Tyrphostins as a promising therapeutic tool in inflammation-related conditions

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Abstract

Introduction
Protein kinases regulate the expression of many genes that are pivotal for inflammation. This review aims to summarise data on a group of small molecules capable to inhibit tyrosine kinases (TYKs), named tyrphostins. They are derivatives of benzylidemalonitrile that decrease tyrosine phosphorylation, thereby affecting not a single mediator, but cell signalling transduction is affected. No single animal model fully represents the human disease, but these models provide valuable tools to understand particular pathways. In this review, we describe some of the recent investigations on tyrphostins as anti-inflammatory agents in vivo in animal models, and in vitro in cell culture systems.

Conclusion
It is anticipated that tyrphostins are suitable for the treatment of inflammation-related diseases. Future research is required to understand the mechanisms of their action to pave way for gaining pharmacological benefits in clinical practice.

Introduction
Kinas play a central role in signal transduction. TYKs catalyse the transfer of the terminal phosphate group of adenosine triphosphate (ATP) to the tyrosine residues on protein substrates, resulting in an activation of the substrate and further participating in the next step of the signalling cascade\(^1\). The substrate is often another kinase or a transcription factor. TYKs are divided into receptor TYKs, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor receptor (FGFR) and non-receptor TYKs, including Src, ablerson murine leukaemia viral oncogene homolog (ABL), focal adhesion kinase (FAK) and Janus kinase (JAK)\(^2\). The receptor TYKs, are not only cell surface transmembrane receptors, but they also act as enzymes possessing kinase activity. Dysregulation of protein kinases is attributable to pathology of a variety of diseases, such as cancer, diabetes, autoimmune, cardiovascular, inflammatory, and nervous disorders. Because protein tyrosine kinases (PTKs) are critical components of cellular signalling pathways, their catalytic activity should be tightly regulated. Recently, over 20 drugs that target kinases have been introduced in clinical practice and many are currently in preclinical studies\(^3\).

The tyrphostins are derivatives of benzylidemalonitrile, where one group in the phenolic moiety is replaced either with other substituted benzenes or with heteroaromatic rings. Also, they can be derivatives of hydroxy-cis-benzylidemalononitriles, in which the malononitrile moiety is fixed relative to the aromatic ring or to the trans position to the benzenemalononitrile has been substituted by ketones and amides\(^5\). This class of low-molecular compounds shows high affinity toward EGF receptor thus, blocking its activity. First investigations on benzylidemalononitrile derivatives started two decades ago, when their ability to compete for the substrate-binding site of receptor TYKs for EGF(HER2) and PDGF was established\(^6\). Except monomer tyrphostins, dimeric molecules linked by various spacers possess significant potential to inhibit EGFR tyrosine kinase activity\(^9\). The results that tyrphostins act as selective anti-proliferative agents suppose their application in several diseases due to hyperproliferation\(^9\) and inflammation\(^10\). Later, a broad spectrum of biological activities have been added like antioxidant, increase of cellular glutathione level, suppression of poly ADP (adenosine diphosphate)-ribose polymerase (PARP) activation\(^11\), as well as these compounds potentiate the expression of some redox-sensitive genes associated to mitogen-activated protein kinase (MAPK), AP-1 and Nrf2 factors\(^12\). TYK inhibitors, except being broadly used in cancer clinical trials\(^13\), have also been evaluated in animal models of type 1 diabetes\(^15\), multiple sclerosis\(^17\), intestinal inflammation\(^18\) and septic shock\(^19\).

A number of studies have shown that in general tyrphostins express their anti-inflammatory activity by modulation of pro-inflammatory gene expression, such as cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS) and several pivotal cytokines (Figure 1). The activation of macrophages by different stimuli-like lypopolisaccharide\(^20\), resulted in tumour necrosis factor-α (TNF-α), interleukine-1β (IL-1β) and eicosanoid production via phosphorylation of TYKs\(^11\), in turn, targeted cell activation by these mediators happens through TYK signalling pathways. Therefore, TYK inhibitors can block the pro-inflammatory effects of cytokines.

