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Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2014, **115**, 499–506 Doi: 10.1111/bcpt.12268

Saquinavir-NO Inhibits IL-6 Production in Macrophages

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(Received 30 October 2013; Accepted 5 May 2014)

Abstract: Covalent attachment of the nitric oxide (NO) moiety to the HIV protease inhibitor Saquinavir (Saq) produced a new chemical entity, named Saquinavir-NO, (Saq-NO) with reduced toxicity and potent immunoregulatory influence on T lymphocytes. In this study, we have compared head-to-head the effects of Saq-NO and Saq on mouse and rat peritoneal macrophage cytokine secretion and NO production upon *in vitro, ex vivo* and *in vivo* conditions. The results demonstrate that Saq-NO, but not Saq, potently decreased interleukin (IL)-10, IL-6 and nitrite accumulation and increased the levels of IL-1β and tumour necrosis factor (TNF) in supernatants of mouse and rat macrophage cultures *in vitro*. Treatment of mice with Saq-NO, but not Saq, inhibited *ex vivo* secretion of IL-6 from macrophages. Consistent with these findings, Saq-NO also reduced blood levels of IL-6 in lipopolysaccharide-treated mice. The observed inhibitory influence of Saq-NO on IL-6 generation in macrophages may be involved in the observed antitumour and immunomodulatory effects of the drug.

Saquinavir (Saq) is the first approved protease inhibitor for the treatment of HIV [1]. Protease inhibitors in combination with nucleoside or non-nucleoside reverse transcriptase inhibitors are the basis for the highly active antiretroviral therapy (HAART) that is efficient in suppression of HIV replication and reduction of clinical manifestations of the disease [2]. In addition to antiviral effects, Saq has also been reported to possess anticancer and immunomodulatory effects [3,4], but also to exert various side effects in patients [5].

It has been previously demonstrated that nitric oxide (NO)-hybridization may reduce toxicity of parental compounds while enhancing the pharmacological potency of the drugs [6]. Along this line of research, a derivative of Saq, named Saquinavir-NO, (Saq-NO, OX1001) was synthesised at OncoNOx (Copenhagen, Denmark) by covalent attachment of NO moiety to Saq. Pre-clinical studies have proven that Saq-NO represents a new chemical entity (NCE) endowen with lower toxicity, equal antiviral and superior antitumour properties than Saq [7–12]. We have also recently shown that Saq-NO exerts a potent immunomodulatory effect on T lymphocytes *in vitro* and that it ameliorates the clinical course of MOG-induced and PLP-induced EAE that represent two well-known pre-clinical models of multiple sclerosis [13].

These data prompted us to study the influence of Saq-NO on macrophages under different experimental conditions. Macrophages play a key role in immunoinflammatory responses by releasing a wide range of soluble pro-inflammatory mediators, including various cytokines and reactive oxygen and nitrogen species, such as NO. Thus, we presently compared head-to-head the influence of Saq-NO and Saq on the secretion of interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor (TNF) and NO from macrophages. Our results prove that, relative to cells untreated or treated with Saq that exhibited a super-imposable profile of cytokine and NO secretion, Saq-NO reduced the production of NO, IL-10 and, in particular, IL-6 and it stimulated that of TNF and IL-1β in rat and mouse macrophages *in vitro*. Saq-NO also inhibited IL-6 secretion in mouse macrophages upon *ex vivo* and *in vivo* conditions.

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Materials and Methods

Reagents. RPMI-1640 medium and foetal calf serum (FCS) were from PAA Laboratories (Pasching, Austria). DMSO, lipopolysaccharide (LPS), interferon (IFN)-γ, haemoglobin and sodium nitroprusside were from Sigma-Aldrich (St. Louis, MO, USA). Saq was purchased from Hoffman-La Roche. Saq-NO was obtained from OncoNOx and was synthesised as described previously [8].

Experimental animals, cells and cell cultures. Experimental animals (C57BL/6 mice and Dark Agouti rats) for most of the experiments were obtained from the Animal House Facility of the Institute for Biological Research 'Siniša Stanković', Belgrade, Serbia. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the institute (App No 2-38/11 – 01-1211). Alternatively, C57BL/6 mice that were used for *in vivo* application of LPS were obtained from Harlan Laboratories (San Pietro al Natisone, Udine, Italy) and were used at the animal house of the Department of Bio-Medical Sciences of the University of Catania (Italy) in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.L. 116/92), as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986) after approval by the local ethical committee. Resident peritoneal cells were collected by peritoneal lavage with 3-ml cold PBS, counted and were seeded in RPMI-1640 culture medium supplemented with 5% (v/v) heat-inactivated FCS into 24-well plates (Sartstedt, Numbrecht, Germany) (1.5×10^6/well). After 2 hr of incubation at 37°C in a humidified

