



National Centre
for the Replacement
Refinement & Reduction
of Animals in Research

The ARRIVE Guidelines

Animal Research: Reporting of *In Vivo* Experiments

The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were developed as part of an NC3Rs initiative to improve the design, analysis and reporting of research using animals – maximising information published and minimising unnecessary studies. The guidelines were published in the online journal *PLOS Biology* in June 2010 and are currently endorsed by scientific journals, major funding bodies and learned societies.

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The guidelines are intended to:

- Improve reporting of research using animals.
- Guide authors as to the essential information to include in a manuscript, and not be absolutely prescriptive.
- Be flexible to accommodate reporting a wide range of research areas and experimental protocols.
- Promote reproducible, transparent, accurate, comprehensive, concise, logically ordered, well written manuscripts.
- Improve the communication of the research findings to the broader scientific community.

The guidelines are NOT intended to:

- Promote uniformity, stifle creativity, or encourage authors to adhere rigidly to all items in the checklist. Some of the items may not apply to all studies, and some items can be presented as tables/figure legends or flow diagrams (e.g. the numbers of animals treated, assessed and analysed).
- Be a guide for study design and conduct. However, some items on the checklist, such as randomisation, blinding and using comparator groups, may be useful when planning experiments as their use will reduce the risk of bias and increase the robustness of the research.

Who are the guidelines aimed at?

- Novice and experienced authors
- Journal editors
- Peer reviewers
- Funding bodies

What kind of research areas do the guidelines apply to?

- The guidelines will be most appropriate for comparative studies, where two or more groups of experimental animals are being compared; often one or more of the groups may be considered as a control. They apply also to studies comparing different drug doses, or, for example, where a single animal is used as its own control (within-subject experiment).
- Most of the recommendations also apply to studies that do not have a control group.
- The guidelines are suitable for any area of bioscience research where animals are used.

How might these guidelines be used?

The guidelines provide a checklist for those preparing or reviewing a manuscript intended for publication.

References

1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLOS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

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Further Information

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	ITEM	RECOMMENDATION
Title	1	Provide as accurate and concise a description of the content of the article as possible.
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.
INTRODUCTION		
Background	3	<p>a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale.</p> <p>b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.</p>
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.
METHODS		
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.
Study design	6	<p>For each experiment, give brief details of the study design including:</p> <p>a. The number of experimental and control groups.</p> <p>b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when).</p> <p>c. The experimental unit (e.g. a single animal, group or cage of animals).</p> <p>A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.</p>
Experimental procedures	7	<p>For each experiment and each experimental group, including controls, provide precise details of all procedures carried out.</p> <p>For example:</p> <p>a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s).</p> <p>b. When (e.g. time of day).</p> <p>c. Where (e.g. home cage, laboratory, water maze).</p> <p>d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).</p>
Experimental animals	8	<p>a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range).</p> <p>b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.</p>

Housing and husbandry	9	<p>Provide details of:</p> <p>a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).</p> <p>b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment).</p> <p>c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.</p>
Sample size	10	<p>a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group.</p> <p>b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.</p> <p>c. Indicate the number of independent replications of each experiment, if relevant.</p>
Allocating animals to experimental groups	11	<p>a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done.</p> <p>b. Describe the order in which the animals in the different experimental groups were treated and assessed.</p>
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).
Statistical methods	13	<p>a. Provide details of the statistical methods used for each analysis.</p> <p>b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron).</p> <p>c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.</p>
RESULTS		
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing (this information can often be tabulated).
Numbers analysed	15	<p>a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%²).</p> <p>b. If any animals or data were not included in the analysis, explain why.</p>
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).
Adverse events	17	<p>a. Give details of all important adverse events in each experimental group.</p> <p>b. Describe any modifications to the experimental protocols made to reduce adverse events.</p>
DISCUSSION		
Interpretation/scientific implications	18	<p>a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature.</p> <p>b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results².</p> <p>c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.</p>
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.

ARRIVE STATEMENT

1. **Title:** Exploration of mitigating effect of colon-specific bioreversible codrugs of mycophenolic acid and aminosugars in experimental colitis model in Wistar rats.
2. **Abstract:** Pharmacological evaluation of synthesized prodrugs were performed using 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) model which efficiently imitates both acute and chronic colitis resembling the human UC. The alleviating effect of synthesized prodrugs as well as standards was evaluated for disease activity score rate and colon/ body weight ratio in TNBS-induced experimental colitis model in Wistar rats. Treatment with synthesized prodrugs showed markedly decrease in the extent and severity of colonic damage with normal morphology and mild lymphocytic infiltrate which might be due to activation of T lymphocyte triggered by immune-stimulation of D-glucosamine as well as D-galactosamine as reported by Sadeghi *et al.*, while colon of animals treated with physical mixture of MPA with aminosugars, appeared congested with ulcerated mucosa. MGLS and MGAS proved to be better than physical mixtures because they were able to release MPA and aminosugars locally in colon in effective concentration for their protective effect while MPA and aminosugars administered orally were unable to reach the colon in required concentration to mitigate colonic inflammation. All groups except DC exhibited normal pancreas morphology. Mild infiltration of inflammatory cells in liver was observed in the groups treated orally with prodrugs and physical mixtures. Out of two prodrugs, MGLS was selected for *in vivo* study. MGLS prodrug showed 68 % release of MPA. *In vivo* studies on MGLS clearly indicates its colon-specific activation after a lag time of 8h which could be ascribed to hydrolytic action of N-acyl amidases found in colon.
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. 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) model efficiently imitates both acute and chronic colitis resembling the human UC.
4. **Objective:** The present work aims at the rational design, synthesis, kinetic studies and pharmacological screening of colon-specific prodrugs of mycophenolic acid for treatment of IBD with following objectives:
 - a. To develop colon targeting chemical delivery systems for the selected drugs.
 - b. To explore the utility of colon- targeting prodrugs of investigational drugs in IBD.

