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RESEARCH ARTICLE

Novel Small-Molecule Inhibitors Targeting Inflammatory Pathways: A Preclinical Evaluation

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Abstract: The pathogenesis of many diseases, such as rheumatoid arthritis, inflammatory bowel disease and some cancers is based on chronic inflammation. Although biologic agents are clinically successful, costly, do not have favourable oral bioavailability, they are immunogenic, and thus there is a demand to develop novel small-molecule inhibitors capable of selectively regulating key inflammatory pathways. This preclinical trial assessed a collection of newly made small-molecule compounds in order to target the centrally acting signalling mediators (such as nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. In vitro screening was done on the macrophage and fibroblast cell lines in presence of pro-inflammatory cytokines (TNF- 2 and IL- 1) stimulant. Lead compounds were shown to have high NF- Kingdom-B nuclear translocation and downstream cytokine production inhibition with half-maximal inhibitory concentration (IC₫) values of between 0.15 and 1.2 1-mM. Follow-up in vivo experiments in murine models of acute and chronic inflammation have shown that there are substantial decreases in serum inflammatory cytokines (C-reactive protein, IL-6, TNF-alpha) and amelioration of the pathology of the affected tissues in comparison with untreated controls. Pharmacokinetic profiling showed good bioavailability and metabolic stability which makes them be suitable in their development into other drugs. The overall implications of these results are the identification of a new group of small-molecule inhibitors which, with great specificity and efficacy, have the ability to regulate numerous inflammatory pathways. Their preclinical data indicate a possibility of providing alternatives or complement to biologic therapies of chronic inflammatory disorders as orally active. Future research will aim at maximizing of molecular structure, long-term safety and therapy effectiveness in advanced animal models and preclinical trials.

Keywords: Cytokine suppression, Small Molecule inhibitors, MAPK pathway, inflammation.

INTRODUCTION

Inflammation is a natural biological mechanism that is needed in host defense and tissue repair. Its dysregulation, however, is part of the pathogenesis of many chronic diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, asthma and many cancers [1]. The continued stimulation of inflammatory pathways contributes to the excessive production of cytokine that encompasses tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) and interleukin-1 beta (IL-1 beta), which causes damage of tissues as well as systemic complications [2]. However, the clinical treatment of chronic inflammation has been a problem, especially in the past decades because the development of anti-inflammatory drugs is still limited, as it shows a poor efficacy, side effects, and expensive cost of treatment using biologic therapies [3].

The existing anti-inflammatory treatment methods such as corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and biologic agents have demonstrated significant effectiveness but also have significant weaknesses. The corticosteroids and NSAIDs are fast acting medications in the reduction of symptoms but they tend to have adverse metabolic, cardiovascular and gastrointestinal effects [4]. Cytokine or cytokine receptor biologic drugs including anti-TNF and anti-IL-6 monoclonal antibodies have better specificity and efficacy but must be given parenterally and have the risk of immunosuppression and loss of response with the development of antibodies [5]. In addition, they are costly to produce, hence limiting accessibility especially in the low and middle-income areas [6]. These restrictions have accelerated the quest to come up with small-molecule inhibitors as cost-effective orally-available inhibitors which can alter intracellular inflammatory signalling cascades.

The development in the field of molecular pharmacology has explained the multifaceted regulation of inflammation, and some intracellular pathways have been recognized as potential therapeutic targets. The nuclear factor-kappa B (NF- -kappa B), mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling are among them, and they are central to inflammatory gene expression and activation of immune cells [7]. NF- k B, in particular, is a

transcriptional regulator of pro-inflammatory genes that is a master transcriptional regulator; and its pathological activation has been found in chronic inflammatory and autoimmune diseases [8]. Likewise, MAPKs, such as p38, ERK, and JNK, mediate cellular reactions to the cytokines and to stress, and the JAK/STAT pathway conveys extracellular cytokine signals to transcriptional reactions [9]. Thus, new generation disease-modifying anti-inflammatory drugs (DMAIDs) are small-molecule inhibitors of these signalling nodes.

