ARRIVE STATEMENT

1. **Title**: Amelioration of hepatotoxicity by bio-cleavable aminothiol chimeras of isoniazid: Design, synthesis, kinetics and pharmacological evaluation

2. **Abstract**: Pharmacological evaluation of synthesized prodrugs was performed on male Wistar rats in a twenty-one day study to assess the hepatoprotective potential. The alleviating effect of synthesized prodrugs as well as physical mixtures was evaluated for liver function markers, antioxidant markers, biochemical parameters and liver histopathology. Treatment with synthesized prodrugs showed prominent decrease in the extent and severity of hepatic damage with normal morphology. Prodrug of isoniazid with N-Acetyl cysteine (NI) exhibited superior abrogating potential compared to other prodrugs and physical mixtures because it was able to restore all the level of enzymes ALT, AST, SOD and GSHPx to desirable levels. All three groups administered with prodrugs exhibited normal hepatic morphology. Mild infiltration of inflammatory cells in liver was observed in the groups treated with physical mixtures. Out of three prodrugs, NI was selected for *in vivo* study. MGLS prodrug showed 52 % release of INH. *In vivo* studies on NI clearly indicates its activation by intestinal amidases.

3. **Background:** Animal studies are to be carried out, to check the effectiveness of synthesized prodrug. In the present study, the species are selected according to the standardized model approved by the scientific community. According to International convention it is necessary to include minimum 6-10 animals in each group to avoid biological variation and to generate sufficient data for statistical evaluation. In the present study six animals were taken in each group. Twenty-one days study for evaluation of hepatoprotective potential is standard model used by scientific community.

4. **Objective**: The present work aims at the rational design, synthesis, kinetic studies and pharmacological screening of hepatoprotective prodrugs of isoniazid (INH) with following objectives:

    a. To develop hepatoprotective chemical delivery systems for the selected drug.
    b. To compare the hepatoprotective potential of these prodrugs with INH.

5. **Ethical statement:** All the experimental procedures and protocols used for *in vivo* release studies were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/PCH/02/2016-17) and were in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India.

6. **Study design: Experimental design**

Table 1 No of animals used for pharmacological evaluation

Sr. No.	Groups	No of Animals
1.	Healthy	6
2.	Isoniazid	6
3.	NI	6
4.	MGI	6
5.	MI	6
6.	Physical Mixture-1	6
7.	Physical mixture-2	6
8.	Physical mixture-3	6
Total no of animals required=48		

7. **Experimental procedures**

➢ **Evaluation of hepatoprotective potential**

Table 2 Experimental procedure

Day 1	Animals will be divided into **08 groups**, each group consisting of six animals. All animals will be weighed. All the animals except the healthy group will be administered with respective doses of INH, prodrugs and physical mixtures calculated on equimolar basis. The healthy control group will be given saline throughout the 21 day study
Day 2	Doses will be administered orally to respective groups
Day 3-day 21	2nd day protocol continued
Day 22	All the animals will be examined for weight loss. Livers of the sacrificed animals will be removed and one part will be fixed in 10% buffered formalin and samples will be sent for microscopic examination and histopathology. Other part of Liver tissues will be washed with normal saline to remove any blood or clots, and will be homogenized on ice in Tris–HCl (5mmol/L containing 2mmol/L EDTA, pH 7.4). Aliquoted samples of the supernatants will be used immediately for the assays of superoxide dismutase (SOD), glutathione peroxidase (GSHPx), malonyl dialdehyde (MDA), ALT, AST, cholesterol and triglyceride.

Assessment of Quantifying Parameters:

- Liver function markers
- Antioxidant markers
- Biochemical parameters
- Histopathological studies

- ***In Vivo* hydrolysis kinetic study**
  - Male wistar rats weighing 180- 220gm will be fasted for 24 hr.
  - The animals were given drug solution in stipulated dose, the quantity depending on body weight of animal.
  - On the 0 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr , 10 hr and 24 hr of treatment 3ml of blood was withdrawn by retro-orbital puncture in EDTA coated tubes and was centrifuged at 5000 rpm at 0-5ºC for 10 minutes.
  - 0.1 ml of supernatant solution of centrifuged blood was added to eppendorf tube and 0.9 ml of methanol added to it for immediate plasma protein precipitation.
  - Solution was vortexed for 2 minutes and then centrifuged at 5000 rpm for 10 minutes at 0-5ºC in order to precipitate solid matter present in biological sample and other impurities.
  - Then 20 µl of supernatant will be injected in HPLC.

