

Two-start design within a Sephadex inflammatory model – A means to generate reliable ED₅₀ data whilst significantly reducing the number of animals used

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ABSTRACT

Pulmonary inflammation disorders represent a major healthcare burden, and novel anti-inflammatory agents are critically needed for the treatment of patients unresponsive to current therapies. *In vivo* animal models play a key role in the preclinical assessment of novel anti-inflammatory compounds. The implementation of streamlined *in vivo* experimental designs that are time- and cost-efficient, while keeping animal usage low, is a key consideration for drug optimization programs. The Sephadex rat model of pulmonary inflammation captures many pathophysiologic characteristics of clinical asthma and allergy, such as eosinophilic infiltration and interstitial edema. Using the *in vivo* Sephadex model, we compared two different study designs that were implemented to screen and select two novel candidate drugs for a drug discovery project. The traditional one-start design, which utilizes few dose-testing groups with many animals per group, was used to select the first candidate drug. Due to tight timelines, the selection process for the second candidate drug had to be optimized, leading to the development of the novel two-start design, an approach whereby dose ranges are optimized in two experimental phases. Here we show that both study designs were comparable in their generation of robust median effective dose values for selected candidate drugs, as represented by similar confidence interval ratios. However, implementation of the two-start design resulted in approximately 50% fewer animals and 50% less time taken to assess the efficacy of an equal number of compounds compared with the one-start design. These results demonstrate that the two-start design is a more efficient experimental approach, and its widespread implementation in drug optimization programs will impact upon the selection process for candidate drugs with regards to time, cost, and animal usage.

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1. Introduction

Preclinical *in vivo* models of pulmonary inflammation are commonly utilized to assess the underlying mechanisms of disease and the impact of pharmacologic intervention [1–3]. Small animal models using allergens, irritants, or other pro-inflammatory stimuli can recapitulate specific features associated with pulmonary conditions, such as airway hyper-responsiveness or obstruction [3]. Due to the relative ease of assessing pulmonary function in rats, these animals have been the mainstay for lung-disease modelling

and anti-inflammatory drug testing in response to a variety of stimuli [2,3].

Sephadex, comprised of cross-linked particles of dextran, is a well established non-allergic stimulus used preclinically to investigate the airway pathophysiology seen in asthma and allergy [1,4]. Due to endogenous hypersensitivity to dextran, intravenous or intratracheal administration of Sephadex in the rat induces a profound and rapid eosinophilic infiltration into lung tissue, accompanied by macrophage, neutrophilic, and T-lymphocytic influx and the formation of pulmonary granulomas [5–7]. Local release of cytokines and other inflammatory mediators contribute to alveolar and bronchial hyper-responsiveness, interstitial edema, and impaired lung function, inducing an inflammatory profile akin to that observed in clinical asthma [1,4–6].

The Sephadex model lends itself well to pharmacologic manipulation due to its reproducibility and rapid induction of

Abbreviations: CD, candidate drug; CI, confidence interval; ED₅₀, median effective dose.

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inflammation within 24 h, and unlike certain allergens such as ovalbumin, pre-sensitization prior to challenge is not required [3,4,6]. In addition to the acute 24 h model, the Sephadex model can be run over longer time courses, for example, over five consecutive days of drug treatment, and can combine investigations of both the potency and systemic effects of anti-inflammatory compounds. As such, this model is appropriate for both low- and high throughput drug testing. Glucocorticoids such as budesonide or dexamethasone can markedly improve the inflammation and edema induced by Sephadex [8,9]. Furthermore, the anti-inflammatory efficacy of candidate drugs (CDs) targeted towards various molecular effectors of the inflammatory pathway is commonly investigated using the Sephadex model [10–13].

Within drug optimization programs, screening and selecting an optimal CD requires extensive work and is often performed during relatively short timelines. In an ideal scenario, the CD is selected from a large number of screened compounds to ascertain the optimal candidate. Therefore, a critical consideration for preclinical CD selection programs is to implement an optimal study design that will generate reliable, reproducible data while maximizing resources [14,15]. Conventional *in vivo* models for CD selection implement a one-start experimental design whereby all animals within a given test group are entered into the study on the same day. Traditionally, this design utilizes few groups with many animals per group because observing significant differences in efficacy between the control and treatment groups is vital for CD selection. However, this design has several limitations, including a reduced ability to generate reproducible median effective dose (ED₅₀) values from the data used to rank candidate compounds, as a fewer number of test doses may need to span a wide range. In addition, the day-to-day variation of results can often necessitate multiple repeat experiments. Generating ED₅₀ values with large confidence intervals (CIs) and having wide day-to-day variation between studies can adversely impact upon animal usage and program cost-effectiveness.

