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Characterization of the Interaction of Indiplon, a Novel Pyrazolopyrimidine Sedative/Hypnotic, with the $GABA_A$ Receptor

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Abbreviations: GABA, γ-Aminobutyric Acid; Indiplon or NBI 34060, N-methyl-N-[3-[3-(2-thienylcarbonyl)-pyrazolo[1,5-alpha]pyrimidin-7-yl]phenyl]acetamide; Ro15-1788, flumazenil; Ro15-4513, Ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate; NBQX = 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide.
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ABSTRACT

Clinically used benzodiazepine and non-benzodiazepine sedative-hypnotic agents for the treatment of insomnia produce their therapeutic effects through allosteric enhancement of the effects of the inhibitory neurotransmitter, γ-aminobutyric acid (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 to rat cerebellar and cerebral cortex membranes with high affinity (Ki values of 0.55 and 0.45 nM, respectively). [^{3}H]Indiplon 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 α1 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 (Foster et al., 2004) as an effective sedative-hypnotic acting through allosteric potentiation of the $GABA_A$ receptor.

INTRODUCTION:

The benzodiazepines produce their therapeutic effects through potentiation of the effects of the inhibitory neurotransmitter, γ-aminobutyric acid (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; Mohler et al., 2002). Different benzodiazepines can enhance GABA-mediated chloride flux to varying maximal degrees, resulting in compounds, which 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 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 "non-benzodiazepines", agents that act as full agonists at the benzodiazepine site, but 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 α, β, γ, δ, ε, ρ, π, and θ. In most native neuronal tissues, two $\alpha_{(1-6)}$ subunits, two $\beta_{(1-3)}$ subunits, and one $\gamma_{(1-3)}$ subunit form the typical $GABA_A$ receptor. The subunits identified as δ, ε, and ρ(1-2) 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.

While 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 α1 β2 and γ2 subunits. The α1 subunit is expressed in most brain regions, with areas of highest receptor density localized to the cerebral cortex, cerebellum and hippocampus. (Duggan and Stephenson, 1990; Fritschy and Mohler, 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; Bohlhalter 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), while 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 $GABA_A$ receptor lies at the interface between the α and γ subunits (Rabow et al., 1995; Sieghart, 1995; McKernan and Whiting, 1996; Sigel and Buhr, 1997). The diverse pharmacological properties of benzodiazepines, such as sedation, muscle relaxation, anxiolysis, anticonvulsant and memory impairment, have been attributed to interaction with these different receptor sub-types (McKernan and Whiting, 1996; Mohler et al., 2002). This has recently been confirmed and extended in an elegant series of experiments using a "knock-in" approach to create mutant mice which have lost benzodiazepine sensitivity in $GABA_A$ receptor subtypes containing specific α-

sub-units (Mohler et al., 2002). This work has shown that certain pharmacological effects of benzodiazepines are a result of an interaction with particular α-sub-units (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-sub-unit-containing $GABA_A$ receptor sub-types, but the "non-benzodiazepine" drugs have preferential affinity for $GABA_A$ receptors containing certain α-sub-units, and in the case of agents such as zolpidem, particularly the α1-containing $GABA_A$ receptors (Crestani et al., 2000).

Indiplon, (NBI 34060; N-methyl-N-[3-[3-(2-thienylcarbonyl)-pyrazolo[1,5-alpha]pyrimidin-7-yl]phenyl]acetamide) is a novel pyrazolopyrimidine (see Figure 1) with sedative and hypnotic properties mediated through potentiation of $GABA_A$ receptors (Foster et al., 2004). The present studies were conducted to define the interaction of indiplon with native $GABA_A$ receptors and to characterize the binding of [^{3}H]indiplon in rat brain tissue.

METHODS

All chemicals and reagents were purchased from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Los Angeles, CA) unless otherwise specifically stated. All animals used in this study were treated in accordance to the Neurocrine IACUC and the NIH Guide for the Welfare of Laboratory Animals.

Synthesis and Radiolabeling of Indiplon

Tritiated indiplon was prepared using an iodinated precursor. The preparation of iodinated indiplon required five equivalents of iodine monochloride in methanol at ambient temperature over 40 hours. The reaction flask required protection from light and an additional 2 equivalents of iodine monochloride was required after 20 hours. A typical work-up afforded an inseparable 2:1 ratio of bis to mono iodinated product in an 84% yield, purified via acetone crystallization. Thus, a mixture of mono and di-iodinated indiplon as the free bases was synthesized in-house to facilitate tritiation. Typical hydrogenation methods failed to afford the desired tritiated product. However, use of sodium borohydride [^{3}H] in methanol in the presence of a catalytic amount of palladium dichloride (Weber *et al.*, 1992) afforded tritiated indiplon (Figure 1). The actual tritiation was performed under contract by American Radiolabeled Chemical Inc. (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 TLC and preparative HPLC. The product (ART 1023) was stored at –20ºC until use.

Membrane Preparation

Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) rats weighing approximately 200 - 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^{o}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^{o}C, the supernatant transferred to a separate tube and centrifuged at 20,000g for 20 min at 4^{o}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^{o}C. Protein concentrations were determined with a Coomassie Plus Protein Reagent kit (Pierce, 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 per well final concentration. This concentration was used in all subsequent binding studies.

Radioligand Binding Assays

Association studies were conducted in order to determine the optimal time of equilibrium of the [^{3}H]radioligand. Membranes were prepared as above and added to a 96-well plate containing either 50 μl 5 nM [^{3}H]indiplon, 1 nM [^{3}H] Ro 15-1788, or 10 nM [^{3}H] Ro 15-4513 (final concentrations) and 50 μl buffer (total binding), or 50 μl of 10 μM triazolam (final concentration) to define the non-specific 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

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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 [^{3}H]indiplon or [^{3}H]Ro 15-1788 and 50 μl buffer, or 50 μl of 10 μM triazolam (to define non specific 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 protein) were incubated with 50 μl of the [^{3}H]-ligand and 50 μl of varying concentrations of unlabelled competitors triazolam, zolpidem (Sigma Chemicals, St Louis), indiplon, (as the free base) and zaleplon (synthesized in house) from 1 pM to 100 μM for a total volume in each well of 200μl. Incubations were carried out for 60 min, as predetermined by the association binding experiments above, at 4°C, and terminated by rapid vacuum filtration onto GF/B filter plates (Whatman, NJ) using a Filtermate 96 Harvester (Packard Instruments, IL).

For saturation analyses, 50 μg membrane protein was incubated with 50 μl of increasing concentrations of [^{3}H]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 above with the inclusion of two additional wash steps in buffer without sucrose prior to 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; Packard) were pretreated with a solution of 1% polyethyleneimine (P3143; Sigma Chemicals) in distilled water for 30 minutes. Filters were pre-rinsed with 200 µl per well of buffer (50 mM Tris-HCl pH = 7.4) using a cell harvester (Unifilter-96 Filtermate; Packard). Membranes were harvested from the assay plate using the cell harvester and washed 3 times with 200 µl of ice-cold buffer (50 mM Tris-HCl pH = 7.4). Plates were dried for 30 - 40 minutes under a constant stream of air (model 1875; Conair). Finally, each well received 50 µl scintillation fluid (Microscint 20; Packard), the plate sealed and monitored for radioactivity using a TopCount (Packard).

