harvester (PerkinElmer Life and Analytical Sciences, Boston, MA). For saturation analyses, 50 μg of membrane protein was incubated with 50 μl of increasing concentrations of 3H radioligand ranging from 100 pM to 30 nM. Nonspecific binding was defined in duplicate wells in the presence of 10 μM triazolam in a final volume of (200 μl) for all radioligands and the bound from free radioligand determined by rapid vacuum filtration as defined.

For “GABA shift” experiments, the membranes were prepared as described above with the inclusion of two additional wash steps in buffer without sucrose before protein determination to wash out endogenous GABA. Competition assays were then carried out in the presence or absence of 100 μM GABA.

Membrane Filtration
Unifilter GF/B filter plates (6005174; PerkinElmer Life and Analytical Sciences) were pretreated with a solution of 1% polyethyleneimine (P3143; Sigma-Aldrich) in distilled water for 30 min. Filters were pre-rinsed with 200 μl/well of buffer (50 mM Tris HCl, pH 7.4) using a cell harvester (Unifilter-96 Filtermate; PerkinElmer Life and Analytical Sciences). Membranes were harvested from the assay plate using the cell harvester and washed three times with 200 μl of ice-cold buffer (50 mM Tris HCl, pH 7.4). Plates were dried for 30 to 40 min under a constant stream of air (model 1875; Coran, East Windsor, NJ). Finally, each well received 50 μl of scintillation fluid (Microscint 20; Packard Instruments Co., Meriden, CT) and the plates sealed and monitored for radioactivity using a TopCount (PerkinElmer Life and Analytical Sciences).

Radioligand Binding Data Analysis
All radioligand binding data analyses were performed using the iterative nonlinear least-squares regression analysis in the curve-fitting program GraphPad Prism (version 3.0 for Windows; GraphPad Software Inc., San Diego, CA). For the radioligand binding experiments (determination of Kd values), including GABA shift assays, the data were routinely fit to single and multiple binding site models and the “fits” were compared using a partial F-test to statistically determine whether a more complex data model was justified with a level of significance of 95%. Hill coefficients (nH) were determined using a four-parameter logistitc equation. Statistical analysis using a one sample t test was run to determine whether the nH values were significantly different from the value of 1. The saturation analysis of [3H]Ro 15-1788 and [3H]Zaleplon yielded Kd values that were equivalent to those determined from association and dissociation (direct kinetic) binding experiments.

Electrophysiology
Drug Solutions. Indipen (synthesized in-house), zolpidem (Sigma-Aldrich), zaleplon (synthesized in-house), and triazolam (Sigma-Aldrich) were prepared as 10 mM or 100 mM DMSO stocks and stored at −20°C. Small aliquots were dispensed so that any given stock was not subject to repeated freeze-thaw cycles. DMSO stocks were serially diluted into external recording buffer to the appropriate test concentrations. The highest concentration of DMSO used was 0.1%, and this was found to not affect GABA currents. GABA (Sigma-Aldrich) was prepared as a 100 mM stock in water and stored at −20°C until use. On each recording day, a fresh 3 μM GABA test solution was prepared in external solution.

Primary Neuronal Cell Cultures. Cerebral cortices from neonatal rats (P0–P1) were dissociated after enzymatic treatment for 30 min at 37°C (Papain dissociation kit; Worthington Biochemicals, Freehold, NJ). Cortical neurons were plated in serum-free medium (BME/B27; Invitrogen, Carlsbad, CA) at low density (2000/well) on glass coverslips containing a feeder layer of cortical astrocytes in 24-well tissue culture trays. On the 4th day in vitro, cultures were treated with 5-fluoro-2′-deoxyuridine (10 μM) and uridine (10 μM). The medium was changed once per week thereafter. Electrophysiological experiments were performed on neurons after 1–3 weeks in vitro.