We have developed a novel alternative experimental design called the two-start method, which is analogous to the adaptive clinical trial [16]. The objective of this method is to generate more robust drug-efficacy data where reproducibility may be an issue, such as with *in vivo* experimental models. A larger number of experimental groups are involved, comprising fewer animals per group. With the Sephadex model, the first start (or initial dose-testing stage) included few test doses that spanned a wide range. This range was then optimized within the second start of the experimental phase to omit doses in which efficacy was not observed and to include additional, incremental doses within the optimal range (Table 1). In addition, two test compounds were started on the same day, allowing control groups to be shared. Results from each start can then be combined to ensure a thorough

testing range and generate reliable and more reproducible ED₅₀ values.

Using the five-day Sephadex inflammatory model, this paper demonstrates how the novel two-start design was used to reduce the number of animals and experiments required to generate reliable ED₅₀ data, compared with the conventional one-start design. ED₅₀ values of a selection of candidate glucocorticoids were determined based on the inhibition of Sephadex-induced lung edema using either the one- or two-start design. The relative robustness and efficiency of both approaches were compared, with the aim of identifying a study design to increase the efficiency of the CD selection process with regards to time, cost, and animal usage.

2. Methods

2.1. Animals

Male Sprague Dawley rats weighing 200–225 g were purchased from Charles River Laboratories International Inc. (Wilmington, MA, USA), randomized at arrival, and housed for ≥5 days before commencement of experimental procedures. Studies were conducted under the AstraZeneca R&D research permit reference no. 31-11684/08 and approved by the Malmö/Lund Ethical Committee for Animal Experiments (221-07) (Sweden).

Animals were housed in plastic cages with aspen bedding and kept under daily observation with access to food (R70 pellet; Lactamin, Lantmannen, Sweden) and water. Housing facilities (AstraZeneca R&D, Lund, Sweden) were maintained at 22 °C with 60% relative humidity and a 12 h (06.00–18.00) light/dark cycle.

2.2. Materials

The following materials were used in this study: Sephadex G-100 superfine (#G10050, batch 096K1778, Sigma–Aldrich, Sweden); IsoFlo®Vet (isoflurane; ORIONPharma, Denmark); NaCl (B. Braun Medical AB, Sweden); pentobarbital sodium (Apoteksbolaget AB, Sweden). Test compounds (glucocorticoids) were from AstraZeneca, Sweden.

2.3. Experimental procedures

2.3.1. Sephadex challenge

Animals were anesthetized with isoflurane (O₂ 2 L/min, N₂O 4 L/min, IsoFlo®Vet 3%) for 3 min, weighed and injected intratracheally with 200 µL of Sephadex 10 mg/mL, to give a final dose of 1 mL/kg. Injections were administered with a puff of air to ensure lung exposure. Control animals were challenged with saline.

2.3.2. Administration of test glucocorticoid compounds

Animals received test compounds by inhalation of dry powder once daily over four consecutive days, with the first administration given 30 min prior to Sephadex challenge (Fig. 1). Animals were terminated at Day 5 with an intraperitoneal injection of

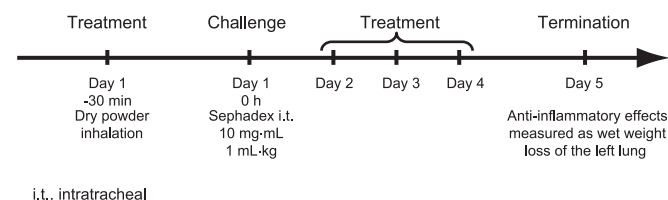


Fig. 1. Experimental schedule of the five-day Sephadex inflammation model.

Table 1
Principles of the two-start design.