Radioligand Binding Data Analysis

All radioligand binding data analyses were performed using the iterative non-linear least squares regression analysis in the curve-fitting program "GraphPad Prism" (version 3.0 for Windows, GraphPad Software, San Diego, CA). For the radioligand binding experiments (determination of Ki 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 logistic equation. Statistical analysis using a one sample t-test was run to determine if the nH values were significantly different from the value of 1. The saturation analysis of [^{3}H]Ro 15-1788 and [^{3}H]indiplon yielded K_D values that were equivalent to those determined from association and dissociation (direct kinetic) binding experiments.

Electrophysiology

Drug Solutions

Indiplon (synthesized in-house), zolpidem (Sigma), zaleplon (synthesized in-house), and triazolam (Sigma) were prepared as 10 mM or 100 mM DMSO stocks and stored at –20°. Small aliquots were dispensed so that any give 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) 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 following enzymatic treatment for 30 min at 37°C (Papain Dissociation Kit; Worthington Biochemical). Cortical neurons were plated in serum-free medium (BME/B27; Gibco) at low density (2,000 per 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 following 1-3 weeks *in vitro.*

Electrophysiological Recording

Coverslips, upon which cells had been plated, were transferred to the recording chamber on an inverted microscope (Olympus IX70) and continuously perfused (1.5-2 ml/min) with control solution at room temperature. The composition of the external solution was (in mM): NaCl (140), KCl (2.5), $CaCl_2$ (2.5), $MgCl_2$ (1.3), glucose (10), HEPES (10) and the pH was 7.3. This was supplemented with 0.3 μM tetrodotoxin to block Na currents and 10 μM NBQX to block AMPA receptor currents. The composition of the internal solution in the recording pipette was (in mM): CsCl (125), NaCl (10), $MgCl_2$ (1), EGTA (5), $CaCl_2$ (0.5), HEPES (10) and the pH was 7.3.

A Multiclamp 700A patch clamp amplifier and pClamp 8 software (Axon Instruments) were used for electrophysiological recording. After gigaohm 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 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. Since 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 (EC_{50} 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 sec (5 times per 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 pre-drug control amplitude, a higher concentration of drug was applied. Each drug concentration was tested on 4-20 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 pre-drug 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 v.8 or GraphPad Prism v.3) and fitted to a sigmoid function:

$$\text{(Logistic 4 parameter)} \quad f(x) = \frac{E\max}{1+\left(\frac{EC_{50}}{x}\right)^{b}}$$

where Emax is the maximum effect, EC_{50} is the concentration of drug that elicited a half-maximal response, x is the drug concentration and b is the Hill slope.

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RESULTS

In order 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 α1, α2, α3 and α5. [^{3}H] Ro 15-4513 is a benzodiazepine partial inverse agonist radioligand, with high affinity for $GABA_A$ receptors containing α1, α2, α3, α5 including α4 and α6. Zolpidem (Ambien™) and zaleplon (Sonata™) are "non-benzodiazepine" sedative-hypnotics, the former showing selectivity towards α1 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 non-benzodiazepine 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 to 10 μM) of indiplon, zolpidem and zaleplon and triazolam. As can be seen in Figure 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 (Figure 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 (refer to Table 2 and Figure 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.

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$GABA_A$ 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 to 10 μM) of triazolam, indiplon, zolpidem and zaleplon. As can be seen in Figure 3A, using the partial inverse agonist, Ro 15-4513 that has high affinity for all 6 alpha subunits of the $GABA_A$ receptor, the inhibition of [^{3}H]Ro 15-4513 binding by triazolam, indiplon, and zolpidem all appeared biphasic indicating binding to two independent sites. While the data for zaleplon did not statistically demonstrate a better fit to a multi-site model, the data in Figure 3A suggests that if higher concentrations of this compound were possible, this interaction would also 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 Ki 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 (refer to Figure 2 and Table 2), suggesting that high affinity values corresponded to an interaction with $GABA_A$ receptors containing the α1 subunit, whereas the low affinity site corresponded to an interaction with $GABA_A$ receptors containing the α6 subunit. The ratio of affinities between the two components was the same for each compound (approx. 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 α6 binding subunit and thus preferentially and selectively labels $GABA_A$ receptors containing the α1 site in the cerebellum at the concentration used (Atack et al., 1999). As clearly demonstrated in Figure 3B, indiplon competed for [^{3}H]Ro 15-1788 binding with high

affinity and this inhibition curve displayed a single component which reached the same maximal inhibition as that for [^{3}H]Ro 15-4513 binding. The level of non-specific binding (from which the specific binding curves were generated) was defined as the binding remaining in the presence of the highest concentration (100 μM) of the non-selective benzodiazepine, triazolam (see Figure 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 100μM GABA. The membranes for this experiment were washed a total of four times during preparation in order to deplete them of endogenous GABA. Benzodiazepine site agonists increase their apparent affinity for the $GABA_A$ receptor in the presence of the neurotransmitter GABA. Both the indiplon and zolpidem inhibition curves were shifted to the left, towards higher affinity by approximately 2-fold. Thus, in the presence of GABA the affinity of indiplon increased from 1.34 nM to 0.63 nM while the affinity for zolpidem increased from 16.1 nM to 7.9 nM, suggesting that both compounds are similarly efficacious as benzodiazepine site agonists and that indiplon has higher affinity than zolpidem (see Figure 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 $GABA_A$ 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 voltage 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 $GABA_A$ receptors (data not shown). Bath application of 300 nM indiplon potentiated the inward current, which reversed upon washout (Fig 5A and 5B). 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 $GABA_A$ receptors. The concentration responses for indiplon, zolpidem, zaleplon, and triazolam on $GABA_A$ currents were further determined and compared. Each concentration of compound was tested at 4-10 different neurons. Each compound produced a maximal potentiation of approximately 200% control (see Figure 5C). The EC_{50} values for potentiation of the chloride current were 11.6 nM, 152 nM and 630 nM for the non-benzodiazepines indiplon, zolpidem and zaleplon respectively, and 26.5 nM for the benzodiazepine triazolam (Figure 5C).

Kinetic analysis of [^{3}H]Indiplon binding to rat cortical membranes.

Time course analyses were performed first in order to determine the time for equilibrium binding of [^{3}H]indiplon. [^{3}H]Indiplon bound rapidly and reversibly to rat cortical membranes. Association experiments revealed that [^{3}H]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 Figure 6 was truncated to 30 minutes to expand the earlier times). The association rate constant K_{+1}, was determined to be 0.149 ± 0.005 $nM^{-1}min^{-1}$ (mean ± SEM; n = 3) assuming pseudo first-order kinetics (see Figure 6). The dissociation rate constant (Figure 6

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inset) was estimated by the addition of 10 μM triazolam after equilibrium binding had been achieved. The dissociation rate constant K_{-1} was determined to be 0.230 ± 0.067 min^{-1} (mean ± SEM; n=3). Applying the equation $K_D = K_{-1} / K_{+1}$, the resulting affinity binding constant for [^{3}H]indiplon was calculated to be 1.54 ± 0.051 nM (mean ± SEM; n=3) which was in agreement with the K_D obtained from direct saturation binding experiments (see also Figure 7). Thus in all subsequent equilibrium experiments, a standard incubation time of 60 minutes was used for [^{3}H]indiplon.

