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Thiazine Dye Antagonism of Opioid Lethality in Mice

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Abstract □ The thiazine dyes, methylene blue and tolonium chloride, were administered subcutaneously alone and in combination to adult male mice in low (0.1 mg) and high (1.0 mg) doses 5 min prior to lethal intraperitoneal doses ($LD_{99.5}$) of morphine, codeine, meperidine, levorphanol, methadone, levopropoxyphene, and propoxyphene. Survivors were recorded at 2- and 24-hr intervals after each opioid challenge. Tolonium pretreatment (0.1 mg) significantly increased the number of survivors that had received lethal doses of morphine and propoxyphene in the 2-hr phase in contrast to methylene blue pretreatment at either dosage level which failed to protect animals against opioid lethality in either the 2- or 24-hr phase. At both the 2- and 24-hr intervals, the low-dosage dye combination (0.1 mg of each dye) significantly protected the animals from lethal doses of morphine, levorphanol, and methadone. The low-dosage dye combination shortened the duration of sleep induced by hexobarbital (100 mg/kg), presumably through inhibition of CNS depression.

Keyphrases □ Thiazine dyes—antagonism of opioid lethality, mice □ Methylene blue—antagonism of opioid lethality, mice □ Tolonium chloride—antagonism of opioid lethality, mice □ Opioids, lethal doses—protection by thiazine dyes

Earlier observations by Harpel and Mann (1, 2) revealed that certain thiazine dyes, methylene blue or tolonium chloride, when administered prophylactically in conjunction with either nalorphine or levallorphan, enhanced the antidotal capabilities of these specific inhibitors against toxic doses of propoxyphene hydrochloride in mice. Similarly, in a pilot study employing methadone hydrochloride in mice, the preadministration of these thiazine dyes alone reduced opioid lethality. Accordingly, the purpose of this investigation was to ascertain the antidotal effectiveness of low (0.1 mg) and high (1.0 mg) doses of methylene blue and tolonium chloride, when administered singly or in an equal dosage combination, prior to lethal doses of several opioid[1] agents in mice. It was also anticipated that insight into the mechanism and extent of protection might be realized by testing the influence of these thiazine dyes upon hexobarbital sleeping time.

EXPERIMENTAL

Adult male mice[2], weighing between 20 and 25 g, were used. Before treatment, the animals were caged in groups of 50 for several days with free access to laboratory chow[3] and water. Immediately prior to experimentation, the animals were placed singly in stainless steel, wire-mesh cages without food and water. In the toxicity studies, however, food and water were provided for mice that were alive 2 hr after opioid injections.

Immediately before each day's testing, drug solutions[4] were prepared with water distilled in this laboratory, with the exception of methadone hydrochloride, which was procured from the manufacturer in vials (10 mg/ml, calculated as the salt).

The opioid drugs and their concentrations were: morphine sulfate, 6.0%; codeine sulfate, 2.0%; meperidine hydrochloride, 2.0%; levorphanol tartrate, 1.6%; methadone hydrochloride, 1.0%; levopropoxyphene hydrochloride, 2.0%; and propoxyphene hydrochloride, 2.0%. The thiazine dyes, methylene blue and tolonium chloride, each were prepared as 0.1 and 1.0% solutions. Sodium hexobarbital was prepared as a 1.0% solution.

A precision timer was used to determine survival and sleeping times, with all measurements being recorded to the nearest minute.

To determine the effects of thiazine dyes, alone and in combination, on opioid lethality and hexobarbital sleeping time, the following pretreatment regimen was employed: (*a*) single-dye pretreatment—methylene blue (0.1 mg), methylene blue (1.0 mg), tolonium chloride (0.1 mg), and tolonium chloride (1.0 mg); and (*b*) multiple-dye pretreatment—methylene blue (0.1 mg) + tolonium chloride (0.1 mg), and methylene blue (1.0 mg) + tolonium chloride (1.0 mg).

Toxicity Studies—The intraperitoneal LD_{50}'s were determined for each opioid drug (Table I) according to the method of Litchfield and Wilcoxon (3). Based upon the data obtained, doses corresponding to 99.5% lethality were selected for use in experimentation.

Each test animal was weighed[5] to the nearest gram, and the proposed pretreatment regimen[6] was performed subcutaneously in the upper left abdomen. When two dyes were administered, the upper right abdomen was used as the second site of injection.

[1] For the purpose of this study, the following agents are designated as opioid drugs: morphine sulfate, codeine sulfate, meperidine hydrochloride, methadone hydrochloride, levorphanol tartrate, propoxyphene hydrochloride, and levopropoxyphene hydrochloride.

[2] Huntingdon Farms, HTF strain.
[3] Purina.
[4] In each case, the salt form was used for the calculation of drug concentration and dose.
[5] Triple-beam Ohaus balance, model 730.
[6] Pretreatment injections were each administered in a volume of 0.1 ml as fixed doses.