Group	1st half of study		2nd half of study		Overall	
	Dose (µg/kg)	N	Dose (µg/kg)	N	Dose (µg/kg)	N
Negative control	—	2	—	2	—	4
Positive control	—	4	—	4	—	8
Dose 1	0.1	4	—	—	0.1	4
Dose 5	—	—	0.3	4	0.3	4
Dose 2	1	4	—	—	1	4
Dose 6	—	—	3	4	3	4
Dose 3	10	4	—	—	10	4
Dose 7	—	—	30	4	30	4
Dose 4	100	4	—	—	100	4
Dose 8	—	—	300	4	300	4
Dose 9	—	—	1000	4	1000	4

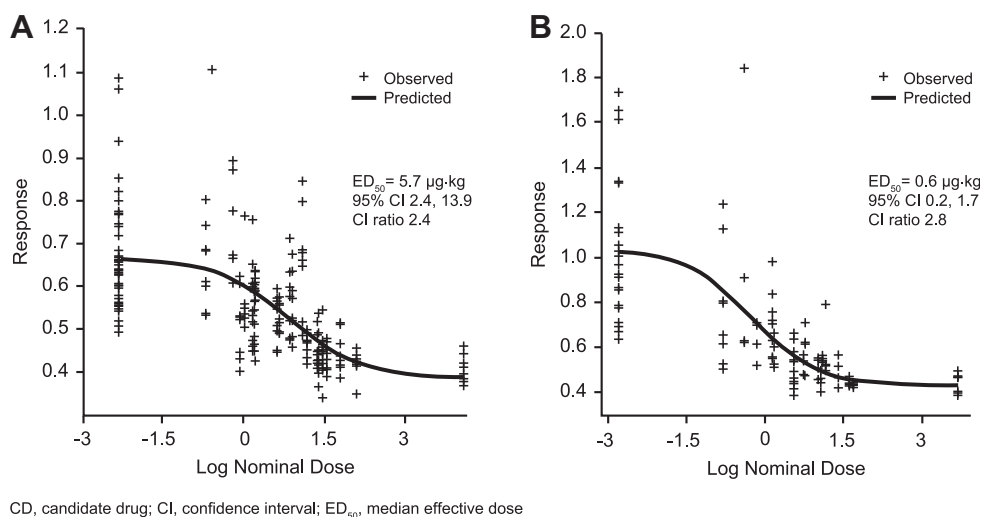


Fig. 2. Comparison of ED₅₀ values for CD1 (A) and CD2 (B).

pentobarbital sodium (10 mL/kg). Inhalation was performed using a nose-only ‘flow-past’ exposure chamber (Münster AG, Switzerland) maintained at 20 ± 4 °C and $50 \pm 5\%$ relative humidity, as described previously [17,18]. The delivered dose was determined by a filter placed within the exposure chamber (AP40, 47 mm, micro-fibre glass; Millipore Inc., USA). The amount of compound detected on each filter was analyzed by high-performance liquid chromatography, and the inhaled dose, body dose, and lung dose per animal was calculated [19] and ED₅₀ values presented as µg/kg lung dose.

2.3.3. ED₅₀ assessments

ED₅₀ values of test compounds were generated by the reduction in Sephadex-induced left-lung edema compared with control animals, as determined by change in weight.

2.4. Statistical analysis

ED₅₀ values were generated by fitting a four parameter logistic curve to data points representing the left-lung weight in individual animals following drug exposure. The negative control group (saline) was assumed to have 100% inhibition, and the positive (Sephadex) control group was assumed to have 0% inhibition. Analysis was performed using R version 2 and XLfit software.

2.5. Comparison of the one-start and two-start design

Compounds A–C tested for CD1 were screened using the one-start experimental design and compounds D–J tested for CD2 were screened using the two-start experimental design. Test compounds within each design were ranked according to ED₅₀ values for inhibition of Sephadex-induced lung edema, determined by change in left-lung weight. The compounds exhibiting greatest efficacy from the one- and two-start designs were selected as CD1 and CD2, respectively.

The robustness of each design was assessed by comparing the CI ratio of ED₅₀ values, determined for CD1 and CD2. The CI ratio is the multiple of the ED₅₀ to give the upper confidence limit, a simple way of describing the precision of an estimated ED₅₀. Two parameters were utilized to compare the efficiency of the two study designs: animal usage, defined as the total number of animals used per compound, and the workload, defined as the number of weeks taken to screen an individual compound.

3. Results

3.1. Comparison of one-start and two-start design

3.1.1. Robustness of study design

The ED₅₀ value for CD1 was 5.7 µg/kg (95% CI 2.4, 13.9), selected by the one-start design from five studies and 19 dose groups. The ED₅₀ value for CD2, selected by the two-start design from two studies and 16 dose groups, was 0.6 µg/kg (95% CI 0.2, 1.7) (Fig. 2). CI ratios were similar for each study design (CD1, 2.4; CD2, 2.8) indicating the ED₅₀s have similar precision.