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atmosphere containing 5% CO_2, peritoneal cells were washed two times with PBS to remove non-adherent cells. The adherent cells were considered as purified peritoneal macrophages (Mf). Mf purity was assessed by flow cytometric analysis on a FACSCalibur flow cytometer (BD Pharmingen) which showed that there were <5% of $CD3^+$ cells and $CD45R^+$ cells in the population purified by the adhesion. Rat Mf were stimulated for 24 hr with LPS (10 ng/ml), and murine Mf were stimulated with LPS (10 ng/ml) + IFN-γ (10 ng/ml) in the absence or presence of Saq or Saq-NO, as indicated in the results.

In vivo treatment with Saq and Saq-NO. C57BL/6 mice were treated for three consecutive days with Saq and Saq-NO (10 mg/kg) or vehicle (2% DMSO in H_2O). On the fourth day, peritoneal cells were collected by peritoneal lavage with 3 ml of cold PBS per animal. Purified cells were either stimulated with LPS (10 ng/ml) + IFN-γ (10 ng/ml) or cultivated without *in vitro* stimulation. Supernatants were collected for measuring nitrite accumulation and IL-1β, TNF, IL-6 and IL-10 production after 24 hr of cultivation.

Alternatively, Saq and Saq-NO were administered to C57BL/6 mice i.p. at the dose of 10 mg/kg for five consecutive days. Control mice were treated with vehicle (2% DMSO in H_2O). LPS was administered i.p. at the dose of 100 μg/mouse 1 hr after the last treatment. Mice were killed 2 hr or 6 hr after LPS challenge. Plasma samples were collected from the mice and used for determination of IL-6 concentration by ELISA.

Cell viability assay. In order to assess the viability of macrophages, crystal-violet (CV) test was applied. At the end of appropriate treatments, Mf were washed with PBS to remove non-adherent dead cells, and the remaining cells were fixed with methanol. After staining with 1% CV solution, the plates were thoroughly washed, and then the dye was dissolved in 33% acetic acid. The absorbance of dissolved dye, corresponding to the number of adherent viable cells, was measured at 570 nm with a microplate reader (LKB 5060-006; LKB, Vienna, Austria).

Assay of NO release. Nitrite accumulation, as an indirect measure of NO release, was determined in cell culture supernatants using the Griess reaction. In brief, triplicate aliquots of cell-free supernatants were mixed with an equal volume of Griess reagent (a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4). The absorbance at 540 nm was determined with a microplate reader (LKB 5060-006) and compared with a standard curve for $NaNO_2$.

ELISA test for determination of cytokines. Cytokine concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anticytokine paired antibodies according to the manufacturer's instructions. Samples were analysed in duplicates for murine IL-10, murine TNF, murine IL-6 and rat IL-6 (R&D, Minneapolis, MN, USA), murine IL-1β, rat IL-1β, rat IL-10 and rat TNF (BD Biosciences, San Diego, CA, USA). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

Statistical analysis. Results are presented as mean ± standard deviation (S.D.) obtained in independent experiments. The significance of the changes was evaluated by Student's t-test (two-tailed distribution, two-sample equal variance), and a p value <0.05 was considered statistically significant.

Results

The effect of Saq and Saq-NO on Mf viability.

In order to assess the effect of Saq-NO on Mf viability, these cells were isolated from rats and mice and activated with LPS or LPS + IFN-γ, respectively. The choice of the stimuli was made according to the previously reported differential responsiveness of mouse and rat macrophages to exogenous activation with either LPS alone or LPS + IFN-γ, respectively [14]. The stimulation lasted for 24 hr in the presence of various single concentrations of either Saq or Saq-NO. Mf viability was significantly affected only by 10 and 20 μg/ml Saq-NO in rat cell cultures (fig. 1A,B). Thus, it is clear that Saq-NO exerts limited effect on Mf viability in the concentrations that have been shown to be effective against malignant cells [12].

The effects of Saq and Saq-NO on Mf production of IL-1β, IL-6, IL-10 and TNF.