The results of structure-activity investigations could very much...
in animal models of acute renal inflammation. The nephroprotective role of AG490 and AG126 has been proven in cyclosporine and cisplatin nephrotoxicity. The action of both substances is realised through inhibiting Janus kinase-2 (JAK2) activation\(^{23}\). AG490 has been tested in Adriamycin-induced nephrotic syndrome, related to strong STAT1, STAT2 and JAK2 phosphorylation\(^{24}\). Treatment with the drug causes inhibition of these events and leads to reduced proteinuria and creatinine levels at late period of disease. The positive effect was associated with less glomerulosclerosis and tubulointerstitial lesions as well as with decreased interstitial infiltration of macrophages and T cells. In a study performed by Chatterjee et al.\(^{25}\), histological observations showed that tyrphostin AG126 (5 mg/kg) reduced the expression of iNOS and nitric oxide (NO) levels in plasma and also suppressed protease-activated receptor (PAR) activation and COX2 expression in ischemia-reperfusion in rat, thus ameliorating renal dysfunction and injury. In rat haemorrhagic shock also, the inhibition of iNOS expression and nitrotyrosine formation by AG126 was observed after intravenous (i.v.) pre-treatment with the drug at a dose of 10 mg/kg prior to the onset of hemorrhage. Hepatic, brain and muscular injury, and to a less extent renal injury was abolished\(^{26}\). AG126 has been examined in another model, that is, splanchnic artery occlusion/reperfusion shock\(^{27}\). This investigation showed that tyrphostin injected intraperitoneal (i.p.) before ischemia (5 mg/kg) reduced TNF-α and IL-1β levels and intracellular adhesion molecule (ICAM)-1 expression in the reperfused ileum attended with improved histological observations. It is known that JAK-STAT signaling events are central in murine hepatic ischemia-reperfusion injury\(^{28}\). In this model, pre-treatment of mice with AG490 (40 mg/kg, i.p.) reduced
neutrophil infiltration and the expression of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6, IL-1β, CXC chemokine ligand (CXCL)-10 and CXCL-2. Decreased cleaved caspase-3 protein expression together with elevated BCL (B-cell lymphoma)-associated X (Bax) expression resulted in a reduced hepatocyte apoptosis and liver injury.

**Tyrophostins in sepsis models**

Despite significant advances in the treatment of sepsis, severe sepsis and septic shock continue to be associated with high morbidity and mortality. It is initiated by infection and can progress to haemodynamic changes, coagulopathic and inflammatory processes, often leading to organ failure. Expression of iNOS in many organs or tissues in septic shock results in an enhanced formation of NO that provoked hypotension, organ injury and impaired host defence. Recently, there has been a growing interest in the use of tyrphostins in septic models as anti-inflammatory agents. The application of AG490 in cecal ligation and puncture (CLP) significantly increased the survival and prevented injury of particular organs. It inhibited the excessive inflammation in lung and spleen, while it had no effect on liver injury. The beneficial response is related to the diminished production of two major participants in sepsis development of TNF-α and high-mobility group box (HMGB)1. The conclusion that tyrophostin has selectively inhibited the canonical nuclear factor-κB (NF-κB) pathway, while the other NF-κB pathways have not been affected, is important for revealing its mode of action.

The i.p. application of AG126 in zymosan-induced multiple organ failure in rats proved its positive effect on lung, liver and intestinal injuries due to a reduced PAR, iNOS and COX2 expression and lowered TNF-α and IL-1β production. In mice, administration of AG490 (5 mg/kg, i.p.) caused an inhibition of the acute phase of zymosan-induced organ dysfunction and revealed a wide range of activities. The substance abolished the elevated biochemical indices for renal and liver failures, attended with decreased levels of TNF-α, IL-6, macrophage inflammatory protein-1α (MIP-1α), regulated upon activation, normal T-cell expressed and secreted (RANTES), α1-antitrypsin and C5AR in kidneys. Simultaneously, AG490 suppressed STAT1 and STAT3 phosphorylations and decreased C5AR expression in kidneys, together with lowered iNOS and TNF-αR expression in liver.

