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Potential Nonequilibrium Analgetic Receptor Inactivators. Further Pharmacologic Studies of *N*-Acylanileridines

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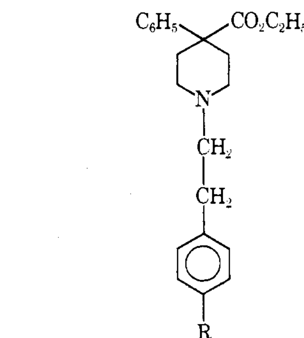
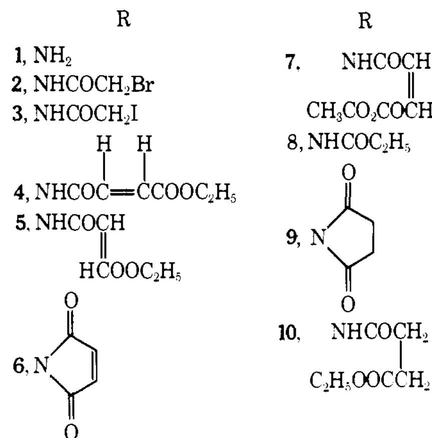
The antagonistic property of ethyl *p*-(4-ethoxycarbonyl-4-phenyl-1-piperidinoethyl)fumaranilate (**5**) was investigated. Compound **5** was found to antagonize morphine analgesia in a complex manner which could not be described as a simple competitive or noncompetitive type. The antagonism, however, lasted for over 6 hr suggesting that **5** has a high affinity for the analgesic receptors. Compound **5** appeared to possess dependence liability in the single-dose suppression test. In the electrically stimulated isolated guinea pig ileum, **5** acted like an agonist. No antagonistic activity of **5** was apparent in the latter two tests.

In a previous communication, we reported the synthesis and analgesic potencies of six *N*-acylanileridines having various alkylating moieties.[1] One compound, namely, ethyl *p*-(4-ethoxycarbonyl-4-phenyl-1-piperidinoethyl)fumaranilate (**5**), appeared to significantly inhibit morphine analgesia. Since the specific narcotic antagonist, naloxone, prevented this inhibition by the anileridine derivative, it was suggested that this compound might have the capacity to alkylate analgesic receptors selectively. A further quantitation of the inhibition of morphine analgesia by **5** is recorded in the present paper.

Since it was of interest to see whether or not the alkylating *N*-acylanileridines could affect narcotic receptors other than those for analgesia, two other pharmacologic parameters were utilized. It is generally known that if animals become physically dependent on one narcotic, they exhibit cross dependence to the other narcotic agents. Taking advantage of this fact, the capacity of the various alkylating *N*-acylanileridines to supress morphine abstinence was assessed. The other parameter employed was the effect of *N*-acylanileridines on the electrically stimulated isolated guinea pig ileum. Studies on the ileum were of interest since it has been demonstrated that the agonistic activity of a series of analgesics in this preparation correlated remarkably well with the analgesic potency in man.[2-4]





Experimental Section

Compounds. All the compounds used in this study were those synthesized and described previously.[1] They were anileridine derivatives containing either various alkylating functions (**2–7**) or nonalkylating groups (**8–10**) that are structurally similar to the alkylating moieties.

Estimation of ED_{50}. Male Sasco mice (Omaha, Neb.) weighing between 20 and 30 g were used in these determinations. The analgesic assay used was a modification of the hot-plate method described by Eddy and Leimbach.[5] The animal responses were made quantal by establishing an end point at the mean peak effect in each group which represented an increase in the reaction time of an individual animal of greater than three standard deviations of the control mean reaction time for all animals used in the group. For example, if an animal initially had a reaction time of 8 sec and the standard deviation for this particular group of animals was 3 sec, a reaction time after drug treatment of >17 sec would be considered a significant increase in the reaction time. An animal having a 10-sec reaction time in this group would be considered a positive responder if the reaction time exceeded 19 sec. The usual control time in these animals was about

