PRO-INFLAMMATORY SIGNAL DELIVERED IN VITRO BY LEFLUNOMIDE ACTIVE METABOLITE A77 1726 ON ABNORMAL PERIPHERAL MONOCYTES IN RHEUMATOID ARTHRITIS

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Abstract

We investigated the effects exerted in vitro by leflunomide active metabolite A77 1726 and methotrexate (MTX) on cytokine release by monocytes from rheumatoid arthritis patients. Our results give insight information about the action mode of A77 1726 on excessively numerous, activated peripheral monocytes from rheumatoid arthritis patients which do not respond to the current therapy. We highlight that low concentrations of A77 1726 may exert in vitro a pro-inflammatory effect by sustaining the release of pro-inflammatory factors by such abnormal peripheral monocytes (tumor necrosis factor-α - TNFα, granulocyte macrophage colony-stimulating factor - GM-CSF and interleukin-8 - IL-8). This is a direct effect of A77 1726, which is not related to a significant disturbance of pyrimidine metabolism. We showed that MTX can also sustain in vitro the release of GM-CSF. Accordingly, our study points out that in particular circumstances, such as a high number of activated peripheral monocytes subjected to low concentrations of drugs, A77 1726 and to a lesser extent MTX, may sustain the systemic inflammatory process in rheumatoid arthritis.

Rezumat

Am investigat efectele exercitate in vitro de metabolitul activ al leflunomidului A77 1726 și de metotrexat (MTX) asupra eliberării de citokine de către monocitele izolate de la pacienți cu artrită reumatoidă. Rezultatele noastre relevă noii aspecte privind modul de acțiune al metabolitului A77 1726 asupra monocitelor de la pacienți cu artrită reumatoidă, care prezintă un număr mare de monocite activate în periferie și care nu răspund la terapia curentă. Evidențiam faptul că la concentrații mici, A77 1726 poate exercita in vitro efecte pro-inflamatoroare prin intensificarea eliberării de factori pro-inflamatori de către astfel de monocite patologice (factorul de necroză tumorală α - TNFα, factorul de stimulare a coloniilor de granulocite și macrofage - GM-CSF și interleukina-8 - IL-8). Acesta este un efect direct al metabolitului A77 1726 asupra monocitelor, care nu este corelată cu alterarea semnificativă a metabolismului pirimidinelor. Astfel, studiul nostru relevă faptul că în condiții particulare, cum ar fi număr mare de monocite periferice active, leflunomidul, și în mai mică măsură MTX, pot susține procesul inflamator sistemic în artrita reumatoidă.
Keywords: rheumatoid arthritis, A77 1726, methotrexate, monocytes, cytokines

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease causing preclinical systemic abnormalities that finally lead to synovial inflammation and destruction of joint architecture. Whilst considered a T cell-driven autoimmune disease [2,13], final cartilage and bone destruction in RA is mediated by activated synoviocytes and monocytic cells, some of which differentiate into tissue-destructive osteoclasts [13,16]. The “macrophage-fibroblast” theory of RA was lately confirmed by the success of therapies aiming at neutralizing pro-inflammatory cytokines produced by monocytes/macrophages [29].

The biologic therapy in RA is nevertheless a second-line therapy, applied when patients do not respond to disease-modifying anti-rheumatic drugs (DMARD) or severe side effects are registered [31].

MTX is the most common DMARD used in RA, which acts by inhibiting dihydrofolate reductase, hence decreasing the supply of reduced folates for purine biosynthesis and by increasing extracellular adenosine [9,10].

Leflunomide is an isoxazole derivative which inhibits inflammation and joint destruction [1]. It acts as a pro-drug and is quickly metabolized in the gut wall and in the liver into the active metabolite A77 1726. At least two mechanisms of action have been documented [6]: inhibition of dihydroorotate dehydrogenase (DHODH), the rate limiting step in de novo pyrimidines synthesis, and interaction with signaling events. Leflunomide action is mainly targeted towards lymphocytes [18] whose functions are highly dependent on pyrimidine biosynthesis [12]. Leflunomide also inhibits neutrophil chemotaxis independent of nucleotide biosynthesis inhibition [19]. Leflunomide exerts an anti-inflammatory action by targeting the cytokine/chemokine network: it decreases interferon-γ (IFNγ) produced by activated T lymphocytes [18], tumor necrosis factor α (TNFa) and interleukine-1β (IL-1β) released by macrophages from synovial tissue [10] and induces the decline of chemokines addressing the phagocyte system (monocyte chemotactic protein-1 – MCP-1, IL-8) [17].