We next investigated whether Saq-NO affected cytokine production in activated Mf. For that purpose, release of cytokines IL-1β, IL-6, IL-10 and TNF from Mf stimulated with LPS or LPS + IFN-γ and cultivated for 24 hr in the presence of various single concentrations of Saq and Saq-NO was determined. Relative to untreated cells or cells treated with Saq, Saq-NO potently reduced secretion of IL-10 and IL-6 (fig. 2) and efficiently stimulated IL-1β (fig. 3A,B) in a dose-dependent manner in mouse and rat Mf. Saq-NO also mildly but significantly

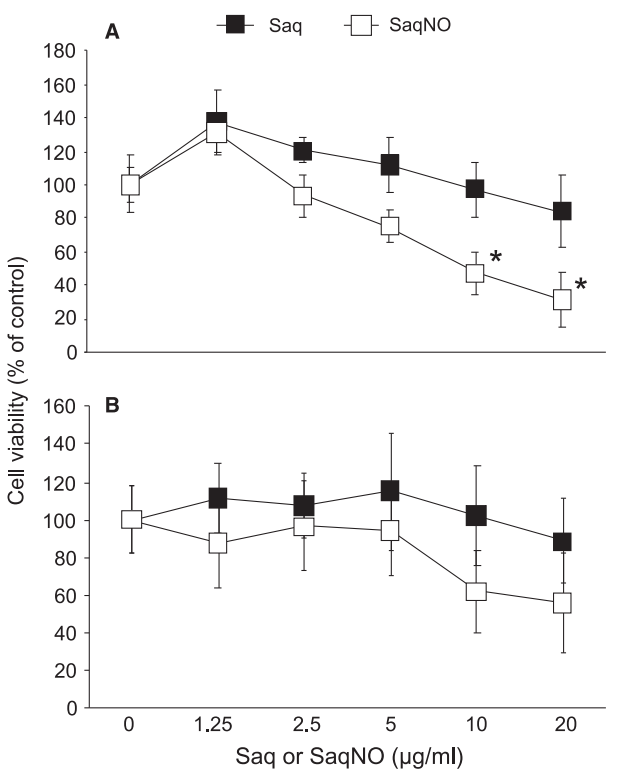

Fig. 1. Viability of rat (A) and mouse (B) peritoneal macrophages stimulated with Saq or Saq-NO. Peritoneal macrophages were obtained from healthy rats (A) and mice (B). The cells stimulated with lipopolysaccharide (LPS) (10 ng/ml) or LPS + interferon (IFN)-γ (10 ng/ml) were incubated with Saq or Saq-NO for 24 hr. Crystal-violet test was performed in order to determine the viability of the cells. Samples from three independent experiments were included in the analysis. *$p < 0.05$ represents statistically significant difference in comparison with control cells (not treated with Saq and Saq-NO).

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stimulated TNF release in rat, but not mouse Mf (fig. 3C,D). In parallel, Saq had minor effect on release of the examined cytokines (figs 2 and 3). Thus, Saq-NO, but not Saq, potently modulated generation of various cytokines from mouse and rat Mf. As there was a possibility that differential effects of Saq-NO and Saq on cytokine production were due to release of NO from Saq-NO, several experiments were performed to test this possibility. Firstly, release of NO from Saq-NO in cell culture medium was measured by Griess reaction. The highest concentration of Saq-NO used in our experiments (20 μg/ml) did not produce measurable accumulation of nitrites after 72 hr of cultivation. Even five times higher concentration of Saq-NO produced very low accumulation of nitrites after 72 hr of cultivation (2.9 ± 1.4 μM, 0.8 ± 1.1 μM and 0.9 ± 0.8 μM for 100 μg/ml, 20 μg/ml and 0 μg/ml of Saq-NO, respectively). Still, to demonstrate further that this trivial release of NO did not contribute to the effects of Saq-NO, a scavenger of NO, haemoglobin (25 μM) was applied to macrophage cultures simultaneously with Saq-NO. As expected, haemoglobin did not affect Saq-NO ability to modulate IL-1β and IL-6 production in macrophages (data not shown). Accordingly, simultaneous treatment of macrophages with Saq and a donor of NO, sodium nitroprusside (200 μM) did not mimic the effects of Saq-NO on the cytokine generation (data not shown). Thus, it can be concluded that the difference in effects of Saq-NO and Saq on cytokine generation in macrophages does not stem from the ability of Saq-NO to release NO.

The effects of Saq and Saq-NO on Mf NO release.

Next, the effect of Saq-NO on NO synthesis in Mf was examined. The cells were stimulated with LPS or LPS + IFN-γ and cultivated in the presence of various single concentrations of Saq or Saq-NO for 24 hr. Unlike Saq, Saq-NO efficiently inhibited NO generation in mouse and rat Mf (fig. 4A,B).

Ex vivo effects of Saq-NO and Saq on IL-1β, IL-6, IL-10 and TNF release from Mf.

