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AHN 086: An Irreversible Ligand of "Peripheral" Benzodiazepine Receptors

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SUMMARY

AHN 086, a derivative of 4′-chlorodiazepam (Ro 5-4864) containing an isothiocyanate moiety, binds irreversibly to "peripheral" benzodiazepine receptors in the kidney with an IC_{50} of ~1.3 nM. Using standard incubation conditions (50 mM potassium phosphate buffer, pH 7.0, 0°), AHN 086 reacted rapidly with "peripheral" benzodiazepine receptors, whereas a time-dependent inhibition of [^{3}H]Ro 5-4864 binding by AHN 086 could be demonstrated by decreasing the pH of the reaction mixture, suggesting the presence of a histidine residue at or near the locus at which AHN 086 reacts. At concentrations up to 1 μM, AHN 086 did not inhibit [^{3}H]flunitrazepam binding to "central-type" benzodiazepine receptors. Thus, AHN 086 appears to be a specific, high affinity, irreversible ligand of "peripheral" benzodiazepine receptors.

Saturable, high affinity recognition sites for benzodiazepines have been identified in peripheral tissues, transformed cells of neural origin, and some areas of the central nervous system (1, 2) with physical and pharmacological characteristics (3–5) that are distinct from the benzodiazepine receptors which were initially described in the central nervous system (5, 6). Although there is unequivocal evidence that the latter sites (CBRs) mediate the principal pharmacologic actions of the benzodiazepines (1, 7–9), the physiologic and pharmacologic functions of the former sites (PBRs) are unknown, although there are indications that the PBRs, at least in the heart, might be associated with a voltage-regulated calcium channel (10).

The development of irreversible ligands has proven invaluable in the characterization, isolation, and purification of CBRs (11–13), opiate receptors (14), and phencyclidine receptors (15). Thus, an attempt was made to prepare a specific, high affinity alkylating ligand of the PBR.

We now report the synthesis and biochemical characterization of AHN 086 (Fig. 1), a specific irreversible inhibitor of radioligand binding to PBRs. AHN 086, which is structurally related to Ro 5-4864, has a high affinity for PBRs (IC_{50} ~1.3 nM) and possesses a pharmacologic profile similar to that of Ro 5-4864.

Materials and Methods

AHN 086, (1-(2-isothiocyanatoethyl-7-chloro-1,3-dihydro-5-(4-chloro-phenyl)-2H-1,4-benzodiazepine-2-one hydrochloride), was prepared in three steps starting with 7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2H-1,4-benzodiazepine-2-one (16), which was reacted with *N*-carbobenzoxy-2-bromoethylamine (17, 18). The resulting intermediate was deprotected with trimethylsilyliodide (19) to give the primary amine which was then reacted with freshly distilled thiophosgene in a two-phase system (20) followed by treatment with ethanolic hydrochloric acid to give AHN 086. The chemical structures and purity of AHN 086 and all intermediates were confirmed by thin layer chromatography, melting point, infrared spectroscopy, nuclear magnetic resonance spectroscopy, mass spectroscopy, and combustion analysis. Experimental details of this synthesis will be described in a subsequent publication.

Male, Sprague-Dawley rats (140–200 g) (Taconic Farms, Germantown, NY) were killed by decapitation. The kidneys were decapsulated, minced with scissors, and homogenized in 20 volumes of ice-cold buffer as specified in the text in a Brinkmann Polytron homogenizer (setting 6–7, 15 sec). This tissue homogenate was centrifuged at 23,000 × *g* for 20 min (4°) and the resulting pellet was resuspended in 100–2,000 volumes of buffer. The brains were removed and processed in the same way as the kidneys. The pellet was resuspended in 50–200 volumes of buffer as indicated in the figure and table legends.