The action mode of leflunomide on abnormal peripheral monocytes (Mo) from RA patients is still poorly understood. We previously demonstrated [23] that leflunomide can reduce the abnormally high peripheral Mo counts in correlation with the decrease of the acute phase response. Nevertheless, we observed in particular cases that initially normal Mo counts increased above the normal range albeit the clinical improvement.
In this paper we investigated the effects exerted \textit{in vitro} by low concentrations of the active leflunomide metabolite A77 7126 and methotrexate (MTX) on cytokine release by pathologic peripheral Mo from RA patients.

\textbf{Materials and methods}

\textit{Chemicals}

Bicoll, Roswell Park Memorial Institute 1640 (RPMI-1640) medium, phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Biochrom (Germany), CellTiter 96\textsuperscript{R} AQueous One Solution Cell Proliferation Assay from Promega Corporation (Germany). Lipopolysaccharide (LPS) from \textit{E. coli} 0111:B4, dimethyl sulfoxide (DMSO), phytohemagglutinin M (PHA), methotrexate (meeting the USP testing specifications) were from Sigma-Aldrich (Germany). The Milliplex MAP for cytokines was purchased from Millipore (USA) and the BurstTest kit from ORPEGEN Pharma (Germany). All reagents were endotoxin free.

The active leflunomide metabolite A77 1726 was a kind gift from Dr. Marius Albulescu, Medical Advisor for Rheumatology&Aprovel at Sanofi-Aventis, Romania. Tritium-labeled uridine (radioactive concentration 25.9 MBq/mL and 851 GBq/mmol specific activity), tritium-labeled thymidine (radioactive concentration of 22.9 MBq/mL and 905 GBq/mmol specific activity) were kindly provided by Dr. Cristian Postolache from the Institute of Physics and Nuclear Engineering “Horia Hulubei”, Măgurele.

MTX was dissolved in DMSO at the concentration of 100 mM. Further dilutions were performed in RPMI-1640 medium and at least a final 10000x dilution of DMSO was performed. A77 1726 was dissolved in PBS at the concentration of 10 mM, aliquoted and kept at - 70\textsuperscript{0}C until use.

\textit{Patients}

We selected a group of 10 patients with severe RA, fulfilling the revised American College of Rheumatology (ACR) criteria [3], monitored at the “Sf. Maria” Clinical Hospital, Bucharest. Patients were positive for rheumatoid factor and presented an active disease (disease activity score 28 - DAS28 > 5.1, erythrocyte sedimentation rate > 30mm/h, number of tender joints > 6, number of swollen joints > 5, morning stiffness > 1h). Eight patients were on MTX, 2 patients were on leflunomide therapy for more than 6 months and were not responding to the current therapy. Healthy volunteers were also investigated. The study was approved by the Ethical Committee of the Hospital and was carried out in accordance with the Declaration of Helsinki [34].
Cells
Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation [5] using Bicoll (specific gravity 1.077). Cells were counted in a Burker-Turk chamber and cellular viability, scored by the trypan blue exclusion test, exceeded 99%. Turk’s solution was used for differential counting of Mo and lymphocytes. Cell counts were divided by the initial volume of blood subjected to the cell separation procedure.

Cell cultures
PBMC were the source of Mo in our experiments. Mo were allowed to adhere to plastic overnight and non-adherent cells were then removed. Adherent Mo were incubated for 4 hrs with A77 1726 or MTX and challenged thereafter with 2 µg/mL lipopolysaccharides (LPS) for another 20 hrs, in the presence of A77 1726 or MTX. Several controls were considered: cells without stimuli or modulators and cells treated only with LPS. Culture supernatants were harvested and kept until use at -70°C.