Electrophysiological Recordings. Coverslips, upon which cells had been plated, were transferred to the recording chamber on an inverted microscope (IX70; Olympus, Tokyo, Japan) and continuously perfused (1.5–2 ml/min) with control solution at room temperature. The composition of the external solution was 140 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 1.3 mM MgCl2, 10 mM glucose, and 10 mM HEPES, and the pH was 7.3. This was supplemented with 0.3 μM tetrodotoxin to block Na+ currents and 10 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benz[4,5]quinoxaline-7-sulfonamide to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents. The composition of the internal solution in the recording pipette was 125 mM CsCl, 10 mM NaCl, 1 mM MgCl2, 5 mM EGTA, 0.5 mM CaCl2, and 10 mM HEPES, and the pH was 7.3.

A Multiclap 700A patch-clamp amplifier and pClamp 8 software (Axon Instruments, Foster City, CA) were used for electrophysiological recording. After gigahm seals were formed between the patch electrodes (1–3 MΩ) and the cell, the whole-cell patch-clamp configuration was established by rupturing the membrane across the electrode tip.

GABA Currents. Once a stable configuration had been achieved, recording was started in voltage-clamp mode, with the cell initially clamped at −70 mV. A pressurized (10 psi) puffer pipette (2–5 μm tip diameter) was positioned near the recorded neuron and GABA (3 μM) was applied by opening a computer controlled solenoid valve for 200 ms. This protocol activated a peak inward current (200–2000 pA) that rapidly decayed. Because the small volume of GABA released from the puffer pipette was rapidly diluted in the external bath, the neurons were exposed to a maximum concentration of <3 μM GABA. This is in the linear portion of the GABA dose-response curve (EC50 6.2 μM; data not shown) and provided a reliable starting point to measure potentiation of the current by positive allosteric modulators. GABA currents were evoked every 12 s (5 times/min) to assure a sufficient sampling of control, drug, and washout responses.

Test substances were applied by bath perfusion. Once a stable baseline of GABA currents was established, the control solution was switched to one containing the appropriate concentration of test substance. The recording chamber volume was approximately 0.5 ml and complete fluid exchange occurred in approximately 1 min. Drugs were applied for 3 min (15 evoked GABA currents), which was sufficient for an equilibrium response to be established. Drugs were washed out for at least 3 min. If the GABA current recovered to predrug control amplitude, a higher concentration of drug was applied. Each drug concentration was tested on 4 to 20 different cells.

Data Analysis. The peak inward current was measured for each puffer application of GABA. The effect of test compounds on the GABA current was measured at the end of the 3-min drug application (average of 3–5 currents) and normalized to the GABA current measured in the predrug baseline (average of 3–5 currents). The responses to each drug concentration from several cells (4–20) were used to plot concentration-response curves (SigmaPlot version 8 or GraphPad Prism version 3) and fitted to the sigmoid function
where $E_{\text{max}}$ is the maximum effect, $E_{50}$ is the concentration of drug that elicited a half-maximal response, $x$ is the drug concentration, and $b$ is the Hill slope.

**Results**

To characterize the interaction of indiplon with native GABA$_A$ receptors, several well characterized pharmacological agents were used (for review, see Barnard et al., 1998). [$^3$H]Ro 15-1788 (flumazenil) is a benzodiazepine site antagonist radioligand with high affinity for GABA$_A$ receptors containing $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$. [$^3$H]Ro 15-4513 is a benzodiazepine partial inverse agonist radioligand, with high affinity for GABA$_A$ receptors containing $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$, including $\alpha_4$ and $\alpha_6$; zolpidem (Ambien) and zaleplon (Sonata) are “nonbenzodiazepine” sedative-hypnotics, the former showing selectivity towards $\alpha_1$ subunit-containing GABA$_A$ receptors, and triazolam (Halcion) is a benzodiazepine sedative-hypnotic.

