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3-AMINO-1,2,4-TRIAZOLE INHIBITS MACROPHAGE NO SYNTHASE [1]

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Summary. Murine macrophages activated by interferon-γ and lipopolysaccharide become leishmanicidal through a process involving L-arginine-derived nitrogen oxidation products. Both nitrite secretion and parasite killing by activated macrophages were inhibited by 3-amino-1,2,4-triazole as well as the related compound, 3-amino-1,2,4-triazine. Moreover, NO synthase activity in cytosolic extracts of activated cells was inhibited by both compounds. 4-amino-1,2,4-triazole, an isomer of 3-amino-1,2,4-triazole, was without effect. Our results suggest that besides its known inhibitory effect on catalases and peroxidases, 3-amino-1,2,4-triazole is an inhibitor of NO synthase. The resemblance between the tautomeric form of 3-amino-1,2,4-triazole and the guanidino group of L-arginine, the natural substrate for NO synthase, might be responsible for the observed inhibition.

We have previously shown that 3-amino-1,2,4-triazole (3-ATOLE), a catalase/peroxidase inhibitor (1,2), prevented lymphokine-induced leishmanicidal activity in murine macrophages (3), consistent with a role of oxygen metabolites in parasite killing. That other mechanisms were also implicated was, however, indicated by the fact that parasite killing could also occur in cells unable to generate superoxide, making the role of oxygen derivatives as leishmanicidal agents a controversial issue (4). Recently, a novel mechanism, based on L-arginine conversion to highly toxic derivatives of the NO type, was shown to be involved in parasite killing (5-7). This led us to study the effect of 3-ATOLE on nitrite production by interferon-γ + lipopolysaccharide- (IFN-γ + LPS) activated macrophages. As shown here, inhibition of leishmanicidal activity induced by 3-ATOLE correlated with an inhibition of nitrite secretion by the activated cells. Experiments performed on cytosolic extracts showed that 3-ATOLE, besides its known inhibitory effect on catalases and peroxidases, is an inhibitor of NO synthase activity.

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Abbreviations : 3-ATOLE, 3-amino-1,2,4-triazole ; 4-ATOLE, 4-amino-1,2,4-triazole; 3-ATINE, 3-amino-1,2,4-triazine; LPS, lipopolysaccharide; IFN-γ, interferon-γ; NGM, N^G-monomethyl-L-arginine; DH, Dulbecco's medium.

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MATERIALS AND METHODS

Mice

CBA (male or female) mice were obtained from IFFA CREDO (Saint-Germain-sur-l'Arbresle, France) and were used between 8 and 18 weeks of age.

Reagents

N^G-monomethyl-L-arginine (NGM) was purchased from Chemical Dynamics Corporation (South Plainfield, New Jersey, USA), LPS (from *E. coli* 0.55-B.5) from Difco Laboratories, (Detroit, MI, USA), 3-amino-1,2,4-triazole (3-ATOLE), NADPH and protease inhibitors from Sigma (Munich, Germany), foetal calf serum (FCS) from Seromed (Munich, Germany), 4-amino-1,2,4-triazole (4-ATOLE) and 3-amino-1,2,4-triazine (3-ATINE) from Aldrich (Steinheim, Germany). IFN-γ (murine; lot no 2309-24) produced by Genentech, Inc., was kindly supplied by Boehringer Ingelheim, Vienna, Austria.

Macrophage cultures

Bone marrow-derived macrophages, obtained as previously described (8),were distributed in Hepes-buffered Dulbecco's medium (DH) supplemented with 10% (v/v) FCS into 96-well microculture plates (Costar 3799, Cambridge, MA, 10^5 cells/well). Three h later, cells were infected with promastigotes of *Leishmania enriettii* (9) at a ratio of 20 parasites per macrophage. The cultures were then incubated at 37 °C for 18 to 20 h to allow for phagocytosis.

Macrophage activation and treatment with inhibitors

Infected macrophages were washed twice and reincubated with IFN-γ with or without added LPS in the presence or absence of 3-ATOLE or related compounds. Twenty four h later, supernatants (100 µl) were harvested for nitrite determination by the Griess reaction as previously described (10). Cells were then washed and lysed with 0.01 % sodium dodecylsulfate (11). The wells were supplemented with M199 medium + 10 % FCS and parasite growth was recorded by measuring [^{3}H]-thymidine uptake (12).

Preparation of macrophage cytosolic extracts (13)

Bone marrow-derived macrophages were cultured in 100-mm plastic Petri dishes (1.0-1.5 x 10^7 cells/dish) with IFN-γ (10-50 U/ml) and LPS (10-30 ng/ml) for 7 h at 37 °C. Macrophages were then washed, resuspended in PBS containing various protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor, 5 µg/ml each of leupeptin, antipain and pepstatin) and sonicated.The extracts were then centrifuged at 11000 x g for 45 min at 4 °C. Supernatant fractions containing 0.1 to 0.6 mg/ml protein (as determined by the method of Baumgarten, 14) were tested for enzyme activity according to Marletta *et al.* (13), in the presence or absence of NGM, 3-ATOLE or related compounds. The reaction was run for