To evaluate the *ex vivo* effects of Saq-NO or Saq on cytokine production in mouse macrophages, the mice were treated with Saq-NO, Saq or vehicle for three consecutive days, and 24 hr after the last application, Mf were isolated from the animals. There was no significant influence of either Saq-NO or Saq on the number of peritoneal cells isolated from mice (6.5 ± 3.0, 8.2 ± 2.1 and $8.8 \pm 3.0 \times 10^6$ in vehicle, Saq and Saq-NO treated mice, respectively). Subsequently, Mf were purified from the isolated cells and cultivated without additional stimulation or in the presence of LPS + IFN-γ for 24 hr. As compared with untreated cells, neither Saq-NO nor

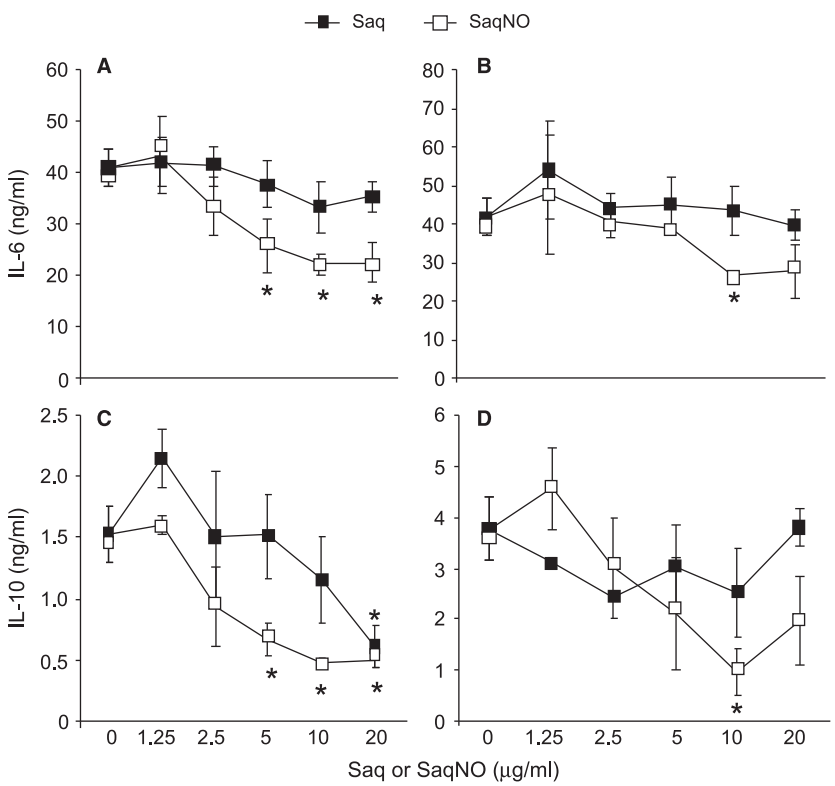

Fig. 2. The effect of Saq and Saq-NO on IL-6 and IL-10 production in rat and mouse peritoneal macrophages. Macrophages purified from peritoneal cells of rats activated with lipopolysaccharide (LPS) (A, C) and mice activated with LPS (10 ng/ml) + interferon (IFN)-γ (10 ng/ml) (B, D) were grown in cell culture for 24 hr in the presence or absence of Saq and Saq-NO. Then, cell-free supernatants were collected, and ELISA was performed for determining the levels of secreted cytokines, IL-6 (A, B) and IL-10 (C, D). Results are presented as mean ± S.D. of results obtained in three independent experiments. *$p < 0.05$ represents statistically significant difference between Saq or Saq-NO-treated and Saq or Saq-NO untreated cultures.

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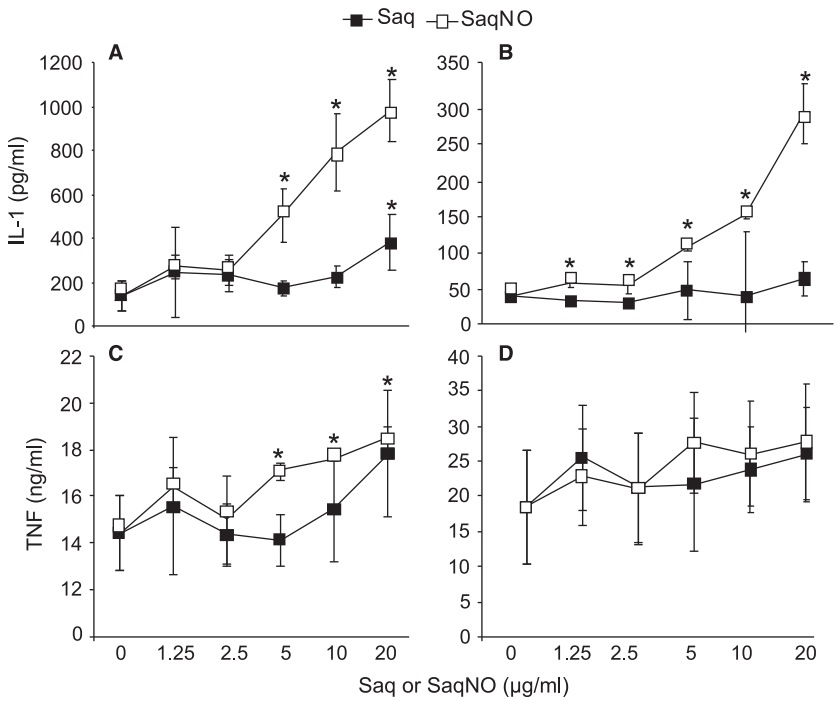