**Affinity of Indiplon for GABA$_A$ Receptors Labeled by [$^3$H]Ro 15-1788 in Rat Brain.** To determine the relative affinities of indiplon and other nonbenzodiazepine agonists to the GABA$_A$ receptor in rat cerebral cortex and cerebellum, membranes were labeled with the benzodiazepine site antagonist [$^3$H]Ro 15-1788 and competed with varying concentrations (100 pM–10 $\mu$M) of indiplon, zolpidem, zaleplon, and triazolam. As can be seen in Fig. 2A, all compounds exhibited concentration-dependent inhibition in the cerebral cortex and were able to effectively compete for the binding of [$^3$H]Ro 15-1788. Triazolam, indiplon, and zolpidem had 125, 38, and 6 times higher affinity, respectively, than the weakest compound, zaleplon. A similar affinity profile was apparent in the cerebellum (Fig. 2B) where triazolam, indiplon, and zolpidem had 101, 38, and 4 times the affinity of zaleplon, suggesting that both tissues discriminate these compounds in an identical manner (Table 2; Fig. 2). All compounds inhibited the binding of [$^3$H]Ro 15-1788 to the same basal level and in a monophasic manner suggesting interaction with a single class of binding site in both tissues.

**GABA$_A$ Subtype Selectivity of Indiplon Revealed by [$^3$H]Ro 15-4513 Binding in Rat Brain.** Rat cerebellar membranes were labeled with the [$^3$H]Ro 15-4513 and competed with varying concentrations (3 pM–10 $\mu$M) of triazolam, indiplon, zolpidem, and zaleplon. As can be seen in Fig. 3A, using the partial inverse agonist Ro 15-4513 that has high affinity for all six $\alpha$ subunits of the GABA$_A$ receptor, the inhibition of [$^3$H]Ro 15-4513 binding by triazolam, indiplon, and zolpidem were all biphasic indicating binding to two independent sites. Whereas the data for zaleplon did not statistically demonstrate a better fit to a multisite model, the data in Fig. 3A suggest that if higher concentrations of this compound were possible, this interaction also would be biphasic. In all four of the compounds tested, the rank order of affinities for the two sites remained the same as those demonstrated for the inhibition of [$^3$H]Ro 15-1788 binding in this tissue. In addition, the $K_i$ values calculated for the high affinity site (using a two-site model) matched those previously obtained from the cerebral cortex and cerebellum using [$^3$H]Ro 15-1788 binding (Fig. 2; Table 2), suggesting that high-affinity values corresponded to an interaction with GABA$_A$ receptors containing the $\alpha_1$ subunit, whereas the low-affinity site corresponded to an interaction with GABA$_A$ receptors containing the $\alpha_6$-subunit. The ratio of affinities between the two components was the same for each compound (approximately 1000-fold).

To further demonstrate the selectivity of indiplon, inhibition curves were constructed using both [$^3$H]Ro 15-4513 and [$^3$H]Ro 15-1788 in the cerebellum. [$^3$H]Ro 15-1788 has low affinity for the $\alpha_6$ binding subunit and thus preferentially and selectively labels GABA$_A$ receptors containing the $\alpha_1$ site in the cerebellum at the concentration used (Atack et al., 1999). As clearly demonstrated in Fig. 3B, indiplon competed for [$^3$H]Ro 15-1788 binding with high affinity, and this inhibition curve displayed a single component that reached the same maximal inhibition as that for [$^3$H]Ro 15-4513 binding. The level of nonspecific binding (from which the specific binding curves were generated) was defined as the binding remaining in the presence of the highest concentration (100 $\mu$M) of the nonselective benzodiazepine, triazolam (Fig. 3B).