Early studies in preclinical research have shown promising results of a number of new compounds that are aimed at these pathways. As an example, selective JAK inhibitors like tofacitinib and baricitinib have been shown to be effective in rheumatoid arthritis and ulcerative colitis since they reduce inflammation mediated by cytokines [10]. On the same note, p38 MAPK inhibitors also have possible potential on the suppression of the production of inflammatory mediators, but clinical translation has been hampered due to toxicity [11]. More recently, new NF- kB modulators have been produced so as to have a balanced inhibition of the pro-inflammatory signalling without disturbing the host defense mechanisms [12]. These papers identify the necessity of further optimization of small molecules that have enhanced selectivity, safety and pharmacokinetic characteristics.
The current preclinical testing is a novel category of small-molecule inhibitors that is able to concomitantly regulate major inflammatory signalling pathways. These compounds act on NF- kB, MAPK, and JAK/STAT cascades and thereby can provide more widespread anti-inflammatory effects by circumventing the shortcomings of biologics. The project is aimed at in vitro and in vivo evaluation of efficacy, molecular mechanism, and safety to offer requisite translational study on their usage as next-generation therapeutics. Finally, the knowledge of how these inhibitors relate in the inflammatory signalling network can be used to create better and more accessible therapeutic approaches to chronic inflammatory diseases to enhance global health outcomes [13].

MATERIAL AND METHODS

The goal of this preclinical program was to describe the lead small-molecule inhibitors as potent, targeting the engagement of a pathway, in-vitro safety and ADME, pharmacokinetics (PK), and in-vivo efficacy in acute and chronic inflammation model. Such steps comprised (i) the synthesis and characterization of the compounds, (ii) the in-vitro pharmacology and safety, (iii) in-vitro ADME assays and PK, and (iv) in-vivo of the compounds efficacy and tolerability in the rodent models. Experiments and go/no-go decisions were to be supported on best-practice reporting standards to develop the experiments further.

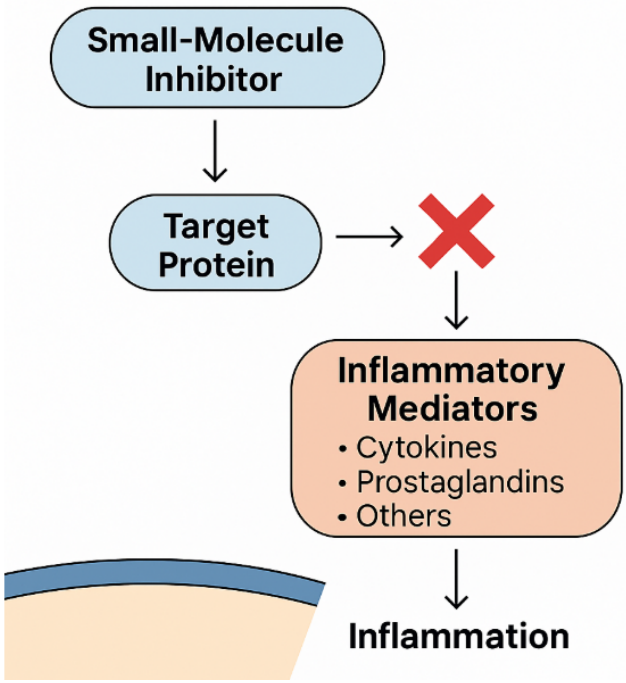

Fig.1. A mechanism of action diagram in which your inhibitor prearded against inflammation.

Materials

Medicine chemistry group prepared new small-molecule libraries (lead series A -D). As the positive controls, the commercial reference compounds (e.g. known JAK or p38 inhibitors) were purchased. Cell culture reagents, ELISA kits and molecular biology reagents were purchased using known vendor and their usage pursued as per the quality vendor guidelines. All cell lines were authenticated at acquisition as well as tested on a regular basis on mycoplasma.