Five tyrophostins (AG126, AG490, AG556, AG1641 and A1) have been thoroughly investigated in lipopolysaccharide (LPS)-induced shock in rats. They abolished the development of liver and pancreatic dysfunctions, expressed as decreased lactic acidosis and hypoglycaemia and plasma TNF-α level. In the lung, the positive effects of AG126 and AG556 have been attributed to the suppressed iNOS and COX-2 protein expression and activity. AG126 and AG556 have been studied in canine *Escherichia coli* sepsis also. While AG556 significantly lowered the serum TNF levels, thus preventing cytokine-induced multiorgan failure and death, AG126 on whole, did not show such therapeutic effect in this model. In these experiments antibiotic therapy presented simultaneously with the tyrophostin therapy, as the increase in survival rate was elevated compared to antibiotic alone. It should be mentioned that after the drug administration, the host defence was not suppressed, because the clearance of bacteria and endotoxin remained unchanged.

It is accepted that endotoxic shock is related with changes of TYK signalling leading to modulation of cardiac action potential duration (APD). Tyrophostin AG556 has been administered once or during 10 days in a guinea pig, with LPS-induced shock. The results from this observation showed that it significantly attenuated the changes in APD, due to the decrease in the plasma NO and the cardiac cyclic guanosine monophosphate (cGMP) production.

**Tyrophostins in neuropathological models**

Available results suggested that tyrophostin AG490 may have therapeutic potential by blocking TYK activities responsible for the development of inflammation concerning central nervous system (CNS). Experimental allergic encephalomyelitis (EAE) is a model resembling human multiple sclerosis. This autoimmune inflammatory demyelinating disease of the CNS is T Helper cell type 1 (Th1)-mediated and IL-12 is a major cytokine, which plays a pathological role. The administration of AG490 in mice suppressed the inflammation through inhibition of IL-12 signalling and prevented the development of the disease.

The entry of activated T lymphocytes into the parenchyma of the CNS is a critical event in the pathogenesis of many experimental rodent models of neuroinflammation when some brain barriers are lost. Tyrophostin AG490 has been able to block lymphocyte adhesion to brain endothelium and prevent the entry of ependymaligenic T cells into the brain in a model of murine encephalomyelitis. AG490 totally blocked the disease in mice, treated with 3 mg of AG490 daily, during 25 days.

Two tyrophostins, AG126 and AG556, have been effective in a model of spinal cord trauma in mice, characterised with massive cell infiltration, cytokine release, apoptosis and tissue injury. Both compounds retarded the development of inflammation and limited tissue lesions. It is considered that such curing effect is a result of lowered myeloperoxidase (MPO) activity and iNOS, nitrotyrosine and PARP expression.

It has been established that the administration of AG126, expresses anti-inflammatory properties in an *in vivo* model of pneumococcal
meningitis. Its mode of action is related to a decrease of TNF-α concentration in the cerebrospinal fluid and inhibition of MAPK phosphorylation in microglial cells.42,43.

Tyrphostins in arthritis and myocarditis models

Much less attention has been paid on the investigation of tyrphostins in chronic inflammation. STAT phosphorylation by activated JAKs is critical for perpetuating inflammation in rheumatoid arthritis (RA). In addition, cytokine-induced JAK/STAT activation can be attended with the activation of other pathways, such as stress-activated protein (SAP)/MAPK and phosphatidylinositol-3-kinase (PI3K)-mediated signalling44. A study by Cuzzocrea et al. has been focused on the effect of tyrphostin AG126 as anti-rheumatic agent45. AG126 successfully ameliorated collagen-induced arthritis (CIA) shown by radiography and histopathological examinations. The mode of anti-inflammatory action of AG126 corresponds to a suppression of iNOS and COX-2 expression, NO production and lowered PARP activity. This mechanism is supported by the in vitro data that AG490 down-regulates STAT1 activation, iNOS expression and NO production by interferon-gamma (IFN-γ) stimulated macrophages.46. The suppression of STAT pathway seemed perspective in fighting joint inflammation having in view that tofacitinib is one of the first JAK inhibitors approved initially as an immunosuppressive agent in renal transplantation, and now is as an immunosuppressive agent in rheumatoid arthritis. It interacts with JAK1 and JAK3 targeting mainly T and B cells.47,48. It will be important to determine whether JAK antagonists are acting principally on T or B cells and which T- and B-cell subsets are involved? Further studies are warranted to better define the role of tyrphostins in the treatment of arthritis using them alone or in combination with conventional drugs.