Table I. Effect of Compound **5** on the ED_{50} of Morphine Sulfate

Pretreatment[a]	ED_{50} of morphine in mg/kg (95% confidence limits)[c]
None	5.45 (4.87–6.11)
10 ml/kg of 40% propylene glycol[b] 2 hr before assay	4.55 (3.14–6.60)
50 mg/kg of **5** 2 hr before assay	10.58 (9.48–11.68)
50 mg/kg of **5** 6 hr before assay	8.65 (7.72–9.69)
100 mg/kg of **5** 3 hr before assay	12.29 (11.17–13.41)

[a]The analgesic effect of **5** had disappeared by the times indicated in the column. Injections were made ip. [b]Propylene glycol (40%) was the vehicle in which **5** was dissolved. [c]Concentrations of morphine sulfate solutions were prepared such that 10 ml/kg was injected sc at each dose level.

7.40 sec and the standard deviation of various groups varied between 2.0 and 3.5 sec. Nonresponding mice were always removed from the heat stimulus before 30 sec to avoid damage to the animals. Ten mice were used at each dose level and at least 30 animals were used to determine each dose–response curve and the ED_{50}. The data were analyzed by the parallel line assay[6] with the aid of a computer.

Suppression of Morphine Abstinence. This was assessed by the single-dose suppression test in morphine-dependent mice as described by Takemori, *et al.*[7] Essentially the method consisted of making mice (male, Simonsen, 20–25 g) physically dependent on morphine by subcutaneous implantation of morphine pellets for 3 days. The pellets were then removed and at the peak of the animals' abstinence, as measured by withdrawal jumping, the test compound was injected to see whether or not the withdrawal sign was partially or completely suppressed. The nonparametric method of Wilcoxon[8,9] for paired differences was used to statistically analyze the number of jumps before and after the test drug. Residual dependence in the same animals was estimated the day after the above test by determining the ED_{50} of naloxone (naloxone-precipitated jumping) by the up and down method for small samples.[10]

Studies on the Electrically Stimulated Isolated Guinea Pig Ileum. All experiments were performed on isolated ilia from male guinea pigs (Oak Crest Rabbitry) weighing between 300 and 500 g. The depressant action of the various *N*-acylanileridines was studied on the contraction of the longitudinal muscle induced by coaxial electrical stimulation.[11] A segment of the intact ileum was used instead of the strip of longitudinal muscle as described by Kosterlitz and Watt.[12] Ilia were set up in 50-ml tissue baths containing Krebs solution kept at 37° and bubbled with 95% O_2 and 5% CO_2. The solution also contained 1.25 μM chlorpheniramine maleate. The contractions were recorded isometrically by a mechanoelectrical transducer (Statham UC 3) and a polygraph (Gilson).

ID_{50} (concentration of the drug which inhibits the maximal contraction of the ileum by 50%) were estimated from dose–response curves using the parallel line assay.[6] The morphine receptor was characterized by the usage of the narcotic antagonist, naloxone, and the determination of pA_2 values. ID_{50} values for the anileridine congeners were determined in the absence and presence of three increasing concentrations of naloxone. pA_2 values which represent the affinity constants of the antagonist for the receptors were estimated by plotting log $(X - 1)$, where X = dose ratio = ID_{50} with antagonist/ID_{50} without antagonist, against −log antagonist concentration (M).[13] The pA_2 values and the slopes of the pA_x plots were compared by the analysis of variance.

Results

Inhibition of Morphine Analgesia by Pretreatment with 5. The estimated ED_{50} values of morphine with and without pretreatment of animals with **5** are recorded in Table I. Pretreatment with the vehicle, 40% propylene glycol, had no effect on the ED_{50}. Pretreatment with 50 mg/kg of **5** 2 hr before the analgesic assay shifted the dose–response curve of morphine to the right in a parallel fashion and the ED_{50} was approximately doubled. The shift in the ED_{50} was still apparent when the animals were pretreated with **5** 6 hr before the assay. However, the effect of **5** may have been wearing off at this time since the difference in the ED_{50} between the groups that were pretreated 2 and 6 hr prior to the assay was of borderline significance as seen by the confidence limits which barely overlap. When the pretreatment dose of **5** was increased to 100 mg/kg, the ED_{50} of morphine appeared to have been shifted slightly more than that observed after pretreatment with 50 mg/kg of **5** but the increase was of borderline significance.