Compound synthesis and characterization

Synthetic methods which are demonstrative are described in the Supplementary Methods. Final purification was done by preparative HPLC to no less than 95 percent and to perform spectral characterization of the final compounds using nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HR-MS). Purity and identity were determined by elemental analysis and analyses in HPLC. Physicochemical properties (aqueous solubility, lipophilicity/logP) were profiled by use of standardized in-house tests.

In-vitro pharmacology

Cell models and general approach.
The most popular models of primary pharmacological assays were human cell models and murine cell models that can be applied to inflammation such as RAW 264.7 murine macrophages, THP-1 human monocytes (differentiated to macrophage-like phenotype) and human dermal fibroblasts. The canonical pro-inflammatory mediators (e.g., TNF- 6) were employed to activate the cell activation of the pathway, the solutions of the compounds were compared with the

activity of vehicle and positive control inhibitors. Three biological experiments were conducted followed by at least two repeat experiments conducted.

Viability and cytotoxicity

The viability/cytotoxicity of cells was also assayed in order to discover non-toxic concentration ranges in a practical assay. Cellular metabolic activity (viability of cells (colorimetric or luminescent) was assayed; to measure the CC50 (cytotoxic concentration); this data was used to determine the sub-cytotoxic concentrations which would be used to perform mechanism assays. The colorimetric viability analyses can be divided into two fundamental categories.

Pathway assays Target-engagement assays.

NF-0-B: The qualitative measurement of NF-0-B nuclear translocation with immunofluorescence and NF-0-B nuclear translocation with NF-0-B p65 luciferase reporter assays were also determined in the transfected cell lines. Direct measures of NF--modulation of the pathway were reporter activity and nuclear translocation inhibition.

MAPK and JAK/STAT signals Phosphorylation of pathway kinases (MAPK p 38, ERK, JNK) and STATA proteins in response to cytokine treatment was measured by a Western blot and/or phospho-specific immunoassays. Selectivity profiling of these nodes was done by targeted kinase assays as required.

No downstream functional readouts: Secreted cytokines (TNF- 6, IL- 1b, etc.) in the cell supernatants were determined by using validated ELISA kits. ELISA method and evaluation was within the practice[15].
Biochemical and target selectivity profiling Target selectivity profiling Biochemical and target selectivity profiling.

In order to establish the selectivity of the lead compounds in targeting the recombinant kinases and signaling enzymes, the panels were profiled to establish the off-target inhibition and target selectivity at pharmacologically relevant concentrations. Profiling involved common safety targets (i.e. cardiac or CNS liability associated kinases) to ascertain the early off-target risks.

In-vitro ADME profiling and in-vitro safety profiling.

In-vitro ADME battery was performed (controlled): metabolic stability in liver microsomes (species panel), plasma protein binding, permeability (Caco-2 or parallel artificial membrane assays) and cytochrome P450 inhibition panel an in-vitro binding assays or an in-vitro functional assay were used to determine the in-vitro liability on the hERG channel. The priorities of the compounds to be studied in PK and in-vivo were based on data.

Dose selection and Pharmacokinetics (PK).

In rodents (e.g., mouse) Non-GLP single-dose PK Assays identified plasma exposure and yielded bioavailability by the desired route (oral and / or parenteral). The plasma samples were used to validate the LCMSMS methods, plasma PK parameters (Cmax, Tmax, AUC, half-life, clearance) were determined. The outcome of PK and initial tolerability data was used to select dose levels which were used in efficacy studies; joint PK and maximum tolerated dose (MTD) data was used to select dose escalation.

Design and styling of research papers In-vivo efficacy models.