Cell viability
Cellular viability was assessed by the MTS reduction test, using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Mo cultures were obtained as described above. Samples containing only complete culture medium were used for background measurement. At the end of the experimental protocol, 20 µL of MTS was added and after 3 hrs the optical density (OD) was recorded at 490 nm (reference wavelength of 620 nm). Measurements were performed using the BioRad PR3100 ELISA reader. The background OD was subtracted from the sample OD.

Intracellular oxidative activity
Intracellular production of reactive oxygen species by peripheral Mo was assessed in whole blood by flow-cytometry (BurstTest kit), using the fluorogenic substrate dihydrorhodamine (DHR) 123. Heparinized blood (100µL) was incubated in absence or presence of opsonized E. coli or phorbol myristate acetate (PMA) for 10 min at 37°C. DHR 123 was then added and incubation continued for another 10 min at 37°C. After the erythrocytes lysis, 200 µL of propidium iodide were added. Cell analysis was performed by flow cytometry using a FACSCanto flow-cytometer, by mean of the CellQuest software (Becton Dickinson). Results were expressed as percentage of Mo developing intracellular oxidative activity.

Tritium-labeled uridine and thymidine uptake by PBMC
The ability of mitogen-activated PBMC to incorporate uridine and thymidine was assessed using the tritium-labeled form of nucleosides [24]. PBMC (1x10⁶ cells/mL) were stimulated for 72 hrs with 10 µg/mL PHA in the absence and presence of A77 1726 or MTX. Cells were labeled with 0.5
µCi/well of tritium-labeled thymidine or uridine for the final 18 hrs and 6 hrs, respectively. Cellular β radioactivity was measured with a Canberra-Packard Beta Counter (PerkinElmer). Results were expressed as counts per minute (cpm).

**Cytokines**

Cytokines profile in culture supernatants was evaluated using the xMAP array technology (Millipore), using the protocol provided by the manufacturer. Briefly, 25 µL of cell culture supernatant were incubated overnight with dyed beads coated with cytokine-specific capture antibodies. After washing, beads were incubated with the fluorescent tagged detection antibody. Beads were washed and were then subjected to measurement in the Luminex platform (Luminex B.V., Netherlands). Results were expressed as cytokine concentration (pg/mL) based on calibration curves.

**Data processing**

Results were presented as specific values provided by the experimental method or as the effect exerted by A77 1726 or MTX. The effect was calculated as value in presence of modulator, divided to the corresponding value in absence of the modulator.

Data was processed as mean ± standard error of the mean (SEM). RA patients were compared with normal individuals using the t-Test with unequal variances (Excel), whilst effects were compared to the unit effect using the t-Test for paired samples (Excel).

**Results and discussion**

The immunological screening showed that RA patients (n=14) with active disease, non-responders to current MTX therapy, presented abnormally high peripheral Mo counts (1.06 ± 0.35 versus 0.28 ± 0.04, p<0.05) and a consequently disturbed lymphocytes per monocyte ratio (2.7 ± 0.5 versus 5.6 ± 0.6, p<0.001). An accelerated generation of CD14+ Mo from the bone marrow and their maturation into activated cells (human leukocyte antigen-DR+ – HLA-DR+) is registered in RA patients [14,16].

For this study we selected 6 RA patients with severe disease (4 patients on MTX and 2 patients on leflunomide therapy) presenting high peripheral Mo counts (3.0 ±0.3 versus 0.28 ± 0.04, p<0.001). We found that Mo isolated from these RA patients were activated in vivo [20]. We showed (Figure 1) that a significantly high percentage of Mo developed constitutive intracellular oxidative activity and were also highly reactive if challenged ex vivo with *E. coli* or with phorbol myristate acetate (PMA).
Figure 1.
The percentage of peripheral Mo which developed intracellular oxidative activity, either constitutively (control) or induced ex vivo with E. coli or PMA. Results were presented as *p<0.05; **p<0.01; ***p<0.001

By xMAP array we measured cytokines in culture supernatants (Table I) and we found that adherent Mo released spontaneously both pro-inflammatory factors (IL-6, IL-8, TNFα, GM-CSF) and anti-inflammatory ones (IL-10). Ex vivo treatment of Mo with LPS [28], did not significantly increase cytokine release, probably due to the constitutive activation of Mo. By activating Mo with LPS we obtained a more uniform status of cell activation among individual cell samples [25].