Saturation Binding Analysis of [^{3}H]Indiplon in rat cortical and cerebellar membranes.

Saturation binding of [^{3}H]indiplon and [^{3}H]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 Figure 7, [^{3}H]Ro 15-1788 binds with high affinity to receptors in both the frontal cortex and the cerebellum (Figure 7A and C). Similarly, [^{3}H]indiplon binding also appeared saturable at 30 nM and bound with high affinity to receptors in both tissues (Figure 7B and D) with similar K_D and Bmax values (see Table 1). It is interesting to note however, that while [^{3}H]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 [^{3}H]Ro 15-1788 in the same preparations (See Table 1). In the cortex, [^{3}H]Ro 15-1788, binds $GABA_A$ receptors containing α subunits 1, 2, 3 and 5 with equal high affinity (~ 1.5nM), and alpha subunits 4 and 6 with lower affinity (30 – 50 nM) (Khan et al., 1996a; Scholze et al., 1996). Since the highest concentration tested for [^{3}H]Ro 15-1788 was approximately 30 nM, occupancy of the lower affinity α4 and α6

containing $GABA_A$ sites will not be strongly represented in these experiments even though a portion of them would be labeled. Much higher concentrations of [^{3}H]Ro 15-1788 would be required to accurately determine the Bmax for these lower affinity sites. Consequently, it appears that the binding sites labeled by [^{3}H]Ro 15-1788 represent a combination of α1, 2, 3 and 5 containing $GABA_A$ receptors. Since the B_{max} value for [^{3}H]indiplon was 50% that of ^{3}H]Ro 15-1788, these data are consistent with the hypothesis that [^{3}H]indiplon preferentially binds $GABA_A$ receptors containing the α1 subunit, which are the dominant species present in these membranes.

Pharmacological properties of binding sites for [^{3}H]Indiplon in rat cerebellum and cortex.

In order to determine whether the binding sites recognized by [^{3}H]indiplon were indeed those of the $GABA_A$ receptor and specifically those containing the α1 subunit, rat frontal cortex and cerebellar membranes were labeled with either [^{3}H]Ro 15-1788 or [^{3}H]indiplon and the pharmacological rank order profile using varying concentrations (100 pM to 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 Ki values, remain virtually identical regardless of the [^{3}H]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 [^{3}H]indiplon binds with high affinity to the $GABA_A$ receptor in brain with an identical and appropriate pharmacological rank order profile for known compounds acting through this receptor and more specifically for $GABA_A$ receptors containing the α1 subunit.

DISCUSSION

In the present studies, we sought to elucidate the interaction of indiplon, a novel pyrazolopyrimidine, with the $GABA_A$ receptor. Radioligand binding experiments in rat brain membranes using well-characterized ligands for the benzodiazepine site on the $GABA_A$ receptor indicated that indiplon had high affinity, with a K_i value that was approximately 50-fold and 10-fold lower than those for zaleplon and zolpidem, respectively. [^{3}H]Indiplon itself proved to be a high affinity radioligand for the benzodiazepine site, which bound to a subset of $GABA_A$ receptors with a pharmacological profile and regional distribution consistent with $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptor.

Several types of experiments confirmed that indiplon has high affinity for the benzodiazepine site on the $GABA_A$ receptor, and is an efficacious potentiator of $GABA_A$ receptors. This was apparent from the inhibition of [^{3}H]Ro 15-1788 binding to rat cerebral cortex and cerebellar membranes (Ki values of 0.55 and 0.45 nM, respectively), from the binding of [^{3}H]indiplon itself in rat cerebral cortex and cerebellar membranes (K_D = 1.01 and 0.53 nM, respectively) and from the potentiation of GABA-evoked chloride currents in rat cultured neurons (EC_{50} = 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 appears to be a high affinity, fully efficacious allosteric potentiator at native $GABA_A$ receptors.

Several lines of evidence suggested that, like zolpidem, indiplon has selectivity for $GABA_A$ receptors containing the α1 subunit. The most direct evidence comes from studies with [^{3}H]indiplon binding which exhibited Bmax values in both the cerebellum and cerebral cortex which were approximately half those for [^{3}H]Ro 15-1788 in the same tissues. This indicates that indiplon binds with high affinity to a subset of $GABA_A$ receptors. However, the monophasic inhibition of [^{3}H]indiplon binding by zolpidem and zaleplon, with Ki values in good agreement with their affinities for $GABA_A$ receptors containing the α1 subunit, strongly suggest that in these experiments, [^{3}H]indiplon labels primarily this subtype of $GABA_A$ receptor. This was supported by experiments in cerebellar membranes. [^{3}H]Ro 15-4513 has very high affinity for all 6 alpha subunits of the $GABA_A$ receptor (Wong and Skolnick, 1992), and since only the α1 and α6 subunits are expressed in rat cerebellum, this radioligand labels both α1 and α6 subunit containing $GABA_A$ receptors in this preparation. Both zolpidem and zaleplon have been reported to have very low affinity for $GABA_A$ receptors containing the α6 subunit (Damgen and Luddens, 1999). The biphasic competition curves for [^{3}H]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 α1 subunit-containing and α6 subunit-containing $GABA_A$ receptors, respectively. From this we infer that indiplon binds to α1 subunit-containing $GABA_A$ receptors with low nanomolar affinity and to α6 subunit-containing $GABA_A$ receptors with micromolar affinity. All

compounds demonstrated about a 1000-fold difference between the activity at the α1 and α6 sites respectively (refer to Figure 3). [^{3}H]Ro 15-1788 has approximately 30 – 50 nM affinity for the α6 site and thus at the concentrations used for cerebellar binding studies (1.5 nM) would not appreciably label this subtype. Thus, the indiplon inhibition appeared monophasic with an affinity virtually identical to the high affinity site of the biphasic competition observed using [^{3}H]Ro 15-4513 (refer to Figure 3B). These data supported the high affinity and selective nature of the binding of indiplon to the $GABA_A$ receptors containing the α1 subunit and while indiplon has some affinity for the α6 subunit, the affinity is 1000-fold weaker, in the micromolar range and not likely to be of any pharmacological consequence. Experiments with recombinant $GABA_A$ receptor subtypes expressed in HEK cells support the conclusions drawn from the present studies that indiplon has high affinity and selectivity for α1 subunit-containing $GABA_A$ receptors (Petroski et al, unpublished data).