**Efficacy of Indiplon in GABA Shift Experiments.** To investigate the efficacy of indiplon as a potentiator of GABA$_A$ receptors, we performed radioligand competition binding experiments with [$^3$H]Ro 15-1788 in rat cerebellar membranes and compared the inhibition profiles of indiplon and zolpidem in the presence and absence of 100 $\mu$M GABA. The membranes for this experiment were washed a total of four times.
during preparation to deplete them of endogenous GABA. Benzodiazepine site agonists increase their apparent affinity for the GABA<sub>A</sub> receptor in the presence of the neurotransmitter GABA. Both the indiplon and zolpidem inhibition curves were shifted to the left, toward higher affinity by approximately 2-fold. Thus, in the presence of GABA the affinity of indiplon increased from 1.34 to 0.63 nM, whereas the affinity for zolpidem increased from 16.1 to 7.9 nM, suggesting that both compounds are similarly efficacious as benzodiazepine site agonists and that indiplon has higher affinity than zolpidem (Fig. 4).

**Effect of Indiplon, Zolpidem, Zaleplon, and Triazolam on the GABA-Induced Chloride Current in Cultured Neurons.** To directly assess the ability of indiplon to potentiate GABA<sub>A</sub> receptor function, we recorded GABA-activated chloride currents from cultured neurons. Application of 3 μM GABA for 200 ms by means of a puffer pipette elicited a transient inward current in cultured neurons vol-

![Fig. 3. Selective discrimination of GABA<sub>A</sub> subtypes in rat cerebellum using [3H]Ro 15-4513. A, inhibition of [3H]Ro 15-4513 binding in rat cerebellum. Biphasic inhibition by indiplon, zolpidem, and zaleplon. B, inhibition of [3H]Ro 15-4513 and [3H]Ro 15-1788 binding in rat cerebellum membranes. The data shown are from a single experiment where each point was performed in duplicate (error bars represent standard deviation) and are representative of at least two independent determinations. Radioligand binding data were analyzed as described in text.](image3.png)

![Fig. 4. Functional agonism determined in the presence of GABA. Inhibition of [3H]Ro 15-1788 binding in rat cerebellum in the presence (filled symbols) or absence (open symbols) of 100 μM GABA. Both indiplon (squares) and zolpidem (circles) demonstrated a 2-fold shift to the left, indicating a higher affinity/potency in the presence of GABA. Experiments were conducted in duplicate, and the Ki values shown are representative of three independent determinations. Radioligand binding data were analyzed as described in text.](image4.png)
age clamped at −70 mV (Fig. 5A, arrows). The current had a reversal potential of 0 mV using a CsCl internal solution and was blocked by 50 μM picrotoxin, indicating that it was mediated by GABA_α receptors (data not shown). Bath application of 300 nM indiplon potentiated the inward current, which reversed upon washout (Fig. 5, A and B). In the absence of puffer applied GABA, indiplon did not activate an inward chloride current, indicating that it is a positive allosteric modulator rather than a direct agonist at GABA_α receptors. The concentration responses for indiplon, zolpidem, zaleplon, and triazolam on GABA_α currents were further determined and compared. Each concentration of compound was tested at 4 to 10 different neurons. Each compound produced a maximal potentiation of approximately 200% control (Fig. 5C). The EC_{50} values for potentiation of the chloride current were 11.6, 152, and 630 nM for the nonbenzodiazepines indiplon, zolpidem, and zaleplon, respectively, and 26.5 nM for the benzodiazepine triazolam (Fig. 5C).

Kinetic Analysis of [3H]Indiplon Binding to Rat Cortical Membranes. Time-course analyses were performed first to determine the time for equilibrium binding of [3H]indiplon. [3H]Indiplon bound rapidly and reversibly to rat cortical membranes. Association experiments revealed that [3H]indiplon reached steady-state equilibrium by 5 min and remained at equilibrium for more than 90 min without any change in the steady-state levels (association data in Fig. 6 was truncated to 30 min to expand the earlier times). The association rate constant \( K_\text{on} \), was determined to be 0.149 ± 0.005 nM^{-1} min^{-1} (mean ± S.E.M.; \( n = 3 \)) assuming pseudo first-order kinetics (Fig. 6). The dissociation rate constant (Fig. 6, inset) was estimated by the addition of 10 μM triazolam after equilibrium binding had been achieved. The dissociation rate constant \( K_\text{off} \), was determined to be 0.230 ± 0.067 min^{-1} (mean ± S.E.M.; \( n = 3 \)). Applying the equation \( K_D = K_\text{off}/K_\text{on} \), the resulting affinity binding constant for [3H]indiplon was calculated to be 1.54 ± 0.051 nM (mean ± S.E.M.; \( n = 3 \)), which was in agreement with the \( K_D \) obtained from direct saturation binding experiments (Fig. 7). Thus, in all subsequent equilibrium experiments, a standard incubation time of 60 min was used for [3H]indiplon.