3.1.2. Efficiency of study design

The three compounds analyzed within the one-start design were screened in a total of seven weeks and utilized 398 rats (Table 2). Using the two-start design, seven compounds were screened in a total of 10 weeks and 439 rats were used (Table 2). Therefore, the average number of screening weeks per compound was 2.3 and 1.4 weeks for the one- and two-start designs, respectively. The average number of rats used in the screening of each compound using the one-start design was 133, whereas an average of 63 rats per compound was used for the two-start design.

To directly compare the efficiency between the study designs after completion of the two CD programs, the number of rats required and weeks spent to screen an equal number of compounds were estimated. The two-start design required 439 rats and 10

Table 2

Total animal usage and workload for each test compound in the one- and two-start design studies.

CD1 (one-start)			CD2 (two-start)		
Compound	Weeks	Rats	Compound	Weeks	Rats
A	1	74	D + E	2	84
A	1	47	F + G	2	90
A	1	57	H + I	2	89
A	1	60	G + J	2	104
A	1	60	J	2	72
B	1	48			
C	1	52			
Total	3	7	7	10	439
No. rats/compound	133		63		
No. weeks/compound	2.3		1.4		

CD, candidate drug.

weeks to screen seven compounds (Table 2). Based on the average rat usage and workload shown in Table 2, to screen seven compounds using the one-start design would require 931 rats and would take 16.1 weeks. However, as scheduling restrictions apply with regards to resource (laboratory, equipment, and personnel) availability, 10 weeks of experimental work required for the selection of seven compounds using the two-start design equated to approximately five months. Accordingly, taking into account these scheduling restrictions, we estimate that to select CD1 from seven compounds using the one-start design, the experimental period would have spanned approximately 10 months. Therefore, the two-start design used 492 fewer rats and reduced the overall time by five months.

4. Discussion

In this paper, we have demonstrated how different study designs can impact upon the number of animals and workload required for the CD selection process in a drug discovery project. Using the five-day Sephadex model of pulmonary inflammation in the rat, we screened for novel candidate glucocorticoids using two different study designs for CD1 and CD2 selection. This allowed for a comparison of the one-start and two-start designs with respect to their robustness and efficiency. Evaluation of efficiency revealed that both the number of rats used and the time taken to determine the efficacy of each compound were reduced by ~50% when implementing the two-start design compared with the one-start approach. Critically, the robustness of the two designs was comparable, reflected by similar CI ratios for respective ED₅₀ values.

Several factors impacted upon the generation of ED₅₀ values for the compound selected as CD1 (compound A), which led to a higher resource utilization for this compound than for all others tested. ED₅₀ estimation for this compound was difficult due to inherent limitations of the one-start design: only a limited number of dose groups were included, generating an ED₅₀ with a large CI. Repeat experiments were therefore required. As this was the first compound representing a new chemical series to be examined in this model, initial pilot experiments using doses that spanned a very wide range were necessary to provide the framework for subsequent efficacy experiments for this and other compounds. Our results suggest that these pilot studies would have been streamlined further had the two-start design been applied. With the two-start approach, the ED₅₀ generated for CD2 (compound G from a different compound series) was confirmed with a narrow CI ratio. This suggests that a robust ED₅₀ value can be generated with fewer studies, by using many dose groups within the two-start design to cover a large dose range. The additional experiments performed within the one-start design did not add value to the ED₅₀ estimate. The two-start design required less study repetition, resulting in reduced variability, robust ED₅₀ evaluation, and a streamlined dose-range finding process. Furthermore, this design provided the added benefit of fewer studies with fewer animals in each dose group, and allowed for the sharing of control and reference groups.

In this case, we show an example where compound A was tested in a 1 start design prior to the testing of other compounds. The actual animal number gains are affected by this testing of compound A. The initial study could have used a two-start design to test compounds A, B and C enabling additional animal numbers to be reduced. In this case, illustrated by a real world example, the timelines to deliver a second drug candidate effectively forced a creative change from one-start studies (used to deliver the initial candidate drug) to two-start studies resulting in efficiency gains and the ability to deliver a second candidate in a shorter time. For subsequent drug discovery projects, using different models and

with different compounds, we initiate dose finding work with two-start designs.