[^{3}H]Indiplon proved to be an excellent radioligand for the characterization of brain membrane receptors. [^{3}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 minutes with the binding being stable for at least 90 minutes. Dissociation was initiated following equilibrium by the addition of triazolam, which effectively dissociated bound [^{3}H]indiplon from the $GABA_A$ receptor with a half-life of approximately 10 min. This clearly demonstrated that the binding of [^{3}H]indiplon to rat brain receptors was of a reversible nature and could be competitively displaced once equilibrium had been achieved.

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Saturation analyses revealed that the binding of [^{3}H]indiplon was saturable and highly specific (specific binding was routinely 80 – 90% of the total binding). In studies directly comparing the saturations of [^{3}H]Ro 15-1788 and [^{3}H]indiplon it was clear that while both compounds bound with high affinity, the receptor density recognized by [^{3}H]indiplon was approximately half that of the antagonist. These data were consistent with the hypothesis that [^{3}H]indiplon was preferentially binding a subset of the $GABA_A$ receptors labeled by [^{3}H]Ro 15-1788.

The actions of indiplon on $GABA_A$ 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 and more potent than zolpidem or zaleplon. The results from the patch clamp experiments on rat $GABA_A$ receptors are in agreement with the results from binding experiments on rat brain membranes.

In conclusion, these studies have shown that the novel pyrazolopyrimidine, indiplon, is a high affinity allosteric potentiator of the $GABA_A$ receptor, acting through the benzodiazepine binding site. Indiplon acts in a subtype selective fashion consistent with selectivity for $GABA_A$ 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 ($t_{1/2}$ = 1hr 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.

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Legends for Figures

Figure 1: Chemical synthesis of [^{3}H]Indiplon:

A mixture of mono and di-iodinated indiplon as the free bases was synthesized in-house to facilitate tritiation. Tritiation was performed as described under contract by American Radiolabeled Chemical Inc. (St. Louis, MO). Each batch of the acquired radiolabel [^{3}H]indiplon, (N-methyl-N-[3-[3-(2-thienylcarbonyl)-pyrazolo[1,5-alpha]pyrimidin-7-yl]phenyl]acetamide) yielded a specific activity of 35 Ci/mmol, with a compound purity greater than 98% as determined by both TLC and preparative HPLC. The product (ART 1023) was stored at –20ºC until use. Abbreviations are: ICl, iodine monochloride; MeOH, methanol; $NaBH_4$, sodium borohydride; $PdCl_2$, palladium dichloride.

Figure 2: Rank order of affinities for [^{3}H]Ro 15-1788 Binding in Rat Brain.

Inhibition of [^{3}H]Ro 15-1788 binding to human $GABA_A$ receptors in rat frontal cortex (A) and cerebellum (B). Competition binding of triazolam, indiplon, zolpidem and zaleplon demonstrated a rank order of affinities: triazolam > indiplon > zolpidem > zaleplon and all compounds competed for the binding of [^{3}H]Ro15-1788 in a monophasic manner to the same baseline in each tissue. The data shown are from a single experiment where each point was performed in duplicate (error bars represent standard deviation), and this experiment is representative of at least three independent determinations (see Table 2 for mean Ki values). Radioligand binding data were analyzed as described.

Figure 3: Selective Discrimination of $GABA_A$ subtypes in Rat Cerebellum using [^{3}H]Ro 15-4513:

(A) Inhibition of [^{3}H]Ro 15-4513 binding in rat cerebellum. Biphasic inhibition by indiplon, zolpidem and zaleplon. (B) Inhibition of [^{3}H]Ro 15-4513 and [^{3}H]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.

Figure 4: Functional agonism determined in the Presence of GABA:

Inhibition of [^{3}H]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.

Figure 5: $GABA_A$ Current From Cultured Cortical Neuron:

A) Concatenated display of 3 μM, GABA-activated currents (200 ms; one out of every three traces shown) before during and after bath application of 300 nM indiplon (indicated by bar) as described. B) Close-up of three superimposed GABA-activated currents (Control, 300 nM indiplon, wash). C) Concentration response curves for indiplon, zolpidem, zaleplon, and

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triazolam. Each data point represents the mean ± SEM of 4-20 determinations from different neurons voltage clamped at –70 mV using an internal solution of CsCl.

Figure 6: Direct Membrane Kinetics of [^{3}H]Indiplon Binding:

Association and dissociation of [^{3}H]indiplon binding to rat frontal cortex membranes. For association experiments, membrane homogenates were incubated at 4°C for various times as described in the Methods. Non-specific binding was defined in the presence of 10 µM triazolam at each time point. The association rate constant (K_{+1}) for [^{3}H]indiplon was determined (assuming pseudo-first order kinetics) by plotting [ln Be/(Be-B)] vs time where, Be, is the amount specific bound (fmol/mg protein) at equilibrium and B is the amount specific bound at any given time point. The association rate constant was calculated from the following equation: [Kob - K_{-1} = K_{+1} • (CL)], where Kob is the slope of the association, K_{-1} is the dissociation rate constant and CL is the concentration of ligand used. **Inset:** Dissociation of [^{3}H]indiplon. Following equilibrium, dissociation of [^{3}H]indiplon was initiated by the addition of 10 µM triazolam and the reaction stopped at various times by rapid vacuum filtration. The specific binding was calculated and the dissociation constant determined from the following equation: [ln B/B_0 = K_{-1} • t], where B is the amount specifically bound at any given time point, B_0 is the specific amount bound at equilibrium and t is time. The data are from a single experiment that was repeated twice with similar results.

Figure 7: Direct Saturation Binding of [^{3}H]Indiplon in Rat Brain:

Saturation and Scatchard analysis (inset) of [^{3}H]Ro 15-1788 (A and C) or [^{3}H]indiplon (B and D) in rat cortical (A and B) or cerebellar (C and D) membranes. Nonspecific binding was defined in the presence of 10 μM triazolam. Scatchard transformations are shown with 95% CI and are the mean of duplicate determinations, which are representative of five independent experiments (see Table 1 for mean K_D and Bmax values). All data were analyzed using the non-linear least squares regression analysis algorithms included in GraphPad Prism (GraphPad Inc, San Diego, CA).

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Table 1: K_D and Bmax determinations for [^{3}H]Ro 15-1788 and [^{3}H]Indiplon in rat frontal cortex and cerebellar membrane homogenates. Values for K_D and Bmax are the mean ± SEM of at least three independent experiments performed in duplicate.

[^{3}H]Label	Tissue	K_D (nM)	Bmax (pmol/mg prot)
Ro 15-1788	Cortex	2.13 ± 0.09	5.08 ± 0.37
	Cerebellum	1.65 ± 0.13	2.30 ± 0.06
Indiplon	Cortex	1.01 ± 0.23	2.58 ± 0.45
	Cerebellum	0.53 ± 0.03	1.16 ± 0.08

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Table 2: Comparison of Ki values (nM) rank order profile of benzodiazepine and non-benzodiazepine sedative-hypnotics in rat frontal cortex or cerebellar $GABA_A$ receptors labeled with either [^{3}H]Ro 15-1788 or [^{3}H]Indiplon. Ki values listed are means ± SEM of at least three independent experiments performed in duplicate. Hill coefficients (nH) are shown in parentheses as mean ± SEM. Statistical analysis using a one sample t-test was run to determine if the nH values were significantly different from the value of 1. No values were significantly different, except for the nH value for [^{3}H] Ro 15-1788 competed with triazolam in the cortex, which gave a p value = 0.01 at a confidence interval of 95%. The reason for this difference is unknown.

