

Screening of N-Benzoyl Isoleucine Methyl Ester (N-bime) for anti-inflammatory analgesic activity and toxicity profile in animals

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ABSTRACT

Background: Pain and inflammation are the basic processes involved in the pathogenesis of many diseases. Non-steroidal anti-inflammatory drugs are often used to treat rheumatic diseases. The study compound N-Benzoyl Isoleucine Methyl Ester (N-bime) is a newly synthesized propionic acid derivative by National Chemical Laboratory, Pune. Since the biological data of this compound is not available, the present study has been planned to screen this compound for anti-inflammatory, analgesic activity and its toxicity profile in animals.

Methods: Single dose toxicity study was carried out in rats. Anti-inflammatory activity was tested by Rat Hind Paw Oedema and Cotton Pellet Implantation method. For Analgesic activity, Acetic acid induced writhing and Tail Pinch method was used. Yeast induced Pyrexia was used for evaluation of anti-pyretic activity. Ibuprofen was the positive control. Data are presented as mean±SEM. Statistical analysis was performed by analysis of variance and students unpaired 't' test.

Results: The test compound N-bime did not show any apparent adverse effects or mortality in the dose range 1mg - 500mg / 100gm body weight in animals. It showed better anti-inflammatory actions in higher doses as compared to Ibuprofen ($p < 0.05$). In acetic acid induced writhing test N-bime offered better protection against writhes, than Ibuprofen. But, both failed to demonstrate analgesic activity in the Tail Pinch method. N-bime showed a gradual decrease in temperature in the anti-pyretic test ($P < 0.001$).

Conclusions: The present study indicates that N-bime does possess anti-inflammatory, analgesic and weak anti-pyretic properties like the NSAIDs. It has proved to be safe in the dose range of 1mg - 500mg / 100gm body weight in rats and mice.

Keywords: Anti-inflammatory activity, Analgesic activity, Anti-pyretic activity, N-Benzoyl Isoleucine Methyl Ester (N-bime), Toxicity profile

INTRODUCTION

Pain and inflammation are the basic processes involved in the pathogenesis of many diseases and relief from the distress and discomfort caused by them is most important for the patient. Pain is an important manifestation of inflammation, as inflammatory cytokines and mediators sensitize primary afferent neurons.¹ Inflammation is mediated by the release of autacoids and a number of mediators such as interleukins 1, 2 and 3, interferon, platelet-derived growth factor etc. Rheumatoid arthritis

(RA) is a chronic, complex, heterogeneous, and widely known autoimmune disease (AD). It is characterized by the presence of long-standing inflammation of the diarthrodial joints resulting in symmetric polyarthritis and synovial membrane hypertrophy with progressive damage to the joints, bone and cartilage destruction, and deformity.² Non-steroidal anti-inflammatory drugs (NSAIDs) such as the propionic acid derivatives which include ibuprofen, naproxen, flurbiprofen and ketoprofen etc. are often used to treat rheumatic diseases and are capable of suppressing the signs and symptoms of inflammation. But, they cause numerous side effects

particularly to the gastro intestinal tract and most of them have a short duration of action.³ Therefore, there is a search for a safer and longer acting agent.

The compound N-benzoyl isoserine methyl ester (N-bime) was a newly synthesized propionic acid derivative by National Chemical Laboratory, Pune. N-bime [(2R,3S)-(-)-N-Benzoyl-3-phenyl isoserine methyl ester], is the synthetic side chain of the anti-cancer drug Taxol molecule present at C13.⁴ The chemical structure of N-bime resembled that of propionic derivative (NSAIDs) and it is also essential of its anti-tumour activity.⁵ Until now, there is no biological data available about the compound and hence, this study was planned to perform acute toxicity profile of (N-bime) in animals so as to determine three likely to be safe and effective doses, which can be employed for further studies. So, the aim of this study was to screen N-bime for anti-inflammatory, analgesic and anti-pyretic activity in animals and to compare anti-inflammatory, analgesic and anti-pyretic activity if any of N-bime with Ibuprofen and to study its toxicity profile.

METHODS

Acute toxicity test

Single dose toxicity study was carried out in male albino rats weighing 150-200gm and mice weighing 18-22gms, in a dose range of 1mg / 100gm upto 500mg / 100gm. body weight. N-bime did not show any apparent adverse effects or mortality when observed for 7 days.⁶ Since the extrapolated dose of ibuprofen from commonly used human doses to animal dose is approximately 20mg / 100gm., three doses of N-bime 10, 20 and 40mg / 100gm were selected for further studies.