Radioligand binding to this tissue suspension was assayed in a volume of 1 ml (2 ml for Scatchard analysis of [^{3}H]PK 11195 binding) consisting of: 0.25 ml of tissue suspension, 0.1 ml of radioligand ([^{3}H]Ro 5-4864, specific activity 78.9 Ci/mmol), in a range from 0.125 to 15 nM for the Scatchard analysis, or [^{3}H]PK 11195 (specific activity 85 Ci/mmol), in a range from 0.125 to 10 nM for the Scatchard analysis (New England Nuclear, Boston, MA) diluted in assay buffer, drugs, and/or the appropriate buffer to final volume. Nonspecific binding was defined using Ro 5-4864 or PK 11195 (final concentration, 10 μM) for [^{3}H]Ro 5-4864 and [^{3}H]PK 11195, respectively. In some experiments,

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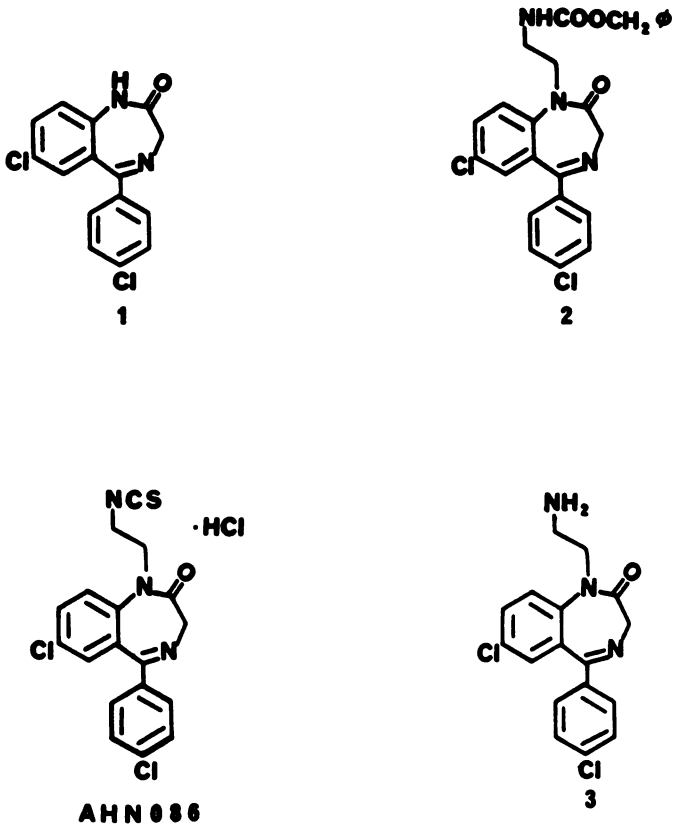
ABBREVIATIONS: CBR, "central-type" benzodiazepine receptor; PBR, "peripheral" benzodiazepine receptor; PK 11195, (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; Ro 5-4864, 4′-chlorodiazepam.



Φ = C_6H_5

Fig. 1. Structure of AHN 086 and its intermediates. AHN 086, (1-(2-isothiocyanatoethyl-7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2H-1,4-benzodiazepine-2-one hydrochloride), was prepared in three steps starting with 7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2H-1,4-benzodiazepine-2-one (**1**), which was reacted with *N*-carbobenzoxy-2-bromoethylamine to give (**2**). This compound was deprotected with trimethylsilyliodide to give the primary amine (**3**). It was reacted with freshly distilled thiophosgene in a two-phase system followed by treatment with ethanolic hydrochloric acid to give AHN 086.

tissue homogenates were preincubated with various concentrations of AHN 086 at 0° for various time intervals, centrifuged (23,000 × *g* for 20 min, 4°), and resuspended from one to six times prior to determination of radioligand binding. Incubations were performed at 0–4° and terminated after 60 min by rapid filtration over Whatman GF/B filters, with three washes (5 ml) of assay buffer using a Brandel M-24R filtering manifold (Brandel Instruments, Gaithersburg, MD). Filters were preincubated with 0.1% polyethyleneimine in distilled water for Scatchard analysis of [^{3}H]PK 11195 binding. In experiments using pH 5 buffer, equal volumes of 50 mM KH_2PO_4 and K_2HPO_4 were adjusted to pH 5.0 with H_3PO_4.

Protein was determined using the Miller modification (21) of the Lowry (22) technique.