Suppression of Morphine Abstinence. All compounds were coded and tested blindly by the experimenter for their capacity to suppress withdrawal jumping in morphine-dependent mice. Morphine clearly suppressed withdrawal jumping in these mice, whereas neither saline nor propylene glycol did (Table II). Way, *et al.*,[14] have demonstrated an inverse relationship between the ED_{50} of naloxone to precipitate withdrawal jumping and the degree of physical dependence in mice. In the present experiments, morphine increased the amount of residual dependence as seen by the lower ED_{50} of naloxone as compared to the control. Anileridine (**1**) and derivatives **2**, **4**, and **5** significantly suppressed the withdrawal jumping and the treated animals exhibited relatively low ED_{50} values of naloxone. Compounds **6** and **7** did not inhibit withdrawal jumping and the residual dependence was not enhanced by these derivatives. The capacity of the anileridine derivatives to suppress withdrawal jumping and increase the degree of physical dependence appeared to correlate with the analgesic potency of the compounds.

Effect of *N*-Acylanileridines on the Electrically Stimulated Isolated Guinea Pig Ileum. The inhibitory effect of the anileridine congeners usually took 10–15 min to become maximal. With compound **2** the maximal effect took up to 30 min. In contrast, the maximal effect of morphine and anileridine was observed within 4 min. The anileridine derivatives were washed out of the bath immediately after noting the maximal inhibition and the ileal twitch height returned to control levels usually within 10 min. After exposure to the higher concentrations of **2** or **3** or after prolonged exposure to these compounds, it took a long time for the ileum to recover its original maximal twitch height in response to the electrical stimulus. After adding to the bath a concentration which inhibited the twitch height by about 80%, it required continual washing of over 2 hr before the original twitch height was restored after exposure to **3** and the twitch height never did recover after exposure to **2**. Even 3.5 hr of continual washing after exposure of the ileum to **2**, the response of the ileum was only 50% of the original twitch height.

The ID_{50} and pA_2 values are recorded in Table III. The ID_{50} values of the anileridine compounds ranged from 4 to 40 times that of morphine and none including anileridine was as potent as morphine in inhibiting the ileal twitches. Inspection of the pA_2 values reveals that all the *N*-acylanileridines have pA_2 values similar to that of morphine–naloxone indicating that all the compounds were probably interacting with morphine receptors. The pA_2 value derived from compound **2** and naloxone was difficult to estimate because **2** had a long duration of action in spite of repeated washings and the ileal response became erratic after the compound was introduced into the bath. If naloxone inhibited the activity of the anileridine compounds in a competitive manner, the pA_x plot should give a straight line with a slope of unity.[13] In the present study, the slopes of all the pA_x plots were straight, did not differ from each other and were very close to the theoretical value of 1.0.

The alkylating anileridine derivatives were next tested to see whether or not they antagonized the action of morphine. A dose–response curve for morphine was first de-

Table II. Single-Dose Suppression Test in Morphine-Dependent Mice

Test drug and dose[a]	ED_{50} of drug, mg/kg[b]	No. of animals	Mean no. of withdrawal jumps per mouse		Wilcoxon analysis for paired difference[c]	Mean ED_{50} of naloxone
			Before	After		
Saline		12	68.5	61.8	NS	1.25
40% propylene glycol		8	66.0	68.5	NS	1.36
Morphine, 10 mg/kg	5.5	6	86.6	8.0	<0.01	0.33
1, 25 mg/kg	4.4	8	70.1	1.2	<0.01	0.32
2, 24 mg/kg	24.0	8	60.1	0	<0.01	0.28
4, 100 mg/kg	46.5	6	73.7	6.6	≦0.05	0.67
5, 50 mg/kg	21.5	15	58.2	13.6	<0.01	0.38
6, 20 mg/kg	>40	6	49.6	48.9	NS	1.30
7, 20 mg/kg	>40	6	48.0	46.0	NS	1.04

[a]The dosages of the test drugs were greater than ED_{90} doses except with compounds **2**, **6**, and **7** where toxicity precluded usage of such doses. [b]The ED_{50} values for analgesia were taken from a previous communication[2] and listed here for ease of comparison and discussion. [c]NS = not significant ($p > 0.05$).