K_i (nM) ± SEM *(nH ± SEM)*

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

Figure 1: Sullivan et al. JPET-2004-071282

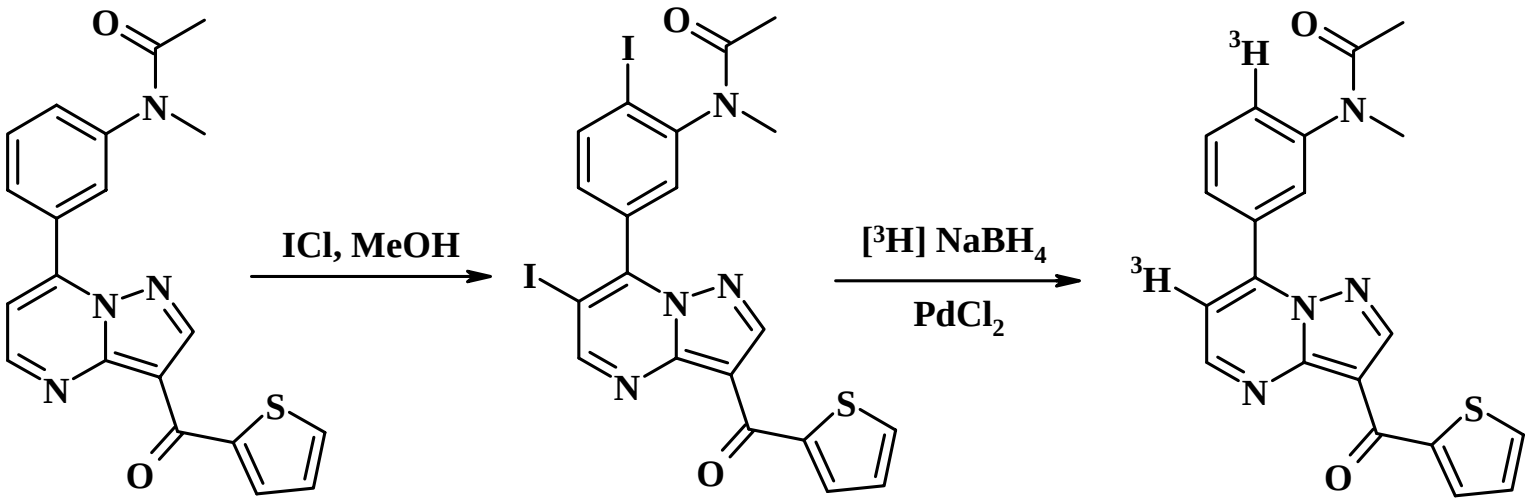

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Figure 2: Sullivan et al. JPET-2004-071282