Table I—Lethal Dosages of Several Narcotic Agents in Mice after Intraperitoneal Injection

Compound	LD_{50}, mg/kg	$LD_{99.5}$, mg/kg	Slope Function[a]
Morphine sulfate	400	820	1.32
Codeine sulfate	143	245	1.23
Meperidine hydrochloride	104	230	1.36
Levorphanol tartrate	118	209	1.25
Methadone hydrochloride	29	62	1.34
Levopropoxyphene hydrochloride	114	185	1.21
Propoxyphene hydrochloride	118	200	1.24

[a] The fold change in dose required to produce a unit change in standard deviation in response along the line.

After 5 min, the animal was injected intraperitoneally with a lethal dose of the opioid and the time of injection was recorded. The survivors, defined as animals that were alive at 2 and 24 hr, were appropriately recorded.

To maintain the 5-min interval between dye and opioid treatments, the number of animals dosed at one time never exceeded 12; at least two of the mice received subcutaneous injection(s) of water (0.1 ml) instead of the dye injection(s) and served as controls.

Animal groups receiving dye–opioid treatments were compared with the proper water-treated controls with respect to incidence of survival. Statistical significance was determined by the use of the χ-square distribution (corrected for continuity). Probability values of 0.05 or less were considered to be significant.

Sleeping Time Studies—Each mouse was weighed and pretreated according to the previously described procedure. Sodium hexobarbital (100 mg/kg ip) was administered 5 min later and the time of injection was noted. The animal was returned to its individual cage and the time of loss of the righting reflex, as well as its subsequent return, was recorded. Sleeping time was considered to be the interval between loss and return of the righting reflex.

To monitor sleeping time properly, the number of animals under observation at a given time never exceeded eight, half of which received injection(s) of water instead of dye(s) and served as controls. In every case, a dye-treated animal was associated with a control. Only control animals that were obtained during the accumulation of a particular dye-treated group were used for comparison with the treated group.

Dye-treated groups were compared with their corresponding controls with respect to duration of sleep. Significance was determined by use of the Student t distribution at the 95% confidence level.

RESULTS

Toxicity Studies—The 2- and 24-hr survival values for each opioid control group are listed in Table II. In each tabulation, the incidence of survival was not greater than 11.1%. The onset of symptoms developed within 5 min from the start of opioid treatment and was characterized by ataxia, a Straub-tail reaction, intermittent convulsions, and prostration, followed by the cessation of respiration. The convulsant episodes were strychnine-like, because they occurred spontaneously and could be initiated by tactile stimuli. The evaluated agents that are considered to be the most potent analgesics clinically (morphine, methadone, and levorphanol) showed less convulsant activity than the weaker analgesics (levopropoxyphene, propoxyphene, meperidine, and codeine). Preadministration with low (0.1 mg) or high (1.0 mg) doses of methylene blue was incapable of protecting the animals from mortality in both the 2- and 24-hr phases (Table II).

Subcutaneous pretreatment with tolonium chloride (0.1 mg) increased the incidence of survival at the 2-hr interval in mice subjected to lethal amounts of either morphine or propoxyphene (Table II). This regimen, however, failed to protect mice against the lethality caused by codeine, meperidine, levorphanol, methadone, and levopropoxyphene after 2-hr.

In the more stringent 24-hr survival phase, tolonium chloride (0.1 mg) was unable to alter significantly the lethality produced by any opioid. Pretreatment with this dye appeared to antagonize morphine lethality (survival = 20%), but comparison with the water-treated controls showed the difference to be nonsignificant ($p = 0.08$).

Opioid antagonism did not occur upon administration of high doses (1.0 mg) of tolonium chloride in the 2- and 24-hr phases.

In both the 2- and 24-hr intervals, the low-dosage dye combination (0.1 mg each) was capable of protecting against fatal doses of morphine, levorphanol, and methadone (Table II). No significant antidotal activity was demonstrated by the multiple-dye pretreatment in the high dosages at either interval.

Sleeping Time Studies—From a total of 216 mice, data were compiled to give mean sleeping time values for each of the six control groups obtained during the accumulation of the various dye-treated groups. The administration of sodium hexobarbital (100 mg/kg ip) elicited hypnosis which lasted approximately 40–60 min.

The preadministration of methylene blue and tolonium chloride (0.1 mg each) was found to decrease significantly the duration of sleep induced by sodium hexobarbital (Table III). The high dose of methylene blue appeared to prolong sleeping time; but when compared with the water-treated controls, the difference was nonsignificant ($p = 0.08$).

DISCUSSION

Isomerism appears to be a factor in determining whether tolonium chloride can antagonize opioid lethality. At the 2-hr inter-

Table II—Effect of Subcutaneous Pretreatment with Methylene Blue and Tolonium Chloride, Administered Singly or in Combination, on Incidence of Survival at 2- and 24-hr Intervals following Intraperitoneal Injection of Lethal Amounts of Opioid Agents