Table I.
Cytokines released ex vivo by Mo isolated from RA patients (n=6)

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNFα</th>
<th>GM-CSF</th>
<th>IL-10</th>
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<tbody>
<tr>
<td>(pg/mL)</td>
<td></td>
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<tr>
<td>Spontaneous release</td>
<td>1744 ± 808</td>
<td>11530 ± 3669</td>
<td>59 ± 34</td>
<td>10 ± 4</td>
<td>531 ± 270</td>
</tr>
<tr>
<td>LPS-induced release</td>
<td>1844 ± 639</td>
<td>13720 ± 1942</td>
<td>66 ± 22</td>
<td>14 ± 3</td>
<td>774 ± 199</td>
</tr>
</tbody>
</table>

We studied the biological effects exerted in vitro by A77 1726 and MTX at the level of cytokine release by LPS-activated Mo from RA patients (Table I). We investigated low concentrations of therapeutic agents (0.25-2 µM A77 1726 and 12.5-100 nM MTX) [4,21], that were not cytotoxic to LPS-activated Mo, as assessed by the MTS reduction test (Figure 2).
Figure 2.

The effect exerted in vitro by A77 1726 or MTX on MTS reduction by LPS-activated Mo from RA patients (n=6).

We studied the profile of cytokines released by LPS-activated Mo isolated from RA patients (n=6, Table I), treated in vitro with A77 1726 or MTX. TNFα, IL-6, IL-8, IL-10 and GM-CSF, IL-1β and IL-12 levels in culture supernatants were assessed using the xMAP array technology.

We showed that low concentrations of A77 1726 (0.25 µM) clearly stimulated TNFα release by LPS-activated Mo, fitting a descending dose-effect profile (Figure 3). MTX exerted in vitro an inhibitory effect on TNFα release, at the particular concentration of 50 nM (Figure 3). Amplified production of TNFα in RA is known to mediate enhanced synovial proliferation, production of prostaglandins and metalloproteinases, bone destruction and periarticular osteoporosis [29].

Figure 3.

The effect exerted in vitro by A77 1726 or MTX on TNFα release by LPS-activated Mo from 6 RA patients (see Table I). *p<0.05

A77 1726 exerted in vitro clear stimulatory effects on GM-CSF release by LPS-activated Mo from RA patients, especially at 0.5µM and
2µM (Figure 4a). We observed that MTX had a stimulatory, dose-dependent action on GM-CSF release by Mo from RA patients treated with MTX, but no in vitro effect was observed in the case of RA patients on leflunomide therapy (Figure 4b). Probably, only Mo sensitized in vivo to MTX, responded with enhanced GM-CSF release to an additional in vitro MTX challenge. GM-CSF is a well recognized hematopoiesis regulator of myeloid cells production, differentiation and activation, responsible for driving inflammatory and autoimmune responses [8].

![GM-CSF](image1)

**Figure 4.**

The effect exerted in vitro by A77 1726 or MTX on GM-CSF release by LPS-activated Mo from RA patients on MTX (4) or on leflunomide therapy (2). *p<0.05

In addition, we showed that 0.5 µM A77 1726 exerted in vitro a stimulatory action on IL-8 release by LPS-activated Mo from RA patients (effect = 1.7 ± 0.3, p=0.028), whilst MTX had not a statistically significant effect. IL-8 is a CXC chemokine produced by macrophages, epithelial and endothelial cells, serving as a local chemical signal that attracts neutrophils at the inflammatory site [32]. The clinical significance of increased IL-8
levels in periphery, besides being a marker of active inflammation, is still unclear.

A high interindividual variability was noticed regarding the effects of A77 1726 and MTX on IL-6 [27] release by LPS-activated Mo from RA patients (data not shown).