Table III. Inhibitory Effect of *N*-Acrylanileridines on the Coaxially Stimulated Isolated Guinea Pig Ileum

Compd	ID_{50} ($\times 10^7$ *M*) ± S.E.[a]	pA_2 ± S.E.[a,b]	Slope of pA_x plot ± S.E.[a,b]
Morphine	1.06 ± 0.20	8.38 ± 0.07	1.12 ± 0.08
1	4.09 ± 0.78	8.25 ± 0.11	1.01 ± 0.06
2	7.25 (6.55, 7.95)	*c*	*c*
3	7.69 ± 1.72	8.01 (7.96, 8.05)	0.96 (0.95, 0.97)
4	20.10 ± 3.99	8.16 (8.05, 8.27)	0.93 (0.92, 0.94)
5	6.37 ± 0.92	8.49 ± 0.13	1.27 ± 0.20
6	22.47 ± 10.01	8.26 ± 0.17	1.01 ± 0.17
7	38.6 (30.0, 47.2)	*d*	*d*
8	6.46 ± 1.06	8.44 ± 0.46	0.74 ± 0.07
9	29.33 ± 6.11	7.81 (7.53, 8.09)	0.98(1.09, 0.87)
10	34.4 (32.2, 36.6)	7.92 (8.21, 7.62)	0.89 (0.77, 1.00)

[a]These values are mean ± S.E. of three to four ilia. Where the number of ilia is less than three, the individual values are listed in parentheses. [b]Analysis of variance of the data revealed that none of the values differed significantly from one another. [c]Unable to determine (see text for explanation). [d]Insufficient amount of compound to make determination.

termined. The bathing medium was then replaced with solution containing the various *N*-acylanileridines and the determination of the morphine dose-response curve repeated. Usually three different concentrations of the anileridine derivatives were used and none of the concentrations exceeded the ID_{60}. These determinations usually took 3 hr; thus, the compounds were in contact with the ileum for that length of time. At the end of this period, bathing solution without anileridine compounds was added to the bath and the dose-response curve of morphine was redetermined.

The inhibition of the twitch caused by the exposure of the ileum to morphine together with compounds **4**, **5**, **6**, or **7** was additive. The dose-response curve of morphine was unaffected when it was determined in the presence of the above anileridine congeners. In these cases, the per cent inhibition was based on the new steady-state twitch height in the presence of the anileridine derivatives. When the compounds were removed from the bathing medium, the twitch height returned to the original normal height within 10 min. When the morphine dose-response curve was redetermined after this time, the curve was again unaffected indicating that there was no long-lasting effects of these anileridine congeners. Compounds **2** and **3** could not be tested for antagonism because the response of the ileum became erratic and unpredictable after long exposure to these derivatives.

Discussion

In the analgesic study, the alkylating anileridine derivative **5** shifted the morphine dose-response curve parallelly to the right as though **5** was inhibiting the analgesic action of morphine competitively. Also, there was no indication that the slope of the curve would change as one might expect if the analgesic receptors were being alkylated. The fact that doubling the dose of **5** did not cause a substantial further shift of the morphine dose-response curve indicates that the antagonism is not a simple competitive one. If the antagonism was competitive, the ED_{50} of morphine after doubling the dose of **5** is predictable from theoretical pA_x plots.[15] The observed experimental ED_{50} value of 12 mg/kg falls short of the theoretical value of about 16 mg/kg. A further increase of the dose of **5** would have aided in clarifying the type of antagonism observed here but, unfortunately, the limit of solubility and short supply of **5** precluded further investigation. Although the antagonism observed with **5** does not appear to be a noncompetitive, irreversible type, the observation that the antagonism lasts over 6 hr suggests that **5** has a high affinity for the receptors.