Fig. 3. The effect of Saq and Saq-NO on IL-1β and tumour necrosis factor (TNF) production in rat and mouse peritoneal macrophages. Macrophages purified from peritoneal cells of rats activated with lipopolysaccharide (LPS) (10 ng/ml) (A, C) and mice activated with LPS (10 ng/ml) + interferon (IFN)-γ (10 ng/ml) (B, D) were grown in cell culture for 24 hr in the presence or absence of Saq and Saq-NO. Then, cell-free supernatants were collected and ELISAs were performed. Results are presented as mean ± S.D. of results obtained in three independent experiments. *$p < 0.05$ represents statistically significant difference between Saq or Saq-NO-treated and Saq or Saq-NO untreated cultures.

Saq had significant effect on the cytokine generation in Mf-stimulated *ex vivo* with LPS + IFN-γ (fig. 5). Interestingly, the treatment of mice with Saq stimulated IL-1β, IL-6 and TNF production, and the treatment with Saq-NO inhibited IL-6 production in Mf cultures that were not additionally stimulated *ex vivo*. In addition, further differences were noted in this assay between Saq-NO and Saq-treated cells, as spontaneous secretion of TNF and LPS + I FN-γ-induced secretion of IL-1β were significantly lower in Saq-NO than in Saq-treated cells (fig. 5A,B). Thus, *in vivo* application of Saq-NO and Saq had opposing effects on the cytokine release from Mf. The only effect observed *in vitro*, that was replicated after *in vivo* application of Saq-NO, was its inhibitory effect on IL-6.

Ex vivo effects of Saq-NO and Saq on NO production in Mf.
The *ex vivo* effect of either Saq-NO or Saq on NO production in Mf was determined. Treatment of the mice with the drugs as well as *ex vivo* cultivation of Mf was performed in the same way as already described for the cytokine measurement. Neither Saq-NO nor Saq had statistically significant influence on NO generation by Mf, irrespectively, on the *ex vivo* treatment (fig. 6). Thus, *in vivo* application of Saq-NO to mice did not affect NO synthesis in Mf.

In vivo effects of Saq-NO and Saq on LPS-induced-IL-6 secretion.
Finally, to test the *in vivo* potency of Saq-NO and Saq to modulate IL-6 generation, the mice were treated with these agents or the vehicle and then injected with LPS to induce cytokine release in the bloodstream. IL-6 was measured in plasma samples. At 6 hr after LPS challenge, IL-6 blood levels were significantly lower in Saq-NO-treated mice as compared to either vehicle- or Saq-treated mice that exhibited super-imposable concentrations (fig. 7). Thus, these results further support that blockade of IL-6 synthesis/secretion represents one of the primary targets of Saq-NO in rodents.

Discussion

In this work, novel immunomodulatory effects of Saq-NO are presented. It is shown that production of various cytokines and NO in mouse and rat macrophages is regulated by Saq-NO *in vitro*. Although the immunopharmacological profile of Saq-NO on the cytokine secretory capacity of rodent macrophages appeared pleiotropic *in vitro* ensuing in inhibition of IL-6, IL-10 and NO, while increasing IL-1β and, more modestly TNF secretion, none of these data with exception of significant suppression of IL-6 secretion were confirmed upon *ex vivo* studies with mouse macrophages. IL-6 secretion was also down-regulated in *in vivo* conditions upon LPS challenge of mice pre-treated with Saq-NO. Thus, inhibition of IL-6 synthesis/secretion from macrophages by Saq-NO is the main outcome of our study. The ability of Saq-NO to down-regulate IL-6 secretion from macrophages may contribute to the immunomodulatory and chemotherapeutic effects of Saq-NO.