For the entire study, the animals (rats and mice) were divided in 5 groups of 10 animals each and were fasted overnight one day before and for 6 hours during study period. The route of administration was oral. Gum acacia (2 ml 6% solution) was the control group, ibuprofen (20mg / 100gm body weight) was used as positive control. N-bime was given in 10mg, 20mg and 40mg / 100gm body weight. Animals were observed every 30 minutes for first 2 hours then hourly for 6 hours and later daily for 7 days.

Anti-inflammatory activity

Acute inflammation: Rat hind paw method.⁷ Male albino rats received the test compound by stomach tube and after 1 hour, 0.2 ml of 1% carrageenin solution was injected in the right hind paw and the same volume of normal saline was injected in the left hind paw. The volume of the paws was measured before and upto 6 hours after the subplantar injection at an hourly interval using a plethysmograph.⁸

The hind paw % oedema was calculated by:

$$\% \text{ Oedema} = \left[\frac{(R - r)}{r} - \frac{(L - l)}{l} \right] \times 100$$

Where,

R= Right hind paw volume
r= Right hind paw initial volume
L= Left hind paw volume
l= Left hind paw initial volume

Percentage odema was calculated for every hour and a graph was plotted and total area under the curve (AUC) was calculated by the trapezoidal rule.⁹

Chronic inflammation

Cotton pellet implantation method¹⁰

Male albino rats weighing 150-200gms were anaesthetized with ether vapours. By dissecting subcutaneous tissue of both the axillae and groins, four pockets were made into which sterilized cotton pellets weighing 10mg each were inserted and closed by taking sutures. The same day onwards respective groups received the drug orally. On the fifth day, the animals were sacrificed and the pockets redissected and pellets removed. After removal of fat and extraneous tissue, the pellets were dried overnight at 60°C in a hot air oven and weighed. The weight of granuloma was calculated by measuring the difference in weights of pellets before and after drug administration.

Analgesic activity

Acetic acid induced writhing

Male albino mice 18-22gm in weight were used.¹¹ After 60 minutes of oral administration of N-bime, acetic acid (0.2ml of 0.6% v/v solution) was injected intraperitoneally in each mouse. The number of writhes, (stretch, torsion to one side, drawing up of the abdomen and opisthotonus, so that the belly of the mouse touches the floor) in each animal were recorded over a period of 30 minutes.

Tail pinch method

Male albino rats weighing 150-200gm, making an attempt to remove the pinch-cork within 30 seconds were selected.¹² N-bime was given orally and the pain threshold was determined by applying the pinch cork (applied 2 cm away from the base of the rats tail), before, 30 and 60 minutes after drug administration. Pain threshold is the time interval between the application of the pinch-cork and the response of the animal (first continuous attempt of removal of the pinch-cork).

Anti-pyretic activity

Yeast induced pyrexia male albino rats weighing 180-200gms.¹³ were used. Fever was induced by 20ml/kg. of a 20% aqueous suspension of Baker's yeast which was injected subcutaneously in the back below the nape of the neck. The animals were fasted for the duration of the experiment (approximately 24 hours). A rectal

thermometer (scale 28 to 42°C) was used to record rectal temperature of the animal, the probe was inserted 4cm into the rectum for 45 seconds. Control temperatures were taken 24 hours after the yeast injection to determine the pyretic response to yeast, which ranged between 1.5-2°C above the pre-yeast injection temperature. Fevered rats were dosed orally with the drug at the 23rd hour post yeast and temperatures were recorded at an interval of 30 minutes for 6 hours. Temperature index (T.I.) was calculated.

$$T.I. = \sum (\text{Observed reading} - \text{Initial temperature})$$

Statistical analysis

Data are presented as means±SEM. The statistical analysis was performed by analysis of variance and students unpaired ‘t’ test to compare the treatment effects.¹⁴

RESULTS

In this study, various models were used to study the effect of N-bime on acute and chronic inflammation in different

animal groups. Table 1 shows carrageenin induced hind paw oedema in different groups of rats. In control group, there was a progressive increase in % oedema in the 3rd, 5th and 6th hour as compared to the positive control group.