Saturation Binding Analysis of [3H]Indiplon in Rat Cortical and Cerebellar Membranes. Saturation binding of [3H]indiplon and [3H]Ro 15-1788 was carried out to determine the \( K_D \) of the radioligands, as well as to determine the relative number of binding sites in the rat cortical and cerebellar membrane preparations. As can be seen in the representative experiments in Fig. 7, [3H]Ro 15-1788 binds with high affinity to receptors in both the frontal cortex and the cerebellum (Fig. 7, A and C). Similarly, [3H]indiplon binding also seemed saturable at 30 nM and bound with high affinity to receptors in both tissues (Fig. 7, B and D) with similar \( K_D \) and \( B_{\text{max}} \) values (Table 1). It is interesting to note however, that although [3H]indiplon binds to a single site in both cortical and cerebellar membranes, the density of receptors in those tissues is only half the number of receptors observed using [3H]Ro 15-1788 in the same preparations (Table 1). In the cortex, [3H]Ro 15-1788, binds GABA_α receptors containing α subunits 1, 2, 3, and 5 with equal high affinity (≈1.5 nM), and α subunits 4 and 6 with lower affinity (30–50 nM) (Khan et al., 1996a; Scholze et al., 1996). Because the highest concentration tested for [3H]Ro 15-1788 was approximately 30 nM, occupancy of the lower affinity α4- and α6-containing GABA_α sites will not be strongly represented in these experiments even though a portion of them would be labeled. Much higher concentrations of [3H]Ro 15-1788 would be required to accurately determine the \( B_{\text{max}} \) for these lower affinity sites. Consequently, it seems that the binding sites labeled by [3H]Ro 15-1788 represent a combination of α1-, 2-, 3-, and 5-containing GABA_α receptors. Because the \( B_{\text{max}} \) value for [3H]indiplon was 50% that of [3H]Ro 15-1788, these data are consistent with the hypothesis that [3H]indiplon preferentially binds GABA_α receptors containing the α1 subunit, which are the dominant species present in these membranes.

Pharmacological Properties of Binding Sites for [3H]Indiplon in Rat Cerebellum and Cortex. To determine whether the binding sites recognized by [3H]indiplon...
were indeed those of the GABA<sub>A</sub> receptor and specifically those containing the α1 subunit, rat frontal cortex and cerebellar membranes were labeled with either [³H]Ro 15-1788 or [³H]indiplon, and the pharmacological rank order profile using varying concentrations (100 pM–10 μM) of triazolam, indiplon, zolpidem, and zaleplon was compared. As can be seen in Table 2, the rank order of affinities, as well as the absolute K<sub>i</sub> values, remain virtually identical regardless of the [³H] label used with a rank order of affinities: triazolam > indiplon > zolpidem > zaleplon. All compounds exhibited inhibition of binding in a monophasic manner characteristic of binding to a single class of sites at this concentration of radioligand in either tissue examined. These data clearly indicate that [³H]indiplon binds with high affinity to the GABA<sub>A</sub> receptor in brain with an identical and appropriate pharmacological rank order profile for known compounds acting through this receptor and more specifically for GABA<sub>A</sub> receptors containing the α1 subunit.