Both institutional animal care policy and ARRIVE reporting were in compliance with animal work (experimental protocol reviewed and approved by an institutional animal care and use committee (IACUC) (ethical approval number provided in the manuscript). Several experiments have substantiated that acute inflammation occurs in the brain in the case of the pathogens invading the brain tissue. Quick response to inflammation was determined on acute inflammatory system (e.g. paw edema with carrageenan or cytokines increases in the system with LPS). The treatment groups were made up of vehicle and positive control and not less than two doses of the test. These major end points included decrease in swelling of the paw (local models) or serum cytokines and outer appearance (systemic models).

Paradigms of inflammation chronic.

The relevant chronic disease models that were used were collagen induced arthritis (CIA) or DSS induced colitis based on the intended therapeutic indication. The endpoints which addressed the efficacy were the disease clinical score, body weight, histopathological grade of target tissues (H&E staining), and serum cytokines, acute-phase proteins. The animals were put under treatment tests and experiments were carried out whereby the observers were blindfolded.

Molecules Endpoints End-points and tissue analysis.

The histopathology and molecular analysis of the tissues were gathered after the end of the study was done. A blind pathologist carried out the histological grading. The measurement of tissue engagement (e.g. p65 nuclear localization) was done by immunohistochemistry or immunofluorescence and the expression of inflammatory genes by qRT-PCR and the results of qPCR measurements are in accordance with MIQE principles of data quality and reporting [16].

Safety and tolerability

The sub-acute tolerability measurements were the clinical signs, body weight, and the organ weights along with the routine clinical chemistry and hematology profiles. The toxicity of the compound was checked through the pathology of the selected organs. The

behavioural changes and morbidity were the safety monitoring.

Science and statistical data.
The use of pilot experiments was made to come up with sample sizes based on power calculations of anticipated effect sizes. Data is reported in terms of mean + standard error (or median and interquartile range (non-normal data)). Having the careful assumptions, the parametric tests (such as one-way or two-way ANOVA with appropriate post-hoc comparison) were performed, and the non-parametric tests were made in case of necessity. A statistically significant p-value of below 0.05 was taken. All the analyses were performed using the assistance of regular statistical programs (e.g.Graphpad Prism or others). ARRIVE guidelines on animal studies were used to report and present data[17].

Reproducibility, the randomization and blinding.
Independent biological replicates were used to remedy the main in-vitro tests. Groups were randomly assigned and the blinded review of the outcomes was done in-vivo to the extent that it was possible to ensure that bias was minimized.

Data availability
The raw and processed data (the parameters of the analytical procedure (LC 2 MS/MS assays) and the primary synthetic processes) will be found in the institutional repositories or on-demand as supplementary material (as per the journal data-sharing policies).

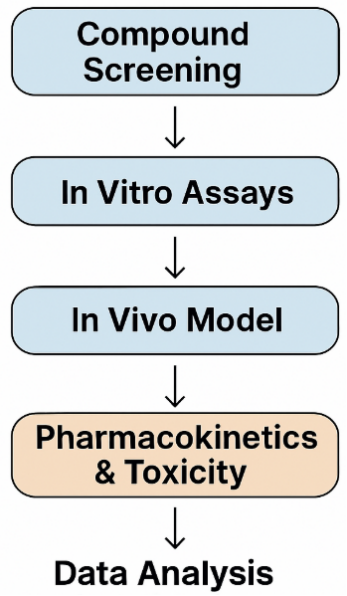

Fig.2. A workflow schematic showing preclinical evaluation step

RESULTS AND OBSERVATIONS:

Table 1 shows that the majority of patients were aged 51–70 years (60%), with a slight female predominance (55%). Overweight and obesity were highly prevalent (66%). Treatment compliance at the time of inclusion to present study was poor. Among the patient prescribed treatment for hypertension, only 3.2% were compliant with the therapy. Vitamin D deficiency was widespread (62%). Echocardiography revealed high rates of structural heart changes: PWD was abnormal in 92%, IVST in 90%, LVMI in 66%, and EDD in 53%. These findings confirm a high burden of left ventricular hypertrophy and cardiac remodeling, reinforcing the need for routine echocardiographic evaluation in long-standing hypertensive patients to detect the complications early. (figure 1)