N-bime in all the three doses decreased % oedema significantly (p<0.05), as compared to the control, the effect being maximum with the 40mg / 100gm. dose (onset at 3 hours). In higher doses (20 and 40mg /100gm) N-bime showed a significant decrease (p <0.05) in the AUC values as compared to the control (gum acacia) group but not with ibuprofen (positive control) group.

Chronic inflammation

Cotton implantation method. Table 2 shows cotton pellet granuloma in rats. The mean weight of cotton-pellet granuloma was 19mg N-bime did produce significant (p<0.001) reduction in weight of granuloma in all the three doses as compared to the control. At 40mg / 100gm. dose N-bime produced significant (p <0.001) reduction in weight of granuloma as compared to ibuprofen.

Table 1: Carrageenin induced hind paw oedema and area under the curve of % swelling in rats over 6 hours.

Groups (dose/100gm)	% Swelling at hours						AUC (% hours)
	1	2	3	4	5	6	
I: Gum acacia	10.47±3.57	19.39±5.41	29.55±4.81	39.92 ±6.55	52.22±7.78	65.59±7.63	36.6±1.64
II: Ibuprofen (20mg)	4.67±1.55	9.39 ±2.21	17.38*±2.28	31.56 ±5.59	29.34*±6.50	29.00**±7.58	21.3±1.64
III: N-bime (10mg)	14.31**±8.92	17.46±3.37	16.71*±1.87	20.14**±5.06	27.07**±1.55	29.11***±5.10	21.5±0.55
IV: N-bime (20mg)	8.66±8.03	15.81±0.87	12.32**±1.01	10.46***°±1.21	11.30***°±2.04	13.72***±6.30	13.3*±3.13
V: N-bime (40mg)	16.44**±6.74	9.56±8.35	9.47**±8.27	8.62***°±7.42	11.90***°±4.13	16.06***±1.73	12.7*±0.19

Values: Mean±SEM
 Ten animals in each group.
 Comparison with Group I : *P <0.05, **P < 0.01, ***P <0.001
 Comparison with Group II : ° P <0.05, °° P <0.01

Table 2: Cotton pellet granuloma in different groups of rats.

Groups (dose/100gm)	I: Gum acacia	II: Ibuprofen (20mg)	III: N-bime (10mg)	IV: N-bime (20mg)	V: N-bime (40mg)
Weight of pellets (mgms.)	19.00±0.38	17.25±1.10	14.42*±1.18	13.92*±0.22	10.42*°±0.08

Values: Mean ± SEM.
 Ten animals in each group.
 Comparison with Group I: *p <0.001
 Group II : °p <0.001

Table 3: Acetic acid induced writhing in different groups of mice.

Groups (dose /100gms)	I: Gum acacia	II: Ibuprofen (20mg)	III: N-bime (10mg)	IV: N-bime (20mg)	V: N-bime (40mg)
Number of writhes in 30 minutes	36.33±2.92	2.17*±0.60	2.00*±1.61	0.33*°±0.33	1.17*±0.60

Values: Mean±SEM
 Ten animals in each group.
 Comparison with Group I : *p <0.001, Group II : °p <0.05

Analgesic activity

Acetic acid induced writhing method. Table 3 shows the results for acetic acid induced writhing method. In the control group the number of writhes induced by acetic acid were 36.33 (mean). Ibuprofen produced a significant (p <0.001) as compared to the control. N-bime did produce significant (p <0.001) reduction in number of writhes in all the three doses as compared to the control. Only in the 20mg / 100gm dose did it produce significant (p <0.05) reduction in number of writhes as compared to ibuprofen. In the 20mg / 100gm. dose N-bime also produced a high percentage of protection against writhing as compared to the control and ibuprofen.

The analgesic activity of ibuprofen is also evident in Table 4 as compared to the control. N- bime also produced a high percentage of protection against writhing as compared to

the control and ibuprofen. In the 20mg / 100gm dose N-bime offered a higher percentage of protection than in the 10mg and 40/ 100gm doses.

Table 4: % Protection against acetic acid induced writhing in different groups of mice.