IL-6 is a small polypeptide of approximately 26 kD molecular weight that is involved in the differentiation and growth of

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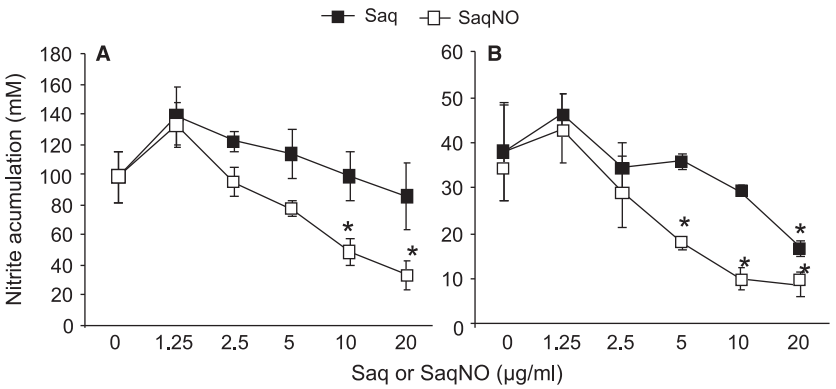

Fig. 4. The influence of Saq and Saq-NO on NO release in rat and mouse peritoneal macrophages. Rat (A) and mouse (B) macrophages were treated with Saq and Saq-NO (1.25–20 μg/ml) in the absence (medium) or presence of lipopolysaccharide (LPS) (10 ng/ml) (A) or LPS + interferon (IFN)-γ (10 ng/ml) (B). After 24 hr, supernatants were collected, and Griess assay was performed. Results are presented as mean ± S.D. of results obtained in three independent experiments. *$p < 0.05$ represents statistically significant difference between Saq or Saq-NO-treated and Saq or Saq-NO untreated cultures.

a variety of cells. It has originally been described as B-cell stimulating factor, hepatocyte stimulating factor and interferon β2, before it was cloned and shown that all these activities were attributable to a single molecule which did not convey antiviral actions [15]. IL-6 binds to a receptor (IL-6R), which consists of the actual cytokine binding part, the IL-6Rα chain, and a second moiety, gp130, which transduces the respective signals into the cell. IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, haematopoiesis, inflammation and tumour genesis. Since the discovery of IL-6, its activities, the IL-6R system and the IL-6 signal transduction mechanism have been further clarified. This led to a new therapeutic approach to block the actions of IL-6 by use of a humanized anti-IL-6R antibody (Tocilizumab) that has been proven to be therapeutically effective for rheumatoid arthritis, systemic juvenile idiopathic arthritis and Castleman's disease [16]. Therefore, the ability of Saq-NO to inhibit IL-6 secretion from macrophages adds this pharmacological property to the previously described immunomodulatory action of Saq-NO on T cells that primarily ensues in inhibition of

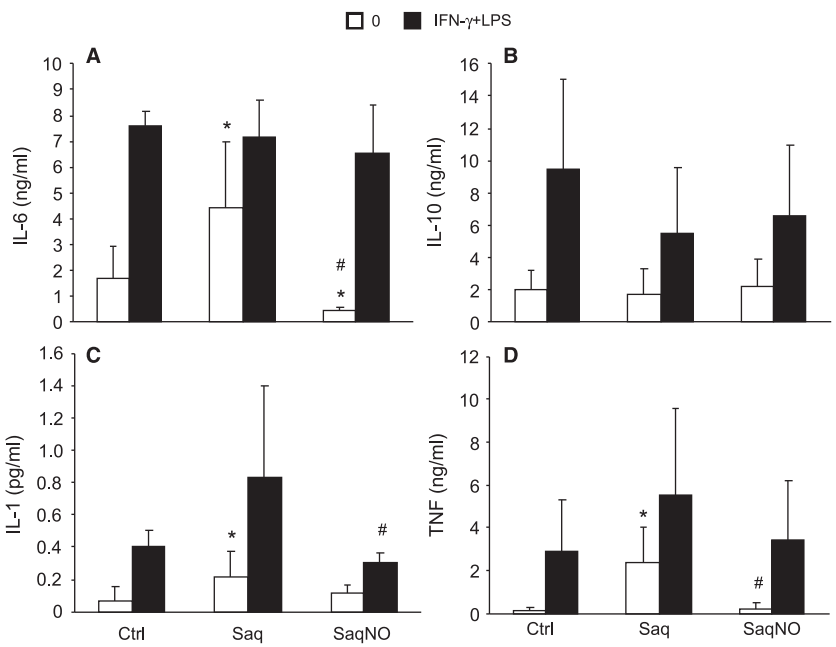

Fig. 5. The effect of *in vivo* application of Saq and Saq-NO on IL-6, IL-10, IL-1 and tumour necrosis factor (TNF) production in mouse peritoneal macrophages. Macrophages were purified from peritoneal cells of mice which were treated i.p. with vehicle (Ctrl) or Saq or Saq-NO for three consecutive days. Mf were activated with lipopolysaccharide (LPS) (10 ng/ml) + interferon (IFN)-γ (10 ng/ml) (B, D) *in vitro* and grown in cell culture for 24 hr. Then, cell-free supernatants were collected, and ELISA was performed for determining the levels of secreted cytokines, TNF (A), IL-1β (B), IL-6 (C) and IL-10 (D). Results are presented as mean + S.D. of results obtained from eight (Ctrl), four (Saq) or seven (Saq-NO) mice per group. *$p < 0.05$ represents statistically significant difference between Saq or Saq-NO-treated and control mice (Ctrl). #$p < 0.05$ represents statistically significant difference between Saq and Saq-NO-treated mice.