Ro 5-4864 was obtained from Hoffman-La Roche (Nutley, NJ). PK 11195 was a gift from Dr. Gerard Le Fur, Pharmuka Laboratories (Gennevilliers, France). 7-Chloro-1,3-dihydro-5-(4-chlorophenyl)2H-1,4-benzodiazepine-2-one was a gift from Dr. Karl Weber, Boehringer Ingelheim.

Results

The IC_{50} values for AHN 086 to inhibit [^{3}H]Ro 5-4864 binding in the brain and kidney (2.1 ± 0.2 nM and 1.2 ± 0.1 nM, respectively) were estimated by extrapolating the observed IC_{50} at different tissue concentrations to an infinite tissue dilution (Fig. 2). Both the pseudo-Hill coefficient and the IC_{50} values in the kidney increased with increasing tissue concentrations. For example, the pseudo-Hill coefficient of AHN 086 ranged from 1.37 ± 0.11 at 200 μg of protein/assay to ~1 for less than 50 μg of protein/assay, and the IC_{50} values were 12 nM and 1.2 nM at 200 and 22 μg of protein/assay, respectively. The pseudo-Hill coefficient in the brain was ~1 for all tissue concentrations tested. The IC_{50} of AHN 086 to inhibit [^{3}H]PK 11195 binding was identical to the value obtained for [^{3}H]Ro 5-4864 (Fig. 3).

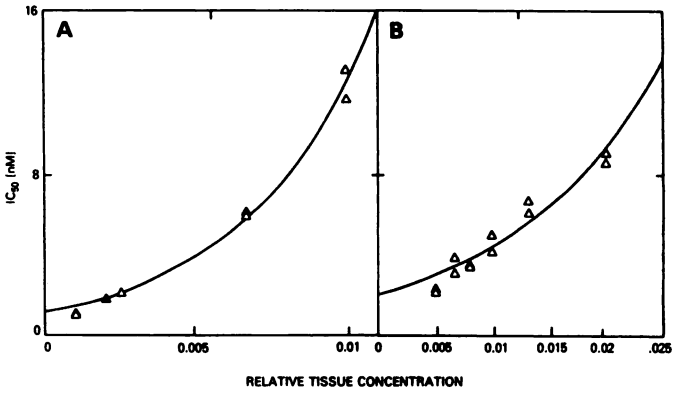


Fig. 2. Determination of the IC_{50} of AHN 086: relationship to protein concentration. Kidney membranes (A) and brain membranes (B) were prepared in 50 mM potassium phosphate buffer (pH 7) as described in the text. The assay volume for the brain membranes was increased to 2 ml. Each *point* is the result of one IC_{50} determination using at least six ligand concentrations performed in triplicate. No single determination of the IC_{50} estimated with a Hill plot had a correlation coefficient of < 0.99. A least squares fit on an exponential equation was performed on these values using the program MLAB. The correlation coefficient for these fits were 0.996 in the kidney and 0.976 in the brain membranes, respectively. ▲, overlapping points.