Glucocorticoids are well documented to reduce the severity and accelerate resolution of acute asthma in many patients, in addition to lowering the chance of relapse [20]. However, for the subset of patients who are non-responsive or develop resistance to current therapies, increasing treatment options is a key clinical priority [21,22]. The Sephadex model utilizing the two-start design is positioned to evaluate an increased number of candidate glucocorticoids, as well as novel compounds directed at other pro-inflammatory molecular targets, which may expedite the availability of new and more effective treatments to patients with asthma. One important benefit of the two-start design for the Sephadex model is that after the first start, we were able to change the doses to ensure the capture of the best data possible, akin to an adaptive design, and don't waste animals even if the initial dose selection in a one-start study was very wrong.

The gains in using a two-start design rather than a one-start design will increase the less reliable the test studies are. The gains are dependent on the lack of reproducibility of the model, but not the window for the efficacy signal, nor the variability of the data. The greater the lack of reproducibility, the greater the gain. Given the complexity and challenges of many *in vivo* studies, the two-start design can be seen to be potentially valuable in a number of areas as reproducibility for *in vivo* pharmacology is generally low.

The two-start principle can also be applied outside of pharmacological work and into other experimental areas. An intuitive argument to understanding the gain relating to lack of reproducibility is as follows. Any experimental model might be fine on a "good" day but not on a "bad" day. Given we cannot determine which days are "good" or "bad", averaging across the two starts minimises the chance of a "bad" day. Statistically, running three-start or four-start experiments would average out any lack of reproducibility even more and give an ultimately more robust and confident determination. For the Sephadex studies described we did not need more than two starts as a two-start design enabled us to reach the level of precision required. For animal studies you need to decide what level of precision you require, for example to guide chemistry in a drug discovery project, or to estimate predicted doses in humans. However, once the level of precision needed is known, the 2-start design enables the determination of the required endpoint and confidence level to be determined with greater efficiency. A separate publication, dealing with the pure statistics underlying the two-start principle is in preparation for publication elsewhere.

The two-start design is akin to adaptive designs. There are "hopping strategy" designs, for example finding a maximum tolerated dose or LD50, which essentially have multiple starts. However, often each such study does not have its own control, so variability associated with studies done on different occasions impacts the end result. The two-start study is a deliberate splitting of a single study into two halves, each with controls and study variability between studies is removed.

There are some experimental limitations for the 2-start study. For example, where the controls cannot be matched for the different starts. This could be the case where the vehicle has to be changed in order to administer different doses of a given drug. If two compounds are to be compared in efficacy in a 2-start design, but need different vehicles for administration then they won't be perfectly matched with respect to controls and the differences in vehicles can impact the results. However, in this case the 2-start design is no more compromised than a 1 start design as there also different vehicles need to be used and always the compound for testing is "tied" to a relevant vehicle. Hence in all *in vivo* studies the vehicle used is attempted to be suitable for a range of test substances.

One key question to ask is whether there are limitations for a two-start design? Where the response is not observed quickly then we may lose the ability to change doses for the second start, but even with the same doses in the starts we will gain if the model has a the lack of reproducibility. Where there is no lack of reproducibility there is no gain, a traditional one-start design would be preferable for pragmatic reasons; the only loss would be in time- and possible resources tied up. Note that we do not have to finish one start before commencing the second start.

The results of the refined two-start experimental design have significant implications for drug optimization programs. A key consideration for *in vivo* preclinical studies undertaken within academia and the pharmaceutical industry is adherence to guidelines for laboratory animal usage, which have evolved according to the '3Rs' principle (replacement, reduction, and refinement) [23,24]. A significant benefit of the two-start design is that control groups are shared across two compounds started on the same day, thereby reducing animal usage. Furthermore, extrapolation of the results reported here reveal that implementation of the two-start design would lead to marked savings of 490 fewer animals and five months in workload compared with using the one-start design, when assessing efficacy of a total of seven candidate compounds. Importantly, not only does this equate to an estimated \$25,000 saving in total costs, but it represents a significant move towards effecting productive experimental models according to the '3Rs' principle. Furthermore, the principle of this design can be applied to other disease models, thereby reducing animal usage, overall costs and time, producing more streamlined and effective *in vivo* pharmacology studies.

Disclosure and conflicts of interest

All authors are full-time employees of AstraZeneca.

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