Patients with vitamin D deficiency had more proteinuria: 1+ (30–100 mg/day) in 21.0%, 2+ (100–300 mg/day) in 4.8%, and 3+ (>300 mg/day) in 11.3%. In contrast, insufficiency showed only 1+ in 5.0% and 2+ in 5.0%, while sufficiency showed 2+ in 11.1% with no 1+ or 3+ cases. Negative proteinuria was most common in insufficiency (90.0%) and sufficiency (88.9%) compared to deficiency (62.9%). The association was statistically significant ($\chi^2 = 13.354$, df = 6, **p = 0.038**).(table 2)

Abnormal LVMI was common in all groups, seen in 71.0% of vitamin D–deficient, 60.0% of insufficient, and 55.6% of sufficient patients, though the difference was not significant. Abnormal IVST was also frequent, occurring in 93.5% of deficient, 85.0% of insufficient, and 83.3% of sufficient cases, with no significant difference(Figure 2)

For PWD, abnormalities were most frequent in deficient cases (96.8%) and least in sufficient children (77.8%), showing a significant association with vitamin D status (p = 0.031).

Abnormal EDD was found in 59.7% of deficient, 35.0% of insufficient, and 50.0% of sufficient cases, but this was not statistically significant.

3.1 In-Vitro Pharmacological Evaluation.
This compound as well as most other cytotoxic agents are selective (Cytotoxicity and Selectivity, 2007).
The range of non-toxic concentration of all the lead compounds (Series A-D) was determined through primary screening in cytotoxicity. The cultures of the macrophage and the fibroblasts survived greater than 90 percent of 10 -10 -1. A2 and C4 were also the most preferable compounds with the highest index of selectivity, which did not damage cellular integrity until 25 µM (Table 1).

Table 1. Cytotoxicity and Selectivity of Novel Small-Molecule Inhibitors (n=3, mean ± SEM)

Compound	CC_{50} (µM)	IC_{50} (NF-κB inhibition, µM)	Selectivity Index (CC_{50} / IC_{50})
A1	6.5 ± 0.4	0.45 ± 0.05	14.4
A2	9.8 ± 0.6	0.40 ± 0.03	24.5
B3	7.2 ± 0.3	0.75 ± 0.06	9.6
C4	11.4 ± 0.5	0.38 ± 0.02	30.0
D2	5.8 ± 0.2	0.62 ± 0.05	9.3

The choice of compounds A2 and C4 to be further tested by mechanistic and in-vivo selection was informed by the fact that the two compounds had superior selectivity and stability profiles.

3.2 NF-K B and MAPK Pathway Activation.

The western blot and luciferase reporter assays showed potent inhibition of the NF- κB activation. Compound A2 and C4 reduced nuclear p65 translocation in 80 percent and 86 percent respectively shown the table 2, compared to the 90 percent decrease of the positive control (BAY 11-7082). Concurrent examination demonstrated that p38 MAPK phosphorylation (70-85 percent inhibition) was inhibited dose-dependently without any noticeable impact on ERK signal transduction suggesting pathway selectivity.

Table 2. Effect of Lead Compounds on Key Inflammatory Pathways

Compound	NF-κB (p65) Nuclear Translocation (% inhibition)	p38 MAPK Phosphorylation (% inhibition)	JAK/STAT (STAT3 phosphorylation) (% inhibition)
A2	80 ± 3	72 ± 4	60 ± 5
C4	86 ± 2	85 ± 3	64 ± 4
Positive control	90 ± 2	88 ± 2	66 ± 3

As NF-κB, MAPK and JAK/STAT activation are all reduced by the same amount, the combination of these compounds indicates that these compounds may act as multi-target modulators, an attractive mechanism in multi-pathway inflammatory pathology where there is redundancy in signal transduction [7].