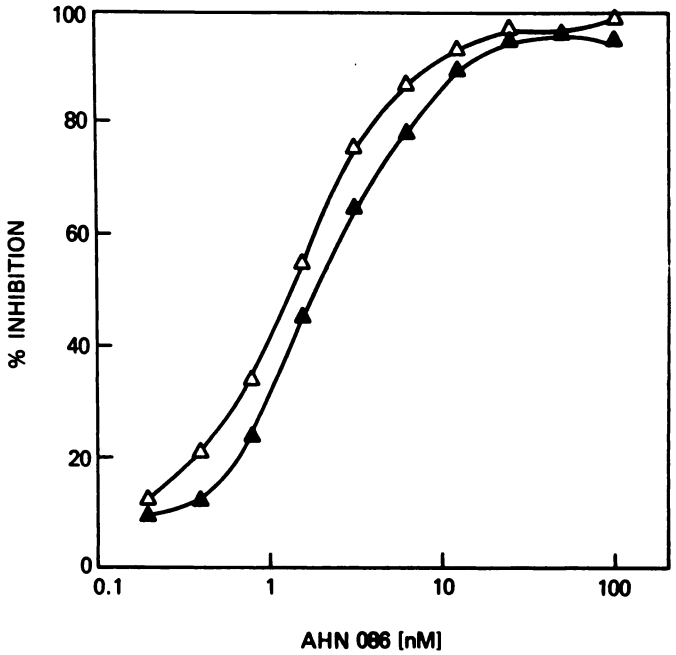


Fig. 3. IC_{50} of AHN 086 for [^{3}H]Ro 5-4864 and [^{3}H]PK 11195. Kidney membranes were prepared as described in the text. The binding assay was performed as described under Materials and Methods. Shown are the means of three experiments in triplicate. The ligand concentrations were 1 nM for both ligands, and the tissue concentration was between 60 and 70 μg/assay tube. △, [^{3}H]Ro 5-4864; ▲, [^{3}H]PK 11195.

Scatchard analysis of [^{3}H]Ro 5-4864 binding to PBRs in kidney membranes after preincubation with AHN 086 followed by extensive washing revealed a concentration-dependent decrease in the B_{max} between 10 nM and 1 μM (Table 1). In contrast, only the K_D of [^{3}H]PK 11195 was affected by up to 100 nM AHN 086 (Table 2). No specific binding of either radioligand was detectable after preincubation of the membranes with 1 μM AHN 086. However, a small, residual amount of [^{3}H]PK 11195 binding (~5% of the B_{max}) to membranes was detectable after incubation with 1 μM AHN 086 that could not be displaced by 10 μM PK 11195 (data not shown). The same amount of [^{3}H]PK 11195 binding was found in membranes heated at 100° for 10 min. Similar findings were also obtained with [^{3}H]Ro 5-4864 (data not shown). A 2-fold decrease in the potency of AHN 086 could be detected by substituting 50 mM Tris-HCl buffer, pH 7.4, for the 50 mM potassium phosphate buffer, pH 7.0, in both kidney and brain membranes (Table 3). AHN 086 at concentrations up to 1 μM had no effect on [^{3}H] flunitrazepam binding in brain membranes (Table 3).

A time course for the inhibition of [^{3}H]Ro 5-4864 was performed to validate the irreversible nature of inhibition by AHN 086. A variety of conditions including: 1) reaction with an excess of cold Ro 5-4864 or PK 11195, 2) increasing k_3 (the reaction rate leading from the reversible to the irreversible receptor ligand complex) by increasing the tissue concentration, or 3) adding secondary butylamine (50 mM) before or after (ranging from 5 to 60 min) the addition of AHN 086 (10 nM)(data not shown) failed to antagonize the effects of AHN 086 under standard incubation conditions and centrifugation to wash out excess of AHN 086. However, reducing the pH of the preincubation buffer and the buffer used for the first two of the six washing steps to pH 5.0 resulted in a time-dependent irreversible inhibition of [^{3}H]Ro 5-4864 binding by AHN 086 (Fig. 4). Using this pH 5 buffer in the preincubation step with AHN 086, a reduction in the rate of alkylation of PBRs by AHN 086 could be observed by adding 10 nM Ro 5-4864 or PK 11195 (Fig. 5). The IC_{50} of Ro 5-4864 was not affected by the decreased pH of the assay buffer, whereas the IC_{50} of PK 11195 increased approximately 2-fold. No effect on the pseudo-Hill coefficient for either ligand could be detected at the reduced pH (Table 4).

TABLE 1

Effects of AHN 086 on [^{3}H]Ro 5-4864 binding to PBR: Scatchard analysis

Kidney membranes, prepared in 50 mM potassium phosphate buffer (pH 7.0), were incubated at a dilution of 1:250 (w/v) with increasing concentrations of AHN 086. Scatchard analyses were performed on these membranes as described under Materials and Methods after four washes of the membranes in 1:250 dilutions. Assays were conducted with 10–20 μg of protein/tube. At least eight ligand concentrations (in triplicate) were used for each determination. Values represent the mean ± SE with the number of determinations as indicated.