Discussion

In the present studies, we sought to elucidate the interaction of indiplon, a novel pyrazolopyrimidine, with the GABA<sub>A</sub> receptor. Radioligand binding experiments in rat brain membranes using well characterized ligands for the benzodiazepine site on the GABA<sub>A</sub> receptor indicated that indiplon had high affinity, with a K<sub>i</sub> value that was approximately 50- and 10-fold lower than those for zaleplon and zolpidem, respectively. [³H]Indiplon itself proved to be a high-affinity radioligand for the benzodiazepine site, which bound to a subset of GABA<sub>A</sub> receptors with a pharmacological profile consistent with GABA<sub>A</sub> receptors containing the α1 subunit. Both GABA shift experiments and patch-clamp recordings of rat cortical neurons in culture suggest that indiplon is a full agonist for the benzodiazepine site on native GABA<sub>A</sub> receptors. Overall, these data are consistent with the in vivo pharmacological profile of indiplon (Foster et al., 2004) as an effective sedative-hypnotic agent acting through the benzodiazepine site on the GABA<sub>A</sub> receptor.

Several types of experiments confirmed that indiplon has high affinity for the benzodiazepine site on the GABA<sub>A</sub> receptor and is an efficacious potentiator of GABA<sub>A</sub> receptors. This was apparent from the inhibition of [³H]Ro 15-1788 binding to rat cerebral cortex and cerebellar membranes (K<sub>i</sub> values of 0.55 and 0.45 nM, respectively), from the binding of [³H]indiplon itself in rat cerebral cortex and cerebellar membranes (K<sub>i</sub> = 1.01 and 0.53 nM, respectively) and from the potentiation of GABA-evoked chloride currents in cultured rat neurons (EC<sub>50</sub> = 11.6 nM). It has long been documented that compounds such as triazolam, zolpidem, and zaleplon all exhibit full agonist activity at the benzodiazepine site using a variety of methods, including potentiation of GABA currents (Im et al., 1993), discriminative stimulus effects in rats and rhesus monkeys (Sanger et al., 1999; McMahon et al., 2002), and positron emission tomographic quantitation (Abadie et al., 1996). Indiplon also exhibited the characteristics of a benzodiazepine site agonist in GABA shift experiments and showed full agonist efficacy in the patch-clamp experiments. Consequently, indiplon seems to be a high-affinity, fully efficacious allosteric potentiator at native GABA<sub>A</sub> receptors.

Several lines of evidence suggested that, like zolpidem, indiplon has selectivity for GABA<sub>A</sub> receptors containing the α1 subunit. The most direct evidence comes from studies with [³H]indiplon binding that exhibited B<sub>max</sub> values in both the cerebellum and cerebral cortex, which were approximately one-half those for [³H]Ro 15-1788 in the same tissues. This indicates that indiplon binds with high affinity to a subset of GABA<sub>A</sub> receptors. However, the monophasic inhibition of [³H]indiplon binding by zolpidem and zaleplon, with
**K_i** values in good agreement with their affinities for GABAA receptors containing the \( \gamma \)1 subunit, strongly suggest that in these experiments, \[^3H\]indiplon labels primarily this subtype of GABAA receptor. This was supported by experiments in cerebellar membranes. \[^3H\]Ro 15-4513 has very high affinity for all six \( \gamma \) subunits of the GABAA receptor (Wong and Skolnick, 1992), and because only the \( \alpha \)1 and \( \gamma \)6 subunits are expressed in rat cerebellum, this radioligand labels both \( \alpha \)1 and \( \gamma \)6 subunit-containing GABAA receptors in this preparation. Both zolpidem and zaleplon have been reported to have very low affinity for GABAA receptors containing the \( \alpha \)6 subunit (Damgen and Luddens, 1999). The biphasic competition curves for \[^3H\]Ro 15-4513 binding in the rat cerebellum for zolpidem and zaleplon; therefore, are consistent with the idea that the high-affinity and low-affinity components represent an interaction of these compounds with \( \alpha \)1 subunit-containing and \( \gamma \)6 subunit-containing GABAA receptors, respectively. From this, we infer that indiplon binds to \( \alpha \)1 subunit-containing GABAA receptors with low nanomolar affinity and to \( \gamma \)6 subunit-containing GABAA receptors with micromolar affinity. All compounds demonstrated about a 1000-fold difference between the activity at the \( \gamma \)1 and \( \gamma \)6 sites, respectively (Fig. 3). \[^3H\]Ro 15-1788 has approximately 30 to 50 nM affinity for the \( \gamma \)6 site and thus at the concentrations used for cerebellar binding studies (1.5 nM) would not appreciably label this subtype. Thus, the indiplon inhibition seemed monophasic with an affinity virtually identical to the high-affinity site of the biphasic competition observed using \[^3H\]Ro 15-4513 (Fig. 3B). These data supported the high-affinity and selective nature of the binding of indiplon to the GABAA receptors containing the \( \alpha \)1 subunit, and although indiplon has some affinity for the \( \alpha \)6 subunit, the