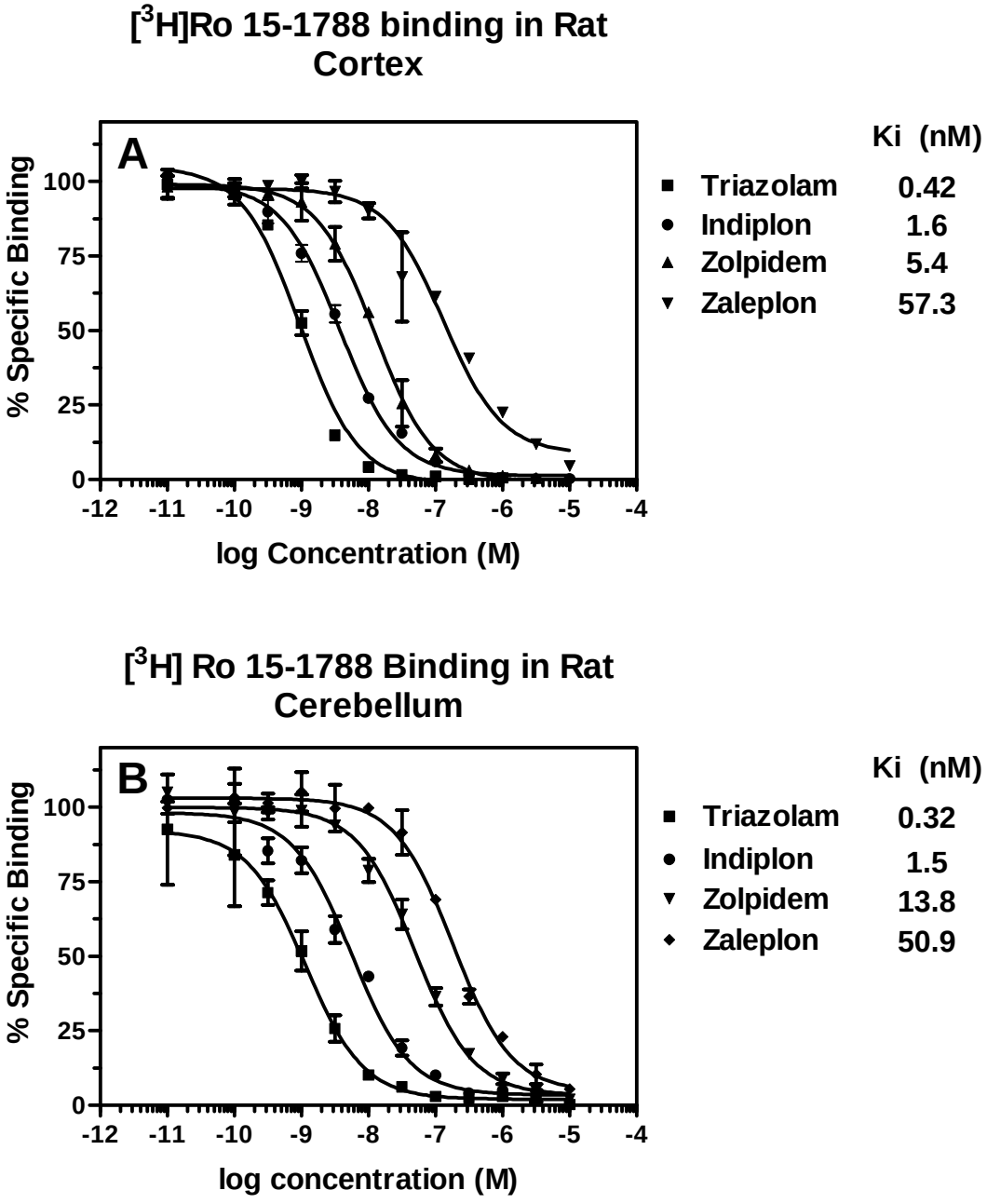

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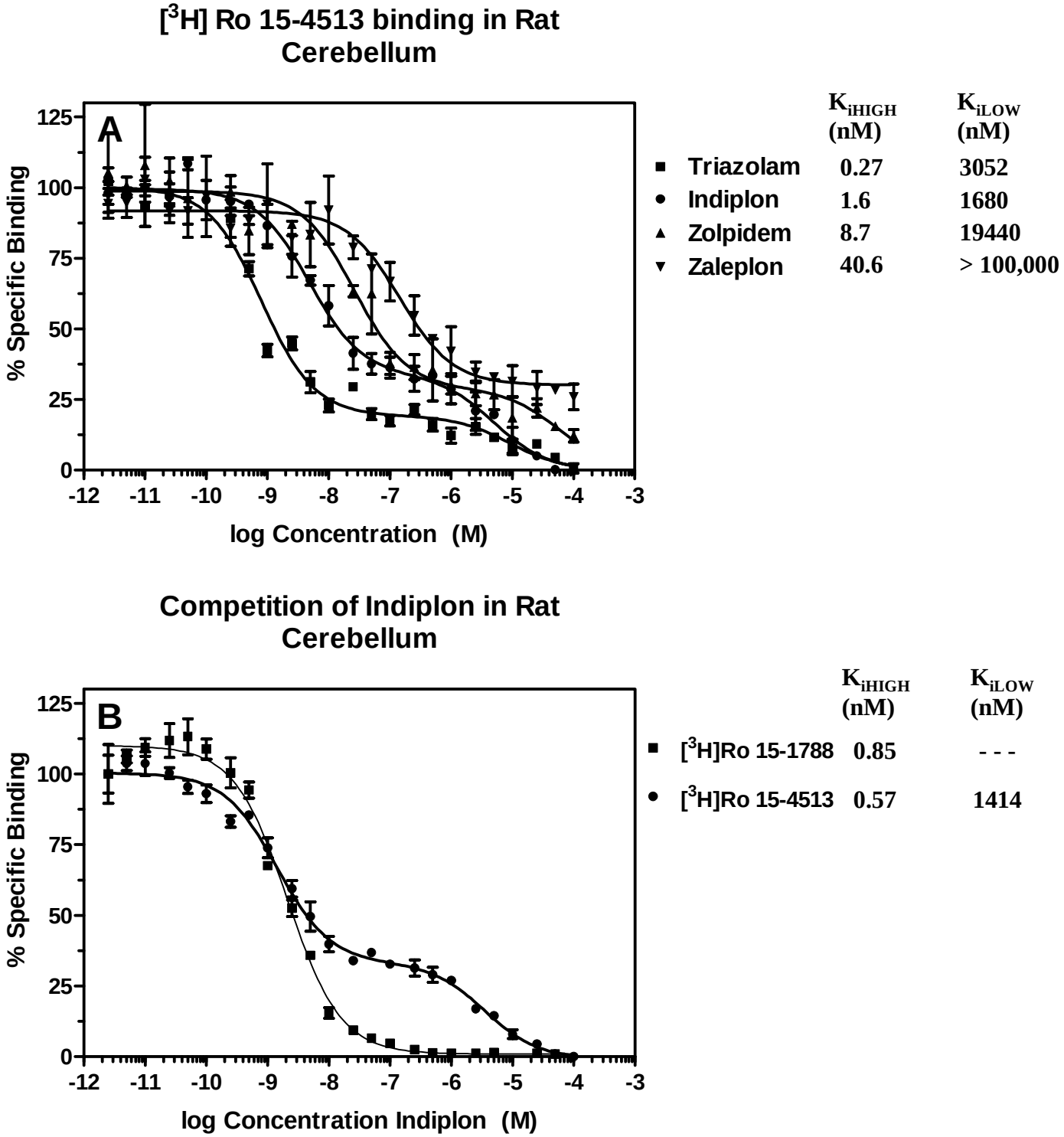
Figure 3: Sullivan et al. JPET-2004-071282



	K_{iHIGH} (nM)	K_{iLOW} (nM)
Triazolam	0.27	3052
Indiplon	1.6	1680
Zolpidem	8.7	19440
Zaleplon	40.6	> 100,000