AHN 086	K_D	B_{max}	% Control (B_{max})	n
	nM	fmol/mg protein		
0	1.23 ± 0.39	5660 ± 550	100	7
10 nM	0.98 ± 0.35	4100 ± 340[a]	72	6
100 nM	2.99 ± 0.72[a]	3100 ± 290[a]	55	4
1 μM		ND[b]	0	10

[a] $p < 0.001$ compared to no AHN 086 using an unpaired t test.
[b] ND, not detectable.

TABLE 2

Effects of AHN 086 on [^{3}H]PK 11195 binding to PBR: Scatchard analysis

Kidney membranes prepared in 50 mM potassium phosphate buffer (pH 7.0) were incubated at a dilution of 1:250 (w/v) with increasing concentrations of AHN 086. Scatchard analyses were performed on these membranes as described in Materials and Methods after four washes of the membranes in 1:250 dilutions. Assays were conducted with 10–20 μg of protein/tube. At least eight ligand concentrations (in triplicate) were used for each determination. Values represent the mean ± SE with the number of determinations as indicated.

AHN 086	K_D	B_{max}	n
	nM	fmol/mg protein	
0	0.72 ± 0.10	5990 ± 1340	6
10 nM	0.64 ± 0.03	6340 ± 1270	6
100 nM	1.39 ± 0.25[a]	8050 ± 2730	4
1 μM		ND[b]	10

[a] $p < 0.001$ compared to no AHN 086 using an unpaired t test.
[b] ND, not detectable.

Discussion

Several lines of evidence suggest that AHN 086, a structural analog of Ro 5-4864, is a specific, site-directed, irreversible ligand of the PBR. Such evidence includes: 1) the potent inhibition of both [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 binding [two high affinity ligands of PBR (2, 4, 23) by AHN 086 without a concomitant effect on radioligand binding to the CBR; 2) the fact that the reaction leading to the irreversible binding could be retarded at pH 5 by either Ro 5-4864 or PK 11195; 3) the fact that the B_{max} of [^{3}H]Ro 5-4864 was reduced followed incubation with AHN 086 concurrent with a modest but significant change in the apparent affinity of the radioligand. Whereas the effects of AHN 086 at concentrations lower than 1 μM on [^{3}H]Ro 5-4864 binding are primarily on B_{max}, the effects of AHN 086 on [^{3}H]PK 11195 binding were manifest as an increase in the K_D value. However, at high concentrations of AHN 086 (100 nM), an irreversible change in the K_D for [^{3}H] Ro 5-4864 was observed. This reduction in the apparent affinity of [^{3}H]Ro 5-4864 accompanying the drop in B_{max} could be attenuated to the presence of a second nucleophile in the vicinity of the receptor, whose alkylation would lead to this increase in K_D. The reaction with this putative group would be at least 1 order of magnitude slower than with the described imidazole system. Computer modelling revealed that the experimental error in the assay system used is too large to permit differentiation between the differences in reaction rate of a one- and a two-site model. The qualitative differences in the rates of inactivation by AHN 086 on [^{3}H]Ro 5-4864 and [^{3}H] PK 11195 binding are not without precedent, since differences in the binding characteristics of these ligands have been shown in a number of systems following covalent modification of the PBR despite the mutually exclusive binding of these radioligands (23–25).[2]

The irreversible nature of AHN 086 binding to PBRs was demonstrated by plotting binding of [^{3}H]Ro 5-4864 versus receptor concentration at near-saturating concentrations of radioligand (Fig. 6). The x axis intercept is significantly different from the origin, which is strong evidence for an irreversible blockade of receptors (20). Furthermore, the effect of AHN 086 on the B_{max} of [^{3}H]Ro 5-4864 persisted through extensive washing of tissues. In contrast, addition of 10 nM Ro 5-4864 elicited an inhibition of radioligand binding that could be reversed by washing ~98% of the control data (results not shown).

Several lines of evidence suggest that histidine is the most likely candidate as the target of the active isothiocyanate moi-

[2] H. Lueddens and P. Skolnick, submitted for publication.