Two tyrphostins, AG126 and AG556, have been comparatively studied in experimental autoimmune myocarditis in rats. This is a T-cell-mediated disorder, TNF-α being one of the major cytokines responsible for the pathological events.49. AG556 significantly attenuated the development of myocarditis and this well correlated with the reduction of TNF-α and IFN-γ production, while AG126 failed to be effective. Prolonged AG556 administration (21 days) prevented the progression of myocarditis without impairment of the immune system.

AG490 in cell culture systems

Tyrphostin AG490 is by far the most extensively studied JAK2 inhibitor; so it deserves to mention the studies on its mechanism of action in vitro. However, its activity also includes blocking of c-Src activity50,51 and cyclin-dependant kinase 2 (Cdk2) activation52. It is considered that AG490 does not affect several protein kinases, such as lymphocyte-specific protein-tyrosine kinase (LCK), LCK/Yes-related novel tyrosine kinase (LYN), Bruton’s tyrosine kinase (BTK), spleen tyrosine kinase (SYK), JAK3 and tyrosine kinase 2 (TYK2)53. Now, it is claimed that AG490 is able to inactivate not only JAK2, but also JAK3 and STAT5a/b signalling pathways in activated T cells.54. AG490 influenced immune response of human T cells in vitro as evident by the results, which showed that tyrphostin prevented IL-2-mediated proliferative growth and expansion55. Simultaneously, AG490 did not influence the activation of zymosan-activated plasma 70 (ZAP70) and p56LCK after anti-CD3 stimulation. This shows that AG490 inhibits the JAK3-mediated type 2 signalling pathway, but it does not affect type 1 pathway.

AG490 has been investigated in IL-6-dependent human multiple myeloma (MM) cell lines. It blocked the glycoprotein 130 (GP130) signalling pathway at the JAK level through inhibition of extracellular receptor kinase 2 (ERK2) and STAT3 phosphorylation, resulting in suppressed cell proliferation and increased apoptosis of cells.56. AG490 suppressed JAK2-STAT3 pathway in human fibroblast-like synoviocytes (FLS) associated with a decrease of mitochondrial ribonucleic acid (mRNA) expression and receptor activator of nuclear factor kappa β ligand (RANKL) production57. Of interest, AG490 significantly increased expression of forkhead box P3 (Foxp3) and lowered expression of co-stimulatory molecules in CD4 T-cells and in bone marrow-derived dendritic cells obtained from mice with type 1 diabetes58.

Conclusion

The development of TYK inhibitors has opened a new trend in cancer therapy followed by intensive investigations on these molecules, which possess relatively selective action and low toxicity. Later on, a series of investigations showed their potential to relief inflammatory symptoms in various animal models. Three tyrphostins, AG126, AG556 and AG490, are broadly studied in various experimental models, which showed affection of many pathways, controlling inflammation (Table 1). It should be mentioned that the doses used are mainly in the intervals between 2.5 mg/kg and 10 mg/kg, which suppose comparable results. There is little information about the specificity and selectivity of tyrphostins. Recent findings summarised in Figure 1, demonstrate that tyrphostins regulate the transcription of multiple proteins that could promote inflammation, dependent on the pivotal cytokines, such as TNF-α, IL-1 and IL-6. STAT phosphorylation is a crucial point targeted by tyrphostins whose inhibition can contribute for the better outcome of inflammatory processes. Due to their significant in vivo activity, tyrphostins might be considered as reasonable candidates for new anti-inflammatory drugs.
Determining their extensive safety profiles will provide a strong foundation for their translation to humans. Data from experiments on long term administration of AG490 in type 1 diabetes model (5 weeks) showed that it did not cause clinical signs of toxicity and changes concerning behaviour, loss of body weight, aggressiveness and hypermobility. In two other studies, 25-day treatment with AG490 or 21-day treatment with AG556 has not been associated with toxicity or side effects. It will be critical to understand how tyrphostins are working and to carefully examine their toxicities. However, final proof of the use of data determined in animal models lies in clinics and clinical data will help shape and define the models.