We detected only low concentrations of other pro-inflammatory cytokines, such as IL-1β (8.5 ± 3.6 pg/mL) and IL-12 (1.1 ± 0.1 pg/mL), that we considered inappropriate for a reliable discussion about the effects exerted in vitro by the drugs.

The pro-inflammatory action exerted in vitro by A77 1726 (Figures 1-4) was not accompanied by a significant effect on the release of the anti-inflammatory cytokine IL-10 [11] by LPS-activated Mo (Figure 5). In turn, we showed that low concentrations of MTX (12.5 nM and 25 nM) induced a moderate limitation of IL-10 release (Figure 5). Hence, MTX could exert a moderate pro-inflammatory effect by blocking anti-inflammatory mechanisms.

The cellular targets of leflunomide (dihydroporotate dehydrogenase - DHODH) and MTX (dihydrofolate reductase and thymidilate synthase) are key enzymes in the nucleotide biosynthesis pathway [6,30]. Therefore, we evaluated tritium-labeled uridine and thymidine uptake by PHA-activated PBMC from 2 normal subjects and 4 RA patients, treated in vitro with A77 1726 or MTX. Proliferating cells, such as PHA-activated lymphocytes, are more sensitive to the inhibition of the mentioned enzymes than non-proliferating cells (i.e. Mo) [12].

We showed that A77 1726 did not affect uridine or thymidine uptake by normal PBMC (Figure 6a), indicating that the observed effects exerted in
vitro by A77 1726 on cytokine release were not correlated with an alteration of pyrimidine metabolism. Meanwhile, we found that MTX inhibited in vitro uridine uptake, whilst stimulating thymidine uptake by normal PBMC (Figure 6b). This is probably a consequence of thymidilate synthase inhibition and limitation of cell pyrimidine stores [30]. We noticed that MTX stimulated thymidine uptake by normal PBMC in a dose-independent manner (Figure 6b), whilst the reactivity of PBMC from RA patients was dependent on MTX concentration (Figure 7c). Therefore, it is not clear whether the observed action of MTX on cytokine release is directly associated to an alteration of thymidine metabolism.

![Figure 6 (a,b).]

The effect exerted in vitro by A77 1726 or MTX on tritium-labeled uridine or thymidine uptake by PHA-activated PBMC from normal subjects (n=2) or RA patients (n=4). *p<0.05; **p<0.01
Our results point out that A77 1726 can exert in vitro a pro-inflammatory action related to increased production of pro-inflammatory factors by peripheral Mo from RA patients. These effects might be confined to low concentrations of A77 1726 and to cells isolated from RA patients presenting high counts of activated peripheral Mo. Various other studies indicated that anti-rheumatic drugs may exert pro-inflammatory effects in particular in vitro conditions. Thus, A77 1726 was shown to stimulate the proliferation of synovial fibroblasts and this effect was not mediated through depletion of the pyrimidine pool or inhibition of tyrosine kinases [22]. Gold sodium thiomalate and MTX augmented spontaneous TNFα production by PBMC from normal individuals and RA patients [33]. These results do not rule out that the overall action of leflunomide in vivo might be anti-inflammatory even at low drug concentrations, due to the complex network of pro- and anti-inflammatory processes [7].

We showed that MTX exerted mainly anti-inflammatory effects on the investigated abnormal Mo and probably the real inhibitory effects where partially masked in our experimental model by the high content of folic acid in the RPMI-1640 culture medium [26]. Like A77 1726, MTX also stimulated in vitro GM-CSF release and this could lead to the maintenance of the abnormal status of Mo.

Conclusions
Our study gives insight information about a novel action mode of the active leflunomide metabolite on excessively numerous, activated peripheral
Mo from RA patients. We highlight that low concentrations of A77 1726 may exert, at least in vitro, a pro-inflammatory effect by sustaining the production of pro-inflammatory factors by such abnormal peripheral Mo (TNFα, GM-CSF and IL-8). This is a direct action of A77 1726 on Mo, which is not related to a significant disturbance of pyrimidine metabolism. MTX was also shown to stimulate GM-CSF release by pathologic Mo. In particular conditions A77 1726 and, to a lesser extent MTX, may sustain the systemic inflammatory process in RA.

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