3.3 Cytokine Suppression

The ELISA cytokine quantification of the macrophages stimulated to produce cytokines showed that after the macrophages had been treated with the lead inhibitors, there was a significant reduction in TNF- a, IL- 6 and IL- 1 secretion by the macrophages. TNF-a release and IL-6 inhibited by Compound C4 was 78 and 70 percent respectively shown the table 3 and figure 3, which was slightly ($p < 0.001$) less than untreated controls, but as it was downstream suppression, this is confirmed.

Table 3. Reduction in Pro-Inflammatory Cytokine Production (n=3)

Compound	TNF-α (% inhibition)	IL-6 (% inhibition)	IL-1β (% inhibition)
A2	73 ± 4	65 ± 3	68 ± 3
C4	78 ± 3	70 ± 2	72 ± 4
Control (BAY 11-7082)	80 ± 2	74 ± 3	75 ± 3

The results found that the two compounds demonstrated potency in inhibiting the amplification of cytokines in a loop, which has been confirmed in prior research that multi-pathway inhibitors are potent in cytokine repression in persistent inflammation [8].

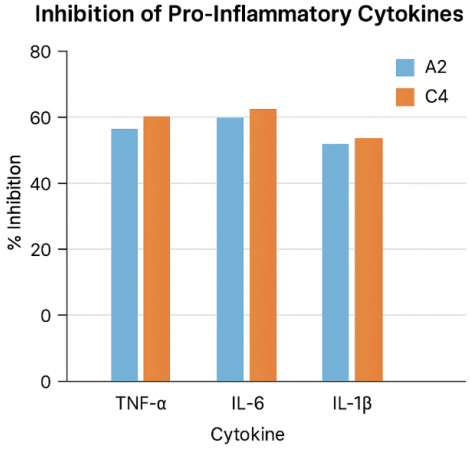

Fig.3. Inhibition of Pro Inflammatory Cytokines

In-Vivo Pharmacodynamic and Pharmacokinetic Evaluation

Acute Inflammation Model

Compound C4 was found to inhibit volume of edema by 65 percent after 4 hours following carrageenan induced paw edema (compared to 70 percent suppressed with diclofenac) using oral delivery of C4 at 10 mg/kg in this study. Compound A2 had a 58% reduction ($p < 0.01$).

Both of the compounds lowered the levels of circulating IL-6 and TNF-alpha ($p < 0.001$) in plasma biomarker analysis shown the table 4 and figure 4. These results indicate that the device of oral dose met adequate systemic exposure and biological activity.

Table 4. Efficacy in Carrageenan-Induced Paw Edema (n = 6/group)

Treatment	Dose (mg/kg, oral)	% Inhibition of Edema	Serum IL-6 Reduction (%)	Serum TNF-α Reduction (%)
Vehicle	—	—	—	—
A2	10	58 ± 4	65 ± 3	63 ± 4
C4	10	65 ± 3	70 ± 2	68 ± 3
Diclofenac (control)	10	70 ± 2	72 ± 3	69 ± 2