8. **Experimental Animals:**

   Male Wistar rats (180-200 g) were purchased from National Toxicology Center, Pune.
   Chemicals:

   Isoniazid, Anaesthetic ether, 10% buffered formalin.

9. **Housing and husbandary:** During the experiment, rats were housed in standard housing conditions like temperature of 25±1 ºC, relative humidity of 45%-55% and 12 h light: 12 h dark cycle. During induction animals had given free access to food pellets (Navmaharashtra Chakan Oil Mills Ltd., Sangli, India) and tap water *ad libitum* during the experiments. Each cage contained 6 rats with bedding prepared by husk.

10. **Sample size:** 48 rats used for carrying out pharmacological evaluation. Each group contained 6 rats such total 08 group prepared.(Table 2)
11. **Allocating animals to experimental groups:** The rats in groups were differentiated by marking head(H), body(B), tail (T), head-tail(HT) , body-tail (BT), none(N).
12. **Experimental outcomes**. Oral administration of INH for twenty-one days resulted in severe hepatotoxicity in rats. Whereas administration of prodrugs and physical mixtures for twenty-one days did not show signs of hepatotoxicity. All the animals were examined for liver function markers, antioxidant markers, biochemical parameters and liver histology. Livers of the sacrificed animals were removed and fixed in 10% buffered formalin and samples were sent for microscopic examination.

13. **Statistical methods:** All data were expressed as mean ± S.E.M.; (n refers to number of animals in each group). Statistical analysis was carried out using one-way ANOVA followed by the Dunnett's post- hoc test. Differences were considered at P <0.001–0.05.

    14-17. Result and discussion: The mitigating effect of synthesized prodrugs as well as physical mixtures was evaluated by estimating aminotransferases, SOD,

GSHPx, MDA, cholesterol and TG in Wistar rats. The hepatoprotective activity of prodrugs was compared with INH. Prodrugs-treated group showed notable reparation of INH-induced hepatotoxicity in Wistar rats as TG and cholesterol level decreased remarkably. Prodrugs were also found to restore the levels of antioxidant enzymes and aminotransferases indicating their hepatoprotective potential.

18. Basline data: Results are represented as follows:

Parameter (units)	H	INH	NI	MGI	MI	INH+NAC	INH+MPG	INH+Met
ALT (U/L)	42±2.20***	144±7.70 #	48±4.50***	56±5.60***	58±4.50***	89±5.40**	93±0.20 **	94±1.40**
AST (U/L)	44±2.10***	148±7.60 #	46±7.30***	64±5.60***	61±5.40***	84±0.20**	85±5.0**	92±6.4**
SOD (U/mg)	48.29±9.09***	14.97±2.44#	45.09 ± 2.29***	34.76 ±2.17**	42.83±7.99****	40.09±5.42***	32.51±6.38**	29.83±3.38*
GSHPx (U/mg)	0.49±0.01***	0.13±0.04#	0.51±0.37**	0.45±0.14**	0.46±0.04**	0.43±0.09**	0.36±0.13*	0.39±0.10*
MDA (U/mg)	36.22±6.18***	66.60±3.06#	42.69±2.94***	40.25±7.73***	50.50±3.01**	47.94±3.59***	45.75±4.01***	52.50±3.01**
Cholesterol (mg/dl)	144.29±2.83***	179.64±3.27#	137.72±6.61***	156.61±1.901**	160.37±10.68**	155.22±4.52***	166.37±2.89*	163.87±3.05**
TG (mg/dl)	190.71±5.03***	227.24±7.20#	181.04±8.282***	193.08±2.71**	191.63±12.39**	187.12±18.44***	200.59±10.28*	194.94±11.48**

19. Generalizability/ Translation: None

20. Details of funding agency: None

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