In the single-dose suppression test, **5** along with **2** and **4** significantly suppressed morphine abstinence suggesting that these congeners may have dependence liability. Usual narcotic antagonists such as naloxone and nalorphine precipitate an increase in the number of withdrawal jumping and markedly decrease the residual degree of physical dependence.[7] However, with **5**, **2**, and **4**, there was no indication of either an increase in withdrawal jumping even after 2 hr of observation or an elevation of the naloxone ED_{50} for precipitated jumping. Thus in this test, these anileridine compounds act like narcotic agonists without visible antagonistic activity. Those compounds which failed to show analgesic effects such as **6** and **7** also failed to suppress morphine abstinence.

In the guinea pig ileum, the ID_{50} observed for morphine was similar to those reported by others.[3,12] Anileridine (**1**) and its derivatives all appeared to interact with receptors similar to those for morphine as seen by the similari-

ty among the pA_2 values. Compound **3** and especially **2** exhibited a prolonged inhibitory action after the higher concentrations and the normal response to electrical stimulation was not restored even after several hours of continual washing. This could be interpreted to mean that long-lasting alkylation may have occurred but the binding may not be specific. The other anileridine derivatives displayed varying degrees of activity with the ID_{50} ranging from 4 to 40 times that of morphine but the activities did not appear to correlate well with the previously reported analgesic ED_{50} values[1] of these compounds. Paton[2] first suggested that in a series of narcotic analgesics there was a correlation between the analgesic potencies in man and the capacity to inhibit the electrically stimulated ileum. Several investigators have since shown a good correlation between inhibitory potencies in the ileum and the analgesic potencies in man[3,4] and in animals.[4,16] Analysis of our data, however, revealed a coefficient of determination (r^2) of only 0.18 with a correlation coefficient (r) of 0.43 between analgesic and ileal inhibitory potencies. Such correlations may be difficult if agonists also possess antagonistic properties. Gyang and Kosterlitz[3] found that certain agonists which are classified as narcotic-antagonist analgesics such as cyclazocine did not fit well into this type of correlation.

None of the anileridine derivatives tested exhibited any antagonism or blockage of the action of morphine in the ileal preparation. The fact that **5** did not show any antagonism is interesting in view of its antagonizing properties against morphine analgesia. This observation together with the comparison of the ID_{50} values may indicate that central analgesic receptors may differ from those in the ileum. Several other differences in the two types of receptors have already been pointed out.[17,18]

It is conceivable that the antagonism of morphine analgesia observed with **5** may actually be some form of acute tolerance since **5** appears to be an agonist without antagonistic properties in the dependent mice and guinea pig ileum. This possibility is unlikely since an acute pretreatment of mice with $>ED_{99}$ doses of other narcotic agonists such as morphine, levorphanol, or methadone does not alter the normal dose–response curve or ED_{50} of these analgesics.[19,20]

In conclusion, among the *N*-acylanileridines, **5** appeared to be the only compound capable of antagonizing morphine analgesia. The data do not permit us to conclude the type of antagonism displayed by **5** but it is not a simple competitive or noncompetitive type. The duration of the antagonism suggests that whatever type of interaction **5** has with the receptors, it is a fairly stable one and may be slowly reversible. Further indication of the strong affinity is the observation that **5** is more difficult to wash off than levorphanol, a potent narcotic agonist, when they are bound to the stereospecific opiate binding material described by Pert and Snyder[21] (Snyder, personal communication). In other systems such as the suppression test and isolated ileum, **5** acted like a pure agonist with no apparent antagonistic properties.

Our success in obtaining a fairly long-lasting analgesic antagonist in the anileridine series has prompted us to synthesize and investigate a series of 3-hydroxymorphinan compounds which would be expected to have much more specific receptor affinity. These studies are now in progress.

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