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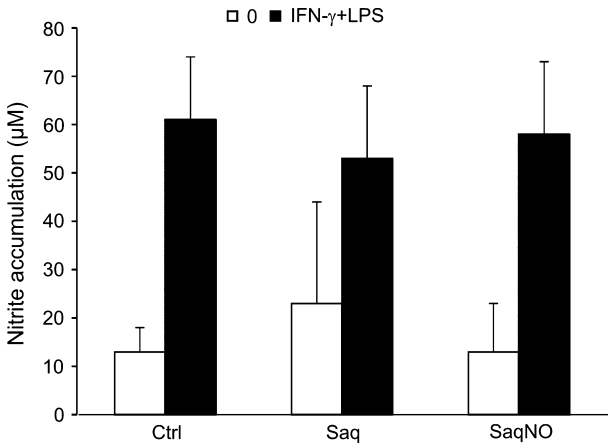

Fig. 6. The influence of Saq and Saq-NO treatment on NO release in mouse peritoneal macrophages. Mice were treated with vehicle (Ctrl) or Saq or Saq-NO (10 mg/kg) for three consecutive days. On the fourth day after the beginning of the treatment, peritoneal cells were isolated and cultivated in the absence (medium) or presence of LPS + interferon (IFN)-γ (10 ng/ml). After 24 hr, supernatants were collected, and Griess assay was performed. Results are presented as mean + S.D. of results obtained from four (Saq), seven (Saq-NO) or eight (Ctrl) mice per group.

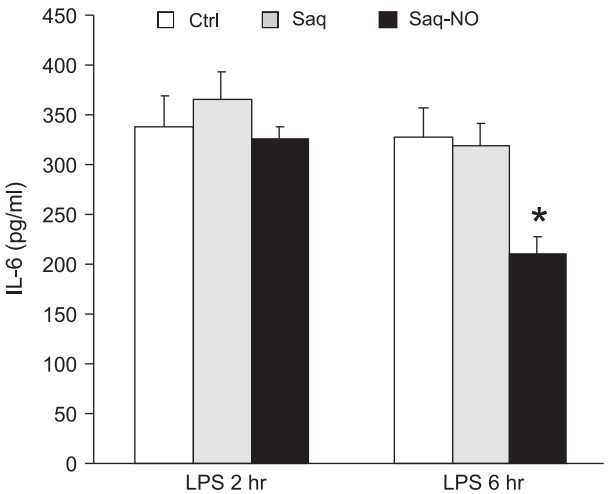

Fig. 7. The influence of Saq-NO and Saq on IL-6 generation in mice treated with lipopolysaccharide (LPS). Mice were treated with Saq or Saq-NO (10 mg/kg) or with vehicle (2% DMSO in H_2O) for five consecutive days. One hour after the last treatment, LPS (100 μg/mouse) was applied. Mice were killed 2 hr or 6 hr after LPS treatment. Cytokines were determined in plasma, and the results are presented as mean + S.D. of values obtained from nine mice per group. $*p < 0.05$ represents statistically significant difference in comparison with control (vehicle-treated) mice and Saq-treated mice.

production of pro-inflammatory cytokines such as IL-17 and IFN-γ [13]. Inhibition of IL-6 production from macrophages adds further value and proof of concept to the pharmacological profile of Saq-NO and further qualifies it as a drug candidate of potential interest for the treatment of autoimmune diseases. Importantly, in our previous study [13], it was also shown that Saq-NO applied *in vivo* inhibited IL-6 blood levels in mice treated with anti-CD3 antibody. Thus, Saq-NO might be particularly efficient in autoimmune and chronic inflammatory diseases in which IL-6 is a dominant pathogenic cytokine, such as inflammatory bowel diseases, diabetes, multiple sclerosis, asthma and rheumatoid arthritis [17].