**Abbreviations list**

APD, action potential duration; CNS, central nervous system; COX2, cyclooxygenase-2; CXCL, CXC chemokine ligand; EGF, epidermal growth factor; JAK, Janus kinase; JAK2, Janus kinase-2; IFN-γ, interferon-gamma; IL-1β, interleukine-1β; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; iv., intravenous; MIP-1α, macrophage inflammatory protein-1α; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; PAR, protease-activated receptor; PARP, poly ADP (adenosine diphosphate)-ribose polymerase; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF-α, tumour necrosis factor-α.

**Table 1. Anti-inflammatory action of AG126, AG556 and AG490 in animal models.**

<table>
<thead>
<tr>
<th>Model</th>
<th>Targeted pro-inflammatory mediators</th>
<th>Host</th>
<th>Route</th>
<th>Dose</th>
<th>Compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> peritonitis</td>
<td>TNF-α</td>
<td>beagle</td>
<td>i.v.</td>
<td>2.5 mg/kg</td>
<td>AG556</td>
<td>[19]</td>
</tr>
<tr>
<td>Endotoxic multiple organ failure</td>
<td>TNF-α, iNOS, COX2</td>
<td>rat</td>
<td>i.p.</td>
<td>5 mg/kg</td>
<td>AG126, AG556, AG490</td>
<td>[34]</td>
</tr>
<tr>
<td>Cecal ligation and puncture</td>
<td>NF-κB, TNF-α, HMGB1</td>
<td>mouse</td>
<td>i.p.</td>
<td>10 mg/kg</td>
<td>AG490</td>
<td>[30]</td>
</tr>
<tr>
<td>Zymosan-induced peritonitis</td>
<td>TNF-α, IL-1β, iNOS, PAR, COX2</td>
<td>rat</td>
<td>i.p.</td>
<td>10 mg/kg</td>
<td>AG126</td>
<td>[31]</td>
</tr>
<tr>
<td>Zymosan-induced peritonitis</td>
<td>TNF-α, IL-6, MIP-1α, RANTES, iNOS, STAT1, STAT3</td>
<td>mouse</td>
<td>i.p.</td>
<td>5 mg/kg</td>
<td>AG490</td>
<td>[32, 33]</td>
</tr>
<tr>
<td>Spinal cord inflammation</td>
<td>iNOS, MPO, nitrotyrosine, PARP</td>
<td>mouse</td>
<td>i.p.</td>
<td>3.5 mg/mouse</td>
<td>AG126, AG556</td>
<td>[41]</td>
</tr>
<tr>
<td>Collagen-induced arthritis</td>
<td>iNOS, NO, COX2, PARP</td>
<td>rat</td>
<td>i.p.</td>
<td>5 mg/kg</td>
<td>AG126</td>
<td>[45]</td>
</tr>
<tr>
<td>Autoimmune myocarditis</td>
<td>TNF-α, IFN-γ</td>
<td>rat</td>
<td>i.p.</td>
<td>0.5 mg/kg</td>
<td>AG556</td>
<td>[49]</td>
</tr>
</tbody>
</table>

*Note: COX2, cyclooxygenase-2; HMGB1, high-mobility group box 1; IFN-γ, interferon-gamma; IL-1β, interleukine-1β; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; iv., intravenous; MIP-1α, macrophage inflammatory protein-1α; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; PAR, protease-activated receptor; PARP, poly ADP (adenosine diphosphate)-ribose polymerase; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF-α, tumour necrosis factor-α.*

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