TABLE 3

Inhibition of radioligand binding to PBR and CBR: Specificity of AHN 086

Membranes were prepared using 50 mM potassium phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 7.4). Values are the mean ± SE of three experiments using at least eight concentrations of AHN 086 (in triplicate) for each determination. The concentrations of radioligands were 1 nM. The tissue concentration was 15 μg/assay.

Buffer	Kidney		Brain		Ligand
	IC_{50}	n_H[a]	IC_{50}	n_H	
	nM		nM		
$K\text{-}PO_4$	1.37 ± 0.15	1.15 ± 0.05	5.28 ± 0.49	1.33 ± 0.16	[^{3}H]Ro 5-4864
Tris-HCl	2.51 ± 0.17	1.36 ± 0.05	12.1 ± 1.04	1.26 ± 0.06	[^{3}H]Ro 5-4864
Tris-HCl	ND[b]	ND	≫1 μM[c]		[^{3}H]Flunitrazepam

[a] Pseudo-Hill coefficient.
[b] ND, not detectable.
[c] No inhibition of [^{3}H]flunitrazepam binding was observed at 1 μM AHN 086. The shift in IC_{50} of AHN 086 in 50 mM Tris-HCl (pH 7.4) is probably due to the presence of high concentrations of Tris cation, which could react with AHN 086.

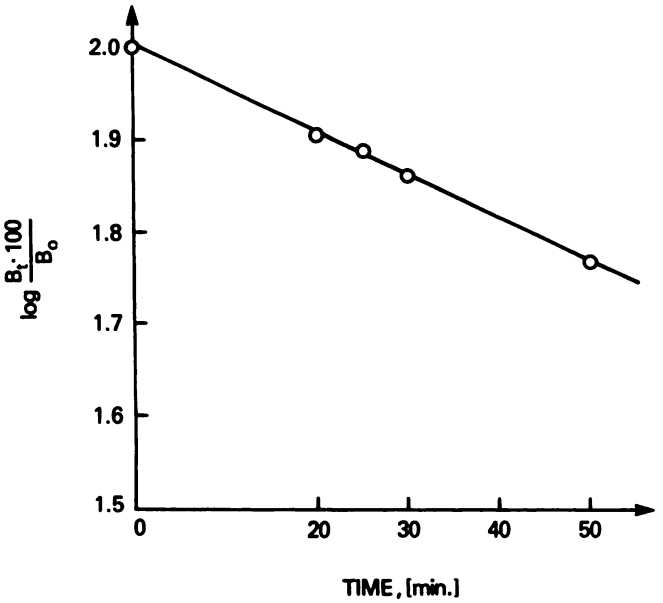


Fig. 4. Time dependency of alkylation by AHN 086. Kidney membranes were resuspended in potassium phosphate buffer, pH 5, in a 1:250 final dilution and incubated for various periods of time with 100 nM AHN 086. [^{3}H]Ro 5-4864 binding (10 nM) to the membranes was measured after two washes at pH 5 and two further washes at pH 7 with a final tissue concentration of 28–30 μg/assay. B_t/B_o = ratio of [^{3}H]Ro 5-4864 bound by tissue preincubated t min with AHN 086 as compared to 0 min preincubation with AHN 086. The time in pH 5 buffer was identical for all assays. The experiment was repeated four times with similar results.