	K_{iHIGH} (nM)	K_{iLOW} (nM)
[3H]Ro 15-1788	0.85	- - -
[3H]Ro 15-4513	0.57	1414

Figure 4: Sullivan et al. JPET-2004-071282

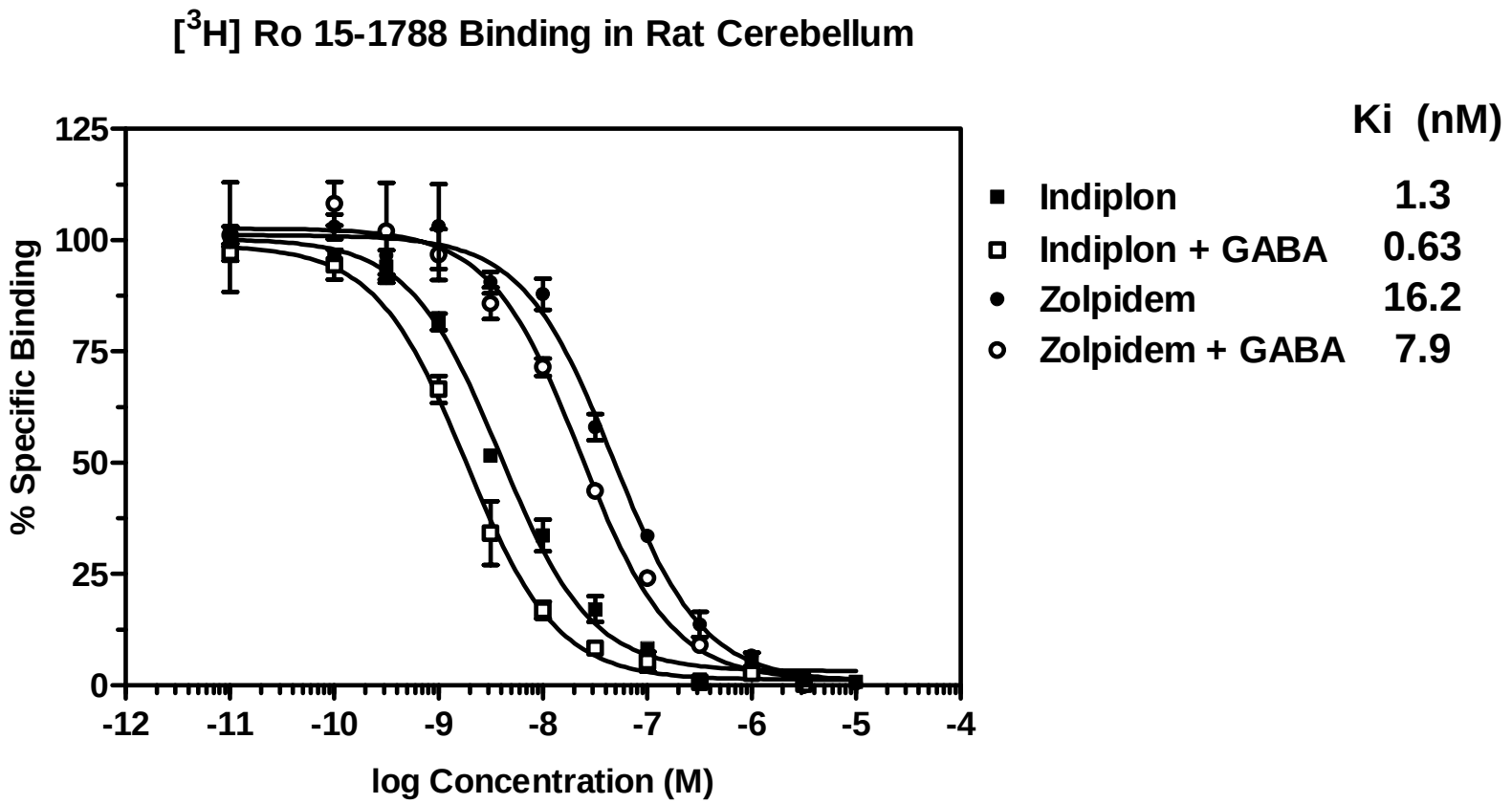


Figure 5A: Sullivan et al., JPET-2004-071282

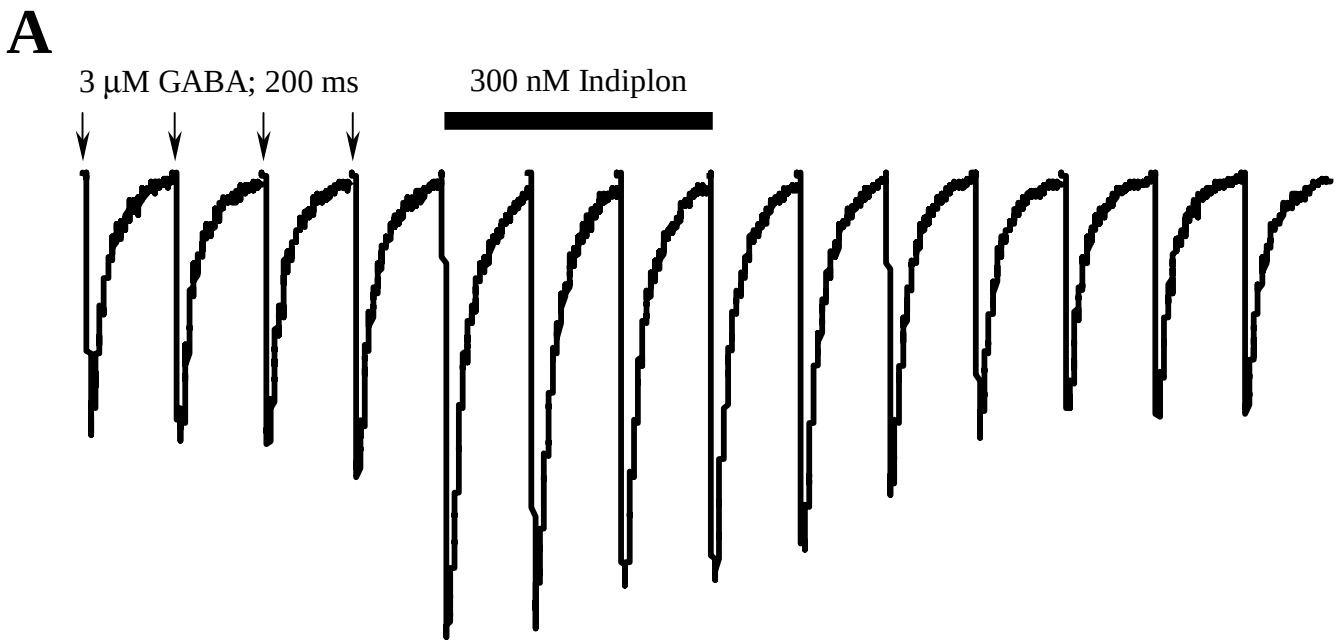


Figure 5B: Sullivan et al., JPET-2004-071282