	Survival, %															
	Water[a], 0.1 ml		Methylene Blue[b]				Tolonium Chloride[b]				Water and Water[a], 0.1 ml Each		Methylene Blue and Tolonium Chloride[b]			
			0.1 mg		1.0 mg		0.1 mg		1.0 mg				0.1 mg Each		1.0 mg Each	
Opioid	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
Morphine	5.6	2.8	10.0	6.7	13.3	13.3	36.7[c]	20.0	13.3	13.3	2.8	0.0	40.0[c]	16.7[c]	6.7	0.0
Codeine	2.8	2.8	6.7	6.7	0.0	0.0	6.7	6.7	3.3	3.3	0.0	0.0	6.7	3.3	10.0	6.7
Meperidine	0.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	3.3	0.0	5.6	0.0	13.3	3.3	6.7	3.3
Levorphanol	5.6	5.6	6.7	0.0	0.0	0.0	13.3	13.3	10.0	6.7	11.1	2.8	36.7[c]	23.3[c]	6.7	3.3
Methadone	8.3	5.6	10.0	6.7	3.3	0.0	6.7	6.7	20.0	10.0	8.3	0.0	26.7[c]	20.0[c]	0.0	0.0
Propoxyphene	5.6	0.0	10.0	6.7	13.3	6.7	30.0[c]	6.7	3.3	3.3	8.3	2.8	13.3	0.0	10.0	3.3
Levopropoxyphene	8.3	0.0	0.0	0.0	6.7	0.0	3.3	0.0	13.3	3.3	5.6	0.0	3.3	0.0	0.0	0.0

[a] Groups of 36 animals. [b] Groups of 30 animals. [c] Compared to water or water and water, χ-square test (corrected for continuity), $p < 0.05$.

Table III—Effect of Subcutaneous Pretreatment with Methylene Blue and Tolonium Chloride, Administered Singly or in Combination, on Duration of Sleep following Intraperitoneal Injection of Sodium Hexobarbital

Treatment[a]	Dose, mg	Mean Sleeping Time, min	*SEM*
Water	—	56.6	2.59
Methylene blue	0.1	52.8	3.00
Water	—	51.5	3.10
Methylene blue	1.0	58.4	2.73
Water	—	54.4	2.19
Tolonium chloride	0.1	52.4	3.68
Water	—	50.0	2.84
Tolonium chloride	1.0	49.9	2.26
Water (two injections)	—	51.6	3.63
Methylene blue and tolonium chloride	0.1 (each)	40.5[b]	2.39
Water (two injections)	—	47.8	2.52
Methylene blue and tolonium chloride	1.0 (each)	46.9	2.19

[a] Water (one or two injections) or thiazine dyes (alone or in combination) administered to groups of 36 animals 5 min prior to sodium hexobarbital (100 mg/kg ip). [b] Compared to water, Student t distribution, $p < 0.05$.

val, lethality from propoxyphene was inhibited, whereas no such effect was seen against levopropoxyphene. This distinction between tolonium antagonism against the *d*- and *l*-isomers of propoxyphene was not apparent at 24 hr. Therefore, tolonium might possess early or transient antagonistic activity that operates mainly against one of a pair of isomers.

Lethal amounts of morphine, methadone, and levorphanol appeared to produce less convulsant activity than the weaker analgesics studied. This observation is in complete agreement with that of Johannesson (4), who noted that toxic doses of morphine in rats caused a flaccid type of respiratory paralysis while death by codeine was always associated with convulsions. Therefore, the possibility was entertained that the thiazine dyes may act in combating narcotic poisoning through the inhibition of opioid-induced depression of the central nervous system (CNS).

In the sleeping time studies, only the low-dosage dye combination significantly reduced the duration of hexobarbital hypnosis. Based upon this finding, it is suggested that protection against the lethality of the potent narcotic analgesics occurred principally through dye interference with opioid-induced CNS depression.

When administered alone or in combination at the high-dosage level, methylene blue and tolonium chloride were unable to prevent opioid lethality. High doses of tolonium chloride are known to decrease the survival time in mice following lethal amounts of propoxyphene, presumably through enhancement of convulsant activity (2). The failure of methylene blue to antagonize opioid lethality may be due to the ability of the dye to produce CNS depression in high doses (5).

Because the precise way in which the opioid drugs act to produce their lethality still is unknown, an attempt to propose a mechanism of inhibition by the dyes becomes futile. Nevertheless, it is of considerable interest to note that morphine, the prototype of narcotic analgesics, and the thiazine dyes, methylene blue and tolonium chloride, show certain dissimilarities with respect to their actions within the CNS. For example, the inhibition of oxidative deamination and the enhancement of dehydrogenase enzymes represent dye actions that are exactly opposite to those of morphine (6, 7). These actions, either of a specific or nonspecific nature, may play a part in the inhibition demonstrated by the "Janus"-type dyes.

SUMMARY

1. The subcutaneous administration of tolonium chloride at the lower dosage level (0.1 mg) significantly reduced the lethality of the opioids, morphine and propoxyphene, at 2 hr after treatment.

2. At the 24-hr interval, toxicity by the clinically potent narcotic analgesics (morphine, levorphanol, and methadone) was antagonized by the low-dosage combination of methylene blue and tolonium chloride (0.1 mg each).

3. Hexobarbital sleeping time was significantly decreased by the low-dosage, multiple-dye pretreatment, presumably through interference with CNS depression.

4. The mechanism by which the thiazine dyes antagonize opioid lethality remains unknown.

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