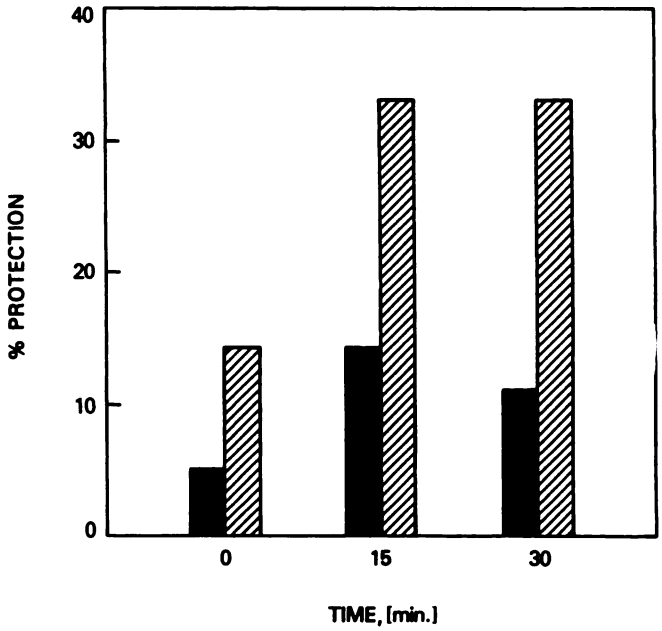


Fig. 5. Alkylation of PBR by AHN 086: protection by Ro 5-4864 and PK 11195. The experimental procedure was as described in the legend to Fig. 4, except that the AHN 086 concentration was 200 nM. Incubations contained either 10 nM Ro 5-4864 (□) or PK 11195 (▨), respectively. The results are expressed as percentage above the corresponding value containing neither Ro 5-4864 nor PK 11195. The experiment was performed three times with qualitatively identical results.

ety of AHN 086. As previously demonstrated, histidine modification of PBRs by diethylpyrocarbonate resulted in a decrease in the B_{max} of [^{3}H]PK 11195, with no significant effect on [^{3}H] Ro 5-4864 binding (25). In the present study, a reduction of 1 pH unit below the pK_a for the imidazole group of histidine increased the IC_{50} of PK 11195 nearly 2-fold but did not affect the potency of Ro 5-4864. These observations are consistent with the greater protection against alkylation by AHN 086 observed with PK 11195 compared to Ro 5-4864 (Fig. 5). In contrast, alkylation of this site by AHN 086 should lead to a greater decrease in the B_{max} of [^{3}H]PK 11195 than of [^{3}H]Ro 5-4864. Although this discrepancy may not easily be resolved until more information is available on the structure-activity relationship of the PBR ligands, it is possible that [^{3}H]Ro 5-4864- and [^{3}H]PK 11195-binding sites are not identical, but overlapping. The histidine group may thus be entirely within the [^{3}H]PK 11195 domain but still susceptible to alkylation by the isothiocyanate group of AHN 086 which is attached to a C_2

TABLE 4

Effect of pH on displacement of [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 binding to PBR by AHN 086

Membranes were prepared as described under Materials and Methods using potassium phosphate buffer (pH 7). Membranes were resuspended in the corresponding assay buffers at pH 5 or 7 to give a final assay dilution of 1:2000 (w/v). Radioligand binding was performed as described under Materials and Methods using the corresponding buffer (pH 5 or 7) throughout the assay. Values represent the mean ± SE of three independent experiments with at least eight concentrations of AHN 086 in triplicate for each determination. The radioligand concentrations were 1 nM.

pH	[^{3}H]PK 11195		[^{3}H]Ro 5-4864	
	IC_{50}	n_H[a]	IC_{50}	n_H
	nM			
5	2.80 ± 0.22[b]	0.97 ± 0.12	1.29 ± 0.16	0.98 ± 0.06
7	1.60 ± 0.27	1.03 ± 0.03	1.17 ± 0.17	0.90 ± 0.04

[a] Pseudo-Hill coefficient.
[b] $p < 0.01$ compared to pH 7 using a t test.