Groups (dose/100gms)	II: Ibuprofen (20mg)	III: N-bime (10mg)	IV: N-bime (20mg)	V: N-bime (40mg)
I: Gum acacia	94.04	94.49	99.08	96.79
II: Ibuprofen (20mg)		7.69	84.62	46.15

Ten animals in each Group.

$$\% \text{ Protection} = 100 - \left[\frac{\text{no of writhes in test}}{\text{no of writhes in control}} \right] \times 100$$

Table 5: Tail pinch method- pain threshold (in seconds) in rats.

Pain threshold (seconds)	Groups				
	I: Gum acacia	II: Ibuprofen (20mg)	III: N-bime (10mg)	IV: N-bime (20mg)	V: N-bime (40mg)
Before	2.31±0.26	4.27±0.89	2.41±0.73	3.22±0.77	2.17±1.23
30 Minutes	0	0	0	0	0
60 Minutes	0	0	0	0	0

Values: Mean±SEM
Ten animals in each group

Table 6: Rectal temperature in different groups of Yeast Induced Pyrexia Rats.

Groups (dose/100gm)	Initial Temp °C	Time post drug (hours)						Temp. Index (T.I.)
		1	2	3	4	5	6	
I: Gum acacia	38.53±0.03	39.33±0.10	39.23±0.10	39.17±0.03	39.13±0.03	39.03±0.03	38.63±0.20	6.70±0.91
II: Ibuprofen (20mg)	38.37±0.10	37.53**±0.12	37.43**±0.13	37.43**±0.13	37.43**±0.13	37.43**±0.13	37.43**±0.13	-10.6**±1.87
III: N-bime (10mg)	39.00±0.03	39.23 ^{ooo} ±0.10	38.97* ^{ooo} ±0.03	38.93* ^{ooo} ±0.10	38.87* ^{ooo} ±0.03	38.77* ^{ooo} ±0.03	38.77 ^{ooo} ±0.03	-0.06* ^{ooo} ±0.03
IV: N-bime (20mg)	38.8±0.20	38.70* ^{ooo} ±0.11	38.57* ^{ooo} ±0.12	38.53* ^{ooo} ±0.13	38.43* ^{ooo} ±0.13	38.30* ^{ooo} ±0.10	38.17 ^{ooo} ±0.10	-4.0* ^{ooo} ±1.8
V: N-bime (40mg)	38.9±0.10	38.57* ^{ooo} ±0.03	38.47* ^{ooo} ±0.03	38.33* ^{ooo} ±0.10	38.13* ^{ooo} ±0.13	38.07* ^{ooo} ±0.10	38.00* ^{ooo} ±0.10	-7.29* ^{ooo} ±1.35

Values: Mean±SEM
Ten animals in each group.
Initial temperature: Temperature before administration of drug (23 hours post-yeast)
Comparison with Group I: *p <0.01, **p <0.001
Group II: °p <0.05, °°p <0.01, °°°p <0.001
Temperature Index = Σ (Observed reading – Initial temperature)

Tail pinch method. Effect of the drugs on pain threshold in rats is shown in Table 5. The pain threshold in control group was zero. Ibuprofen and N-bime (in all the three doses) did not produce an increase in the pain threshold.

Table 6 shows change in rectal temperature after drug administration in different groups of yeast induced pyrexia in rats. The control (gum acacia) group showed an increase in rectal temperature (39.33°C in the first hour) than the initial temperature throughout the study period of 6 hours.

Ibuprofen produced an immediate decrease in rectal temperature within one hour which persisted throughout the study period of 6 hours as compared to control ($p < 0.001$). N-bime however, showed a significant decrease ($p < 0.001$) in rectal temperature in all the three doses as compared to control. This decrease however, was very slow ($0.10^{\circ}\text{C}/\text{hour}$). The decrease in temperature was more and persistent with $40\text{mg}/100\text{gm}$ dose ($p < 0.001$). Ibuprofen caused more reduction in temperature than N-

bime ($p < 0.001$). In the control group, temperature index was 6.7. Both ibuprofen and N-bime (all the three doses) showed a significant ($p < 0.001$) decrease in temperature index as compared to control. However, reduction was greater with ibuprofen than with N-bime.

Results of the study have been summarized in Table 7.

Table 7: Results of various animal models used for Screening of N-bime at a glance.