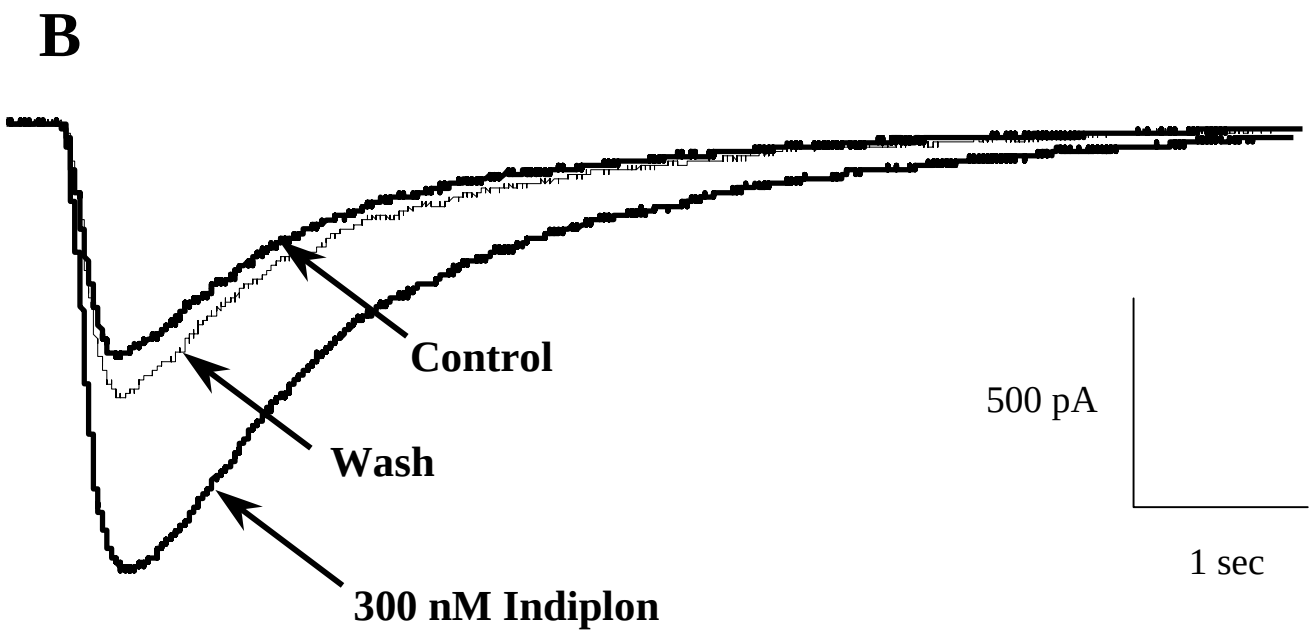


Figure 5C: Sullivan et al. JPET-2004-071282

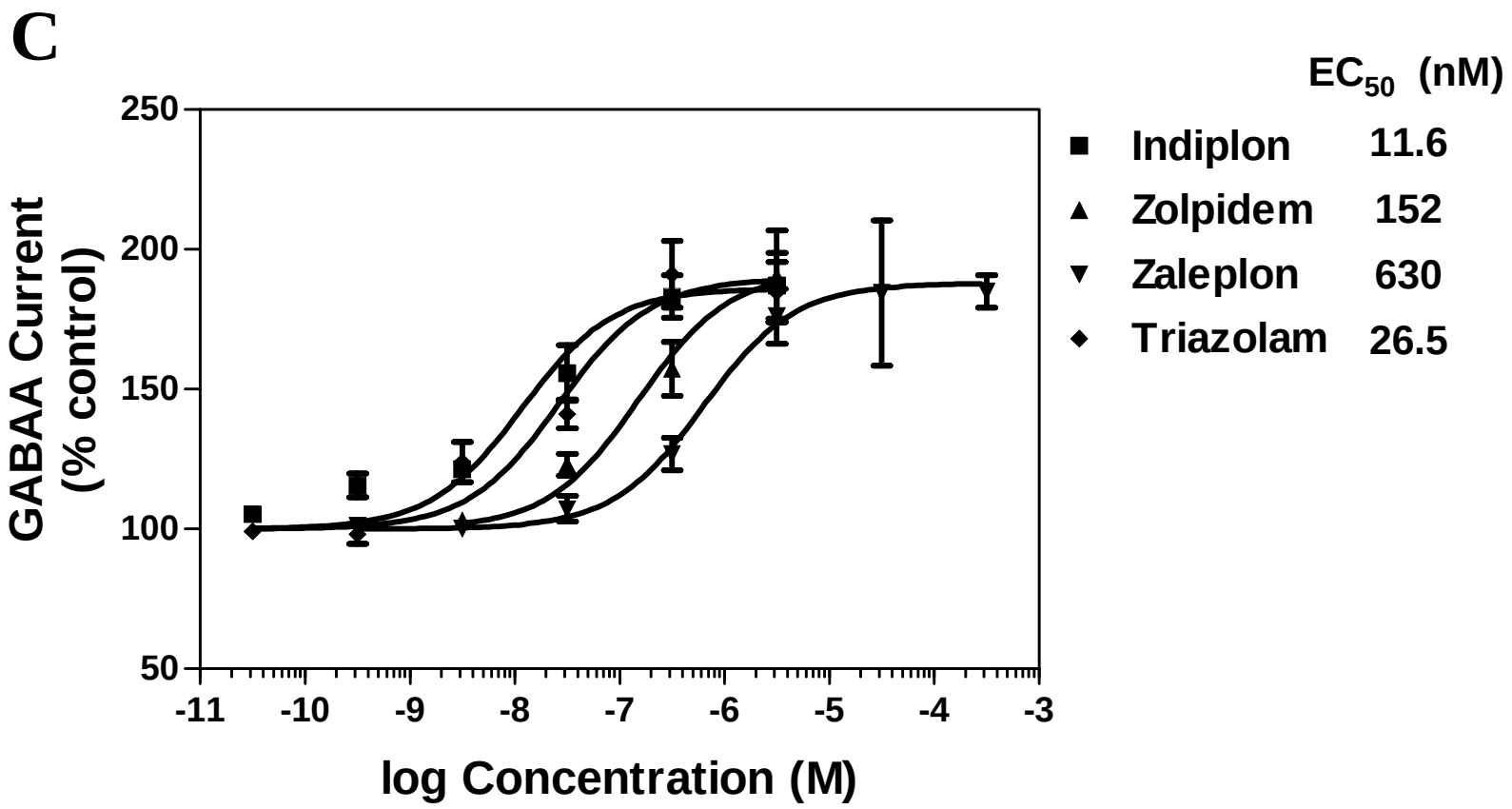


Figure 6: Sullivan et al. JPET-2004-071282

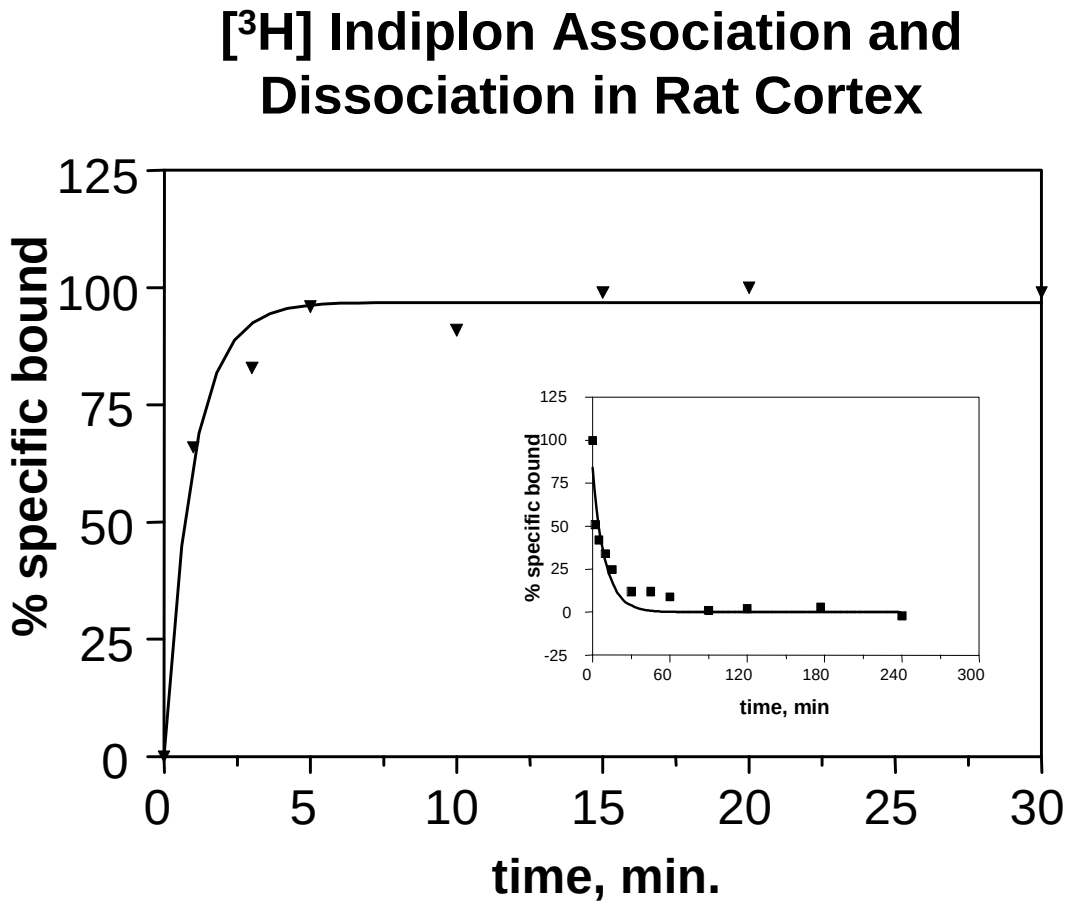


Figure 7: Sullivan et al. JPET-2004- 071282