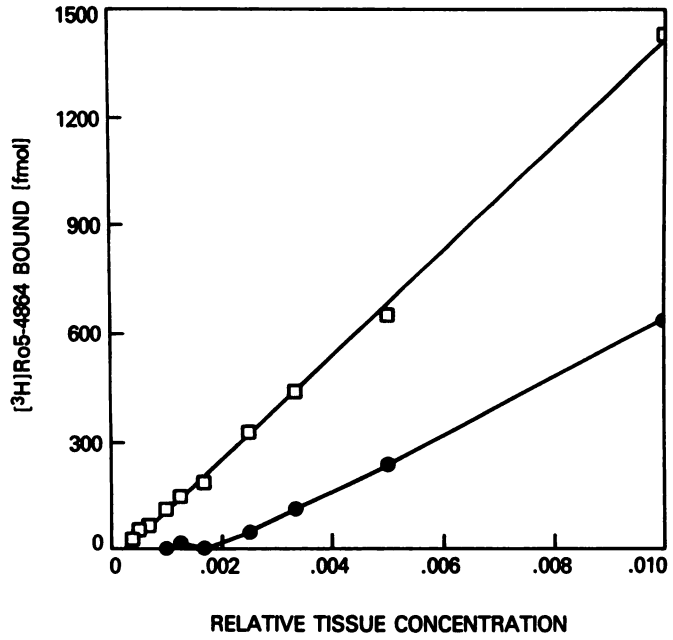


Fig. 6. Irreversibility of AHN 086. Kidney membranes were prepared as described in the text. Increasing concentrations of tissue and 20 nM [^{3}H] Ro 5-4864 were co-incubated in the presence (●) or absence (□) of 10 nM AHN 086. The concentration of [^{3}H]Ro 5-4864 used was sufficient to approximate the B_{max} values for all tissue concentrations. The difference between the x axis intercepts corresponds to the portion of receptors irreversibly blocked by this AHN 086 amount (i.e., equivalent to 31 μg of protein). Qualitatively identical results were obtained using 1 nM AHN 086, which blocked receptors equivalent to 3.4 μg of protein.

spacer arm. A similar qualitative difference in the binding of two ligands to one site has been described after covalent modification of the CBRs (11, 13, 26).

According to Kitz and Wilson (27), a reaction leading to an irreversible inactivation of a binding site follows the scheme:

$$L + R \underset{}{\overset{K_i}{\rightleftharpoons}} LR \xrightarrow{k_3} L^*R$$

where L is the irreversible ligand, R is the receptor, LR is the reversible receptor-ligand complex, L^*R is the irreversible complex, K_i is the dissociation constant of the reversible complex, and k_3 is the rate constant for the formation of the irreversible complex. If the excess of the irreversible ligand is removed or reduced prior to the assay and the inhibitor concentration is $[L] \gg [R]$, then:

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_i}{k_3} \times \frac{1}{[L]}$$

As AHN 086 is an irreversibly binding ligand, no estimate of the K_i or the reaction leading to the irreversible complex can be made unless a time course of inhibition can be obtained. The observation that no measurable time course of inhibition could be obtained under standard incubation conditions (i.e., pH 7) can be explained by the fact that at pH 5, $t_{1/2}$, the extrapolated time at which half of the receptors are blocked, is ~70 min. Assuming that the isothiocyanate moiety reacts only with the uncharged imidazole group of histidine, reducing the pH by 2 units will result in ~1% of the uncharged histidine being available at pH 5 compared to pH 7. Lowering the pH to 5 by itself does not affect binding of Ro 5-4864 (Table 4), so that only k_3 is affected by the pH change. Thus, the reaction leading to the irreversible complex at pH 7 may be too fast to be detectable under the separation method employed. Furthermore, secondary butylamine at concentrations of up to 50 mM was ineffective in blocking the action of AHN 086 at pH 7, although it was shown to alter the migration (R_F) of AHN 086 on thin layer chromatography in a chloroform phase within minutes, demonstrating an immediate reaction with the isothiocyanate moiety to give the corresponding thiocarbamate (date not shown). Since the pK_a of secondary butylamine is 10.78 ± 0.01 (determined by titration with HCl at 20°), insufficient quantities of this strong nucleophilic free base would be present in an aqueous phase of pH 7 to significantly react with AHN 086. According to the Henderson-Hasselbalch equation, 99.998% of the secondary butylamine will be in its protonated form at pH 7.0, leaving 8.3 μM out of 50 mM to react with AHN 086. This still amounts to an excess of ~30,000-fold over the receptor concentration used in these assays.

Thus, several lines of evidence suggest that AHN 086 is a high affinity ligand and, as an irreversible ligand of PBRs structurally related to Ro 5-4864, it may prove useful for the purification and isolation of the PBRs and the determination of the physiologic function of these sites.

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