Groups (dose/100mg)	Animal models				
	Acute inflammation	Chronic inflammation	Analgesic activity		Anti-pyretic activity
			Writhing	Tail pinch	
I: Gum acacia	-	-	-	-	-
II: Ibuprofen (20mg)	+++	-	+	-	++ immediate
III: N-bime (10mg)	+	+	+	-	+ slow
IV: N-bime (20mg)	++	+	++ °	-	+ slow
V: N-bime (40mg)	+++ over 6 hours	++ °	+	-	+ slow

No significant effect

+ Significant effect in comparison with control

° Significant effect in comparison with ibuprofen

DISCUSSION

Toxicity profile

The test compound N-benzoyl isoserine methyl ester (N-bime) was considered safe to be given in the dose range of $1\text{mg}-500\text{mg}/100\text{gm}$ body weight in rats and mice as it did not produce any toxic effects and nor was there any mortality over a period of 7 days. The doses 10mg , 20mg , and 40mg were selected for further studies.

Anti-inflammatory test

1. *Acute inflammation:* Carrageenin induced hind paw oedema in rats.

Gum acacia (control) did not produce any anti-inflammatory activity. Although ibuprofen produced a decrease in % oedema in the 3rd, 5th and 6th hour as compared to the control, it failed to produce a significant decrease in AUC as compared to the control (Table 1).

N-bime produced a significant decrease in % oedema in all the three doses and a significant decrease ($p < 0.05$) in AUC in higher doses (20mg and 40mg dose) as compared to the control, which could be due to its longer duration of action. The test can be divided into three phases of time: 0- 90 minutes; 90- 150 minutes and 150- 360 minutes, wherein different mediators of inflammation are released, they are

histamine and 5-hydroxy-tryptamine; kinins and prostaglandins.

Since the % oedema was well controlled by N-bime (especially in the higher doses) it could be assumed that the compound acts by inhibiting the synthesis of enzymes similar to the NSAIDs.

2. *Chronic inflammation:* Cotton pellet granuloma in rats.

As expected, in this test too, the control did not show any anti-inflammatory activity. Surprisingly, ibuprofen did not produce significant anti-inflammatory activity as compared to the control. (Table 2).

N-bime once again produced significant ($p < 0.001$) anti-inflammatory activity as compared to the control in all the three doses. However, it was only the $40\text{mg}/100\text{gm}$ dose that produced significant anti-inflammatory activity as compared to ibuprofen.

Thus, N-bime has shown activity in the acute and chronic models of inflammation. It has proved to possess better anti-inflammatory actions in the higher doses of 20 and $40\text{mg}/100\text{gm}$, than the $10\text{mg}/100\text{gm}$ dose.

Analgesic activity

1. *Acetic acid induced writhing:* The control did not show any analgesic activity. Once again, as expected ibuprofen

produced a significant ($p < 0.001$) analgesic activity as compared to the control (Table 3). It also offered a high percentage of protection against writhes (Table 4).

Although the test compound too produced a high % of protection against writhes and produced significant ($p < 0.001$) analgesic activity in all three doses as compared to the control, it did so better in the lower dose of 20mg / 100gm, than 40mg/100gm dose.

2. *Tail Pinch Method:* Since this method is used for screening opioid analgesics it was expected for ibuprofen not to produce any analgesic activity. The test compound failed to produce any analgesic activity indicating that its mechanism of action could be like that of ibuprofen.

Anti-pyretic test

Yeast induced pyrexia in rats: Control temperatures varied minimally, usually ranging between ± 0.2 of the mean control value for the study. Ibuprofen showed an immediate anti-pyretic activity within 30 minutes of administration (Table 6).

As compared to the control, the N-bime did possess significant ($P < 0.001$) anti-pyretic activity. However, there was a gradual decrease in rectal temperature showed by ibuprofen.¹³

Thus, the present study indicates that N-bime does possess anti-inflammatory, analgesic and weak anti-pyretic properties like the NSAIDs. It has proved to be safe in the dose range of 1mg-500mg / 100gm body weight in rats and mice.

CONCLUSION

The test compound, N-benzoyl isoserine methyl ester (N-bime) is a newly synthesized (by NCL, Pune) propionic acid derivative. Acute toxicity studies on this test compound did not show any apparent adverse effects or mortality in this dose range. Thus, to conclude, this study reveals anti-inflammatory, analgesic and anti-pyretic activity of N-bime in mice and rats in the doses of 10, 20 and 40mg / 100gm body weight. However, it is suggested that this being a preliminary study, further studies with this compound are needed for confirmation of these findings.

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