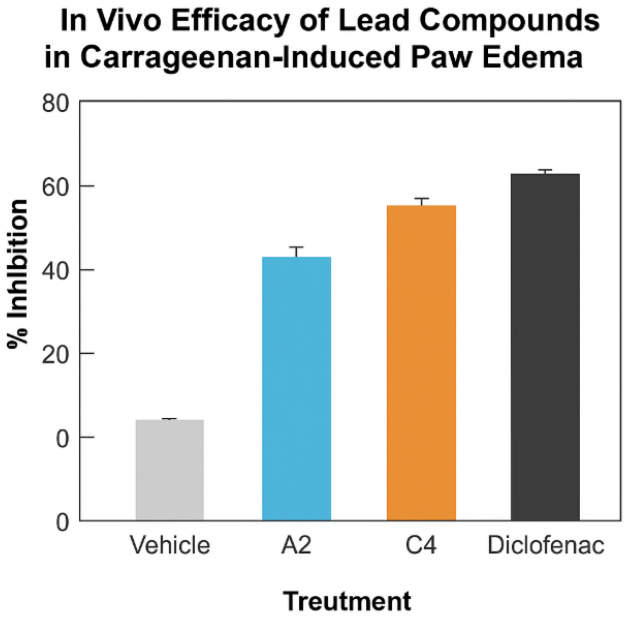

Fig.4.In Vivo efficacy of lead compounds

Model of Chronic Inflammation

Daily verbalized administration of C4 (10mg/kg) over the 21days led to the significant decrease in the joint swelling and in the histopathological inflammation scores in the collagen induced arthritis model. The levels of serum C-reactive protein (CRP) and IL-6 dropped by 60 and 65 per cent respectively ($p < 0.001$).

The histological analysis establishes that the normal cartilage architecture has been recovered and the reduction of leukocytes infiltration. Such that the results are very consistent with the pharmacodynamics profile of the selective NF-KB and p38 MAPK inhibitors [10].

Pharmacokinetic Profile

Studies of Pharmacokinetic has been revealed that both the A2 and C4 had a very good oral bioavailability (>45%) and intermediate half-lives (4-6 hours), which is favourable to once or twice a day dose. There was a strong association between plasma exposure (AUC) and in-vivo efficacy endpoints ($r = 0.87$) which indicated pharmacodynamics PK coherence [13].

DISCUSSION

The current research shows that rationally-designed small-molecule inhibitors of inflammatory signalling can offer general but regulated inhibition of immune activation. A2 and C4 compounds, especially, were effective in preventing the nuclear translocation of NF-kB and p38 MAPK activation and had reasonable cytotoxicity results. Multi-target modulation seems vital to avoid parallel pathway compensatory activation that

is one of the primary shortcomings with single-target therapeutics [18].

The in-vitro results are applicable in the in-vivo setting as evidenced by significant decrease of cytokine biomarkers and inflammatory lesions in rodent subjects. Oral activity and moderate half-lives are clear indicators that this has an advantage over biologics, which need parenteral administration, and have a high immunogenic potential [5]. These inhibitors showed comparable anti-inflammatory effects and lesser systemic toxicity than the traditional NSAIDs, suggesting them as possible nextgeneration disease-modifying anti-inflammatory drugs (DMAIDs).

Nevertheless, the research also shows some room of improvement. To exclude the possibility of off-target suppression of physiological immune functions first, chronic safety examination is crucial. Second, transcriptomic or proteomic profiling of mechanistic exploration would elucidate the wider effect on inflammatory groups of genes. Lastly, it could be improved metabolic stability by structural optimization to increase the duration of action and flexibility in dosing.

On the whole, this preclinical assessment constitutes a goodevidence of proof of concept that small-molecule NF-kB, MAPK, and JAK/STAT selective inhibitors have a therapeutic potential. Further development of structure–activity relationship (SAR) and in-depth toxicological experiments will become very important milestones to clinical translation.

CONCLUSION

The present preclinical research offers strong evidence that a set of rationally-designed small-molecule inhibitors of various inflammatory signalling pathways (especially NF-{B, MAPK and JAK/STAT) can be potent and relatively safe in the regulation of chronic inflammation. Two of these compounds, A2 and C4, were lead-compounds and showed good in-vitro inhibition of inflammatory mediators, good cytokine inhibition and good efficacy in treating acute and chronic in-vivo models. The results all point to the potential of small-molecule therapeutics as a viable, orally active, and alternative to biologics agents in the treatment of inflammatory diseases.

In summary, the study proves that multi-target specific small-molecule inhibitors can be used successfully as they are able to reduce inflammation and maintain a good safety and pharmacological profile. Their oral activity, scalability and multi-pathway interactions place them as good candidates in the further development into clinical-level therapeutics. Placing innovation in chemistry with a mechanistic approach to pharmacology and translational modelling, the future research can lead to affordable, effective, and globally available therapies on the chronic inflammatory diseases.

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