Macrophages are among the major players in innate immunity, as well as an important antigen presenting, effector and regulatory cells in adaptive immunity. Depending on their activity, they can inhibit cell proliferation, induce tissue destruction and support T cell immunity (M1 macrophages) or they can promote cell proliferation, tissue repair and humoural immunity (M2 macrophages) [18]. This M1/M2 polarisation is highly important for various diseases that are immune-mediated or immune-related, including cancer [19,20]. Cytokine generation by macrophages contributes to the functions of cancer cells. Concentrations of Saq-NO that affected macrophage viability were the most effective in modulation of release of the examined cytokines. This could be explained by the effect of Saq-NO on a signalling cascade that is common for cell death induction and cytokine release stimulation, such as S6 kinase signalling that was shown previously important for regulation of IFN-γ and IL-17 production in T cells [13]. Still, Saq-NO regulated cytokines and NO in concentrations that did not affect viability of macrophages, but that were previously shown effective against transformed cells [10,12]. This is in agreement with our previous observation of the absence of toxic effect of Saq-NO on astrocytes and fibroblasts *in vitro* [8]. Also, this implies that antitumour effects of Saq-NO could be simultaneously achieved through direct influence on cancer cells and indirectly through its effects on macrophages.

Tumour-associated macrophages (TAM) predominantly belong to M2 macrophages, and they are the main inflammatory population of the stroma of numerous tumours [21]. They perform various functions to promote tumours, including angiogenesis stimulation, matrix remodelling and adaptive immunity suppression. Indeed, increased number of TAM in cancer has been correlated to poor patient prognosis [21,22]. An important way in which TAM contribute to tumour growth and progression is through release of pro-inflammatory cytokines, including IL-6, TNF and IL-1β [23]. Although Saq-NO stimulated TNF and IL-1β production *in vitro*, such effects were not observed upon *ex vivo* conditions, and the biological relevance of these findings is therefore unknown. In addition, the stimulatory effect of Saq-NO on TNF secretion was only observed *in vitro* from mouse but not rat macrophages and it was of modest magnitude. On the other hand, IL-6 generation was consistently inhibited upon *in vitro, ex vivo* and *in vivo* conditions by Saq-NO.

Although IL-6 is easily induced in macrophages by LPS and therefore is a typical cytokine produced by M1 macrophages, it also performs M2 macrophage functions. Accordingly, circulating levels of IL-6 have been reported as prognostic indicator of survival and metastasis in human cancers [24,25]. Importantly, IL-6 pro-proliferative and antiapoptotic effects on tumour cells are mediated through activation of signal transducer and activator of transcription 3 (STAT-3) pathway, which has been identified as the leading signalling

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pathway for promotion of tumour growth, survival and invasion [26]. In particular, recent evidence indicates a key role of IL-6 and STAT3 in the pathogenesis of colitis-associated cancer that is the most serious complication of inflammatory bowel diseases. There, IL-6 is mainly produced by tumour-infiltrating myeloid cells, and it promotes survival and proliferation of tumour-initiating cells derived from the intestinal epithelium [27]. These effects of IL-6 are mainly STAT3-dependent, and blockade of STAT3 signalling in intestinal epithelial cells significantly reduces colitis-associated cancerogenesis. Importantly, a critical role for IL-23 and its downstream cytokines IL-17 and IL-22 were identified in the development of colitis-associated cancers [27]. As both IL-6 and IL-17 generation are targets of Saq-NO, these data qualify this compound of particular interest for the treatment of inflammatory bowel diseases and prevention of colitis-associated cancers.

Moreover, IL-6 has been shown to promote M2 macrophages, thus providing a positive regulatory feedback that ensures continuous support for tumour growth [28]. As a proof of importance of IL-6 for cancer progression, siltuximab, a chimeric anti-IL-6 antibody, as well as a high-affinity fully humanized anti-IL-6 monoclonal antibody (mAb 1339) have been shown beneficial in treatment of human cancers either as single agents or in combination with other anticancer drugs [29,30]. Thus, by inhibiting IL-6 secretion from macrophages, Saq-NO might decrease IL-6 level in tumour microenvironment and in that way it could contribute to tumour inhibition.

It is important to underline that the observed *in vitro* effects of Saq-NO were of significantly greater magnitude than the effects of Saq, while *in vivo* Saq and Saq-NO even exhibited contrasting effects. These results further support the concept that although Saq-NO is based on Saq with whom it shares various properties, this agent represents a NCE endowed with numerous properties that are distinct from those of its parental drug. Although NO moiety is the single structural difference between Saq and Saq-NO, here we show that NO release from Saq-NO is not responsible for its effects on the cytokine production in macrophages. Indeed, the amount of NO released from Saq-NO is very low, and it has been previously shown that it is not relevant for antitumour properties of the agent [8,31]. In conclusion, Saq-NO is a novel and potent immunomodulatory agent and further studies regarding its influence on immune cells and immune-mediated and/or immune-related diseases are warranted.

Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Serbia (173013 and 173035).

Disclosure of Interest

FN is CSO and co-founder of OncoNOx.

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