- c. To compare the effectiveness of these prodrugs with standard drugs in TNBS-induced colitis.
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/15/2014- 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 control	6
2.	Disease Control	6
3.	Mycophenolic acid	6
4.	Mycophenolate mofetil (Standard)	6
5.	MGLS	6
6.	MGAS	6
7.	D-glucosamine	6
8.	D-galactosamine	6
9.	Physical Mixture-1	6
10.	Physical mixture-2	6
Total no of animals required=60		

7. Experimental procedures

➤TNBS induced ulcerative colitis

Table 2 Experimental procedure for TNBS induced ulcerative colitis

Day 1	Starvation will be started at 10.00 a.m. Animals will be divided into 10 groups , each group consisting of six animals. All animals will be examined for weight loss, stool consistency and rectal bleeding.
Day 2	Starvation will be continued and the animals will be examined for weight loss, stool consistency and rectal bleeding.
Day 3	All animals except that of the healthy control group will be catheterized 8 cm intra-rectally and 0.25 mL of TNBS in ethanol was injected into colon <i>via</i> rubber canula (dose; 100 mg/kg of body weight of TNBS in 50% v/v ethanolic solution). Each animal after TNBS administration was maintained in a vertical position for 30 sec and returned to its cage. The animals of all

	groups will be examined for weight loss, stool consistency and rectal bleeding.
Day 4	All the animals will be examined for weight loss, stool consistency and rectal bleeding.
Day 5	4 th day protocol continued.
Day 6	Oral or rectal drug intervention, once daily. The healthy control group will be given saline throughout the 11 day study while colitis control group received saline after induction of colitis. All the animals will be examined daily for weight loss, stool consistency and rectal bleeding.
Day 7	6 th day protocol continued.
Day 8	6 th day protocol continued.
Day 9	6 th day protocol continued.
Day 10	6 th day protocol continued.
Day 11	All the animals will be examined for weight loss, stool consistency and rectal bleeding and will then sacrificed. Colon to body weight ratio of these animals will be calculated. Colons, livers and pancreas of the sacrificed animals will be removed and fixed in 10% buffered formalin and samples will be sent for microscopic examination and histopathology.

Assessment of Quantifying Parameters:

- Clinical activity score
- Colon to body weight ratio
- 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 0-5°C 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 0-5°C 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 (200-230 g) were purchased from National Toxicology Center, Pune.
Chemicals:

2, 4, 6-trinitrobenzene sulfonic acid (TNBS), Anaesthetic ether, 10% buffered formalin.

9. **Housing and husbandary:** During the experiment, rats were housed in standard housing conditions like temperature of 25 ± 1 °C, 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:** 60 rats used for carrying out pharmacological evaluation. Each group contained 6 rats such total 10 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.** Induction of colitis lead to severe inflammation. All the animals were examined for weight loss, stool consistency and rectal bleeding and then sacrificed. Colon to body weight ratio of these animals were calculated. Colons, livers and pancreas of the sacrificed animals were removed and fixed in 10% buffered formalin and samples will be sent for microscopic examination and histopathology.
13. **Statistical methods:** All data were expressed as mean \pm S.E.M.; (n refers to number of animals in each group). For disease activity score rate, statistical differences between

groups were calculated by Two-Way ANOVA followed by Bonferroni's test whereas statistical analysis of ulcerogenic activity 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 standards was evaluated for disease activity score rate and colon/ body weight ratio in TNBS-induced experimental colitis model in Wistar rats. The anti-colic activity of prodrugs was compared with MMF and MPA. Till 5th day the disease activity score increased rapidly and consistently for all TNBS-treated groups. From day 6 to 10, standards MMF and MPA, prodrugs, carriers and physical mixtures were administered orally to animals. Full blown colonic inflammation was evidenced by the high disease activity score (3.30 ± 2.0) in colitis control group. Prodrugs- treated group showed remarkable decrease in disease activity score rate (99-100%) that was comparable with MMF- treated groups. Results of animal study revealed that disease activity-lowering effect of both prodrugs was significantly superior to MPA (77%), individual carriers (61-69%) and physical mixtures (88%). All groups except DC exhibited normal pancreas morphology. Mild infiltration of inflammatory cells in liver was observed in the groups treated orally with prodrugs and physical mixtures.

18. Baseline data: Colon to body weight ratio for each rat (n=6) is represented as follows:

Groups Rats	DC	MMF	MPA	MGLS	MGAS	GL	GA	GL+MPA	GA+MPA
H	0.061	0.021	0.031	0.026	0.029	0.039	0.033	0.037	0.042
B	0.050	0.020	0.036	0.020	0.039	0.045	0.038	0.032	0.032
T	0.071	0.0232	0.032	0.022	0.019	0.0435	0.0292	0.042	0.0512
HT	0.061	0.021	0.032	0.025	0.0384	0.038	0.053	0.038	0.0412
BT	0.060	0.0226	0.032	0.020	0.039	0.025	0.047	0.032	0.032
None	0.069	0.021	0.034	0.022	0.019	0.0435	0.0492	0.042	0.0512

19. Generalizability/ Translation: For colitis induction purpose instead TNBS, Dextran sulphate sodium also being used widely in Swiss albino rats.

20. Details of funding agency: For procurement of animals, chemicals and glasswares utilized for animal activity, funds were provided by DST's Women Scientist Scheme (WOS-A) funding agency[SR/WOS-A/ LS-1115/2014].



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