![Table 1](https://jpet.aspetjournals.org/article-pdf/317/5/543/17555177/17555177.pdf)

**TABLE 1**

<table>
<thead>
<tr>
<th>(^3H) Label</th>
<th>Tissue</th>
<th>( K_D ) (nM)</th>
<th>( B_{\text{max}} ) (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro 15-1788</td>
<td>Cortex</td>
<td>2.13 ± 0.09</td>
<td>5.08 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>1.65 ± 0.13</td>
<td>2.30 ± 0.06</td>
</tr>
<tr>
<td>Indiplon</td>
<td>Cortex</td>
<td>1.01 ± 0.23</td>
<td>2.58 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>0.53 ± 0.03</td>
<td>1.16 ± 0.08</td>
</tr>
</tbody>
</table>

\( K_D \) values in good agreement with their affinities for GABAA receptors containing the \( \alpha \)1 subunit, strongly suggest that in these experiments, \[^3H\]indiplon labels primarily this subtype of GABAA receptor. This was supported by experiments in cerebellar membranes. \[^3H\]Ro 15-4513 has very high affinity for all six \( \alpha \) subunits of the GABAA receptor (Wong and Skolnick, 1992), and because only the \( \alpha \)1 and \( \gamma \)6 subunits are expressed in rat cerebellum, this radioligand labels both \( \alpha \)1 and \( \gamma \)6 subunit-containing GABAA receptors in this preparation. Both zolpidem and zaleplon have been reported to have
affinity is 1000-fold weaker, in the micromolar range, and not likely to be of any pharmacological consequence. Experiments with recombinant GABA<sub>A</sub> receptor subtypes expressed in human embryonic kidney cells support the conclusions drawn from the present studies that indiplon has high affinity and selectivity for α1 subunit-containing GABA<sub>A</sub> receptors (R. E. Petroski, J. E. Pomeroy, R. Das, H. C. Bowman, and A. C. Foster, unpublished data).

[<sup>3</sup>H]Indiplon proved to be an excellent radioligand for the characterization of brain membrane GABA<sub>A</sub> receptors. [<sup>3</sup>H]Indiplon was found to bind rapidly, saturably, reversibly, and with high affinity to receptors either in the frontal cortex or in the cerebellum. Kinetic analyses for association of the label confirmed that the radioligand bound in a reversible and time-dependent manner, reaching equilibrium within 5 min with the binding being stable for at least 90 min. Dissociation was initiated after equilibrium by the addition of triazolam, which effectively dissociated bound [<sup>3</sup>H]indiplon from the GABA<sub>A</sub> receptor with a half-life of approximately 10 min. This clearly demonstrated that the binding of [<sup>3</sup>H]indiplon to rat brain receptors was of a reversible nature and could be competitively displaced once equilibrium had been achieved.

Saturation analyses revealed that the binding of [<sup>3</sup>H]indiplon was saturable and highly specific (specific binding was routinely 80–90% of the total binding). In studies directly comparing the saturation of [3H]Ro 15-7988 and [3H]indiplon, it was clear that although both compounds bound with high affinity, the receptor density recognized by [<sup>3</sup>H]indiplon was approximately one-half that of the antagonist. These data were consistent with the hypothesis that [<sup>3</sup>H]indiplon was preferentially binding a subset of the GABA<sub>A</sub> receptors labeled with either [3H]Ro 15-1788 or [3H]indiplon.

TABLE 2

Comparison of K<sub>i</sub> values (nM) rank order profile of benzodiazepine and nonbenzodiazepine sedative-hypnotics in rat frontal cortex or cerebellar GABA<sub>A</sub> receptors labeled with either [3H]Ro 15-1788 or [3H]indiplon. K<sub>i</sub> values listed are means ± S.E.M. of at least three independent experiments performed in duplicate. Hill coefficients (n<sub>H</sub>) are shown in parentheses as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>[3H] Label</th>
<th>Ro 15-1788</th>
<th>Indiplon</th>
<th>Zolpidem</th>
<th>Zaleplon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>Ro 15-1788</td>
<td>0.55 ± 0.07 (1.7 ± 0.2)</td>
<td>1.8 ± 0.06 (1.1 ± 0.1)</td>
<td>11.2 ± 4.6 (0.9 ± 0.1)</td>
<td>68.5 ± 29.5 (0.9 ± 0.1)</td>
</tr>
<tr>
<td></td>
<td>Indiplon</td>
<td>0.34 ± 0.02 (1.2 ± 0.1)</td>
<td>1.7 ± 0.30 (1.2 ± 0.1)</td>
<td>12.6 ± 2.7 (1.0 ± 0.1)</td>
<td>81.2 ± 10.7 (0.9 ± 0.2)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Ro 15-1788</td>
<td>0.45 ± 0.17 (1.5 ± 0.5)</td>
<td>1.2 ± 0.22 (1.1 ± 0.1)</td>
<td>10.3 ± 1.6 (0.9 ± 0.1)</td>
<td>45.5 ± 4.8 (0.9 ± 0.1)</td>
</tr>
<tr>
<td></td>
<td>Indiplon</td>
<td>0.31 ± 0.05 (1.0 ± 0.1)</td>
<td>1.2 ± 0.32 (1.0 ± 0.1)</td>
<td>13.5 ± 4.3 (0.8 ± 0.1)</td>
<td>59.8 ± 12.2 (0.8 ± 0.1)</td>
</tr>
</tbody>
</table>

The actions of indiplon on GABA<sub>A</sub> receptor function were further characterized by electrophysiological experiments on neocortical neurons in culture. Indiplon was shown to be a positive allosteric modulator of GABA-activated chloride currents with lower EC<sub>50</sub> values than zolpidem or zaleplon. The positive allosteric modulator of GABA-activated chloride currents was further characterized by electrophysiological experiments on rat brain membranes.

In conclusion, these studies have shown that the novel pyrazolopyrimidine, indiplon, is a high-affinity allosteric potentiator of the GABA<sub>A</sub> receptor, acting through the benzodiazepine binding site. Indiplon acts in a subtype-selective manner consistent with selectivity for GABA<sub>A</sub> receptors containing the α1 subunit. These in vitro data are consistent with the in vivo pharmacology of indiplon (Foster et al., 2004), where indiplon acts as an effective sedative-hypnotic, which also possesses anxiolytic and anticonvulsant properties. These features, combined with a short half-life (t<sub>1/2</sub> = 1 h after oral dosing in mouse and rat; Foster et al., 2004), have made indiplon an attractive candidate as an improved sedative-hypnotic agent to treat insomnia, a concept that is currently being evaluated in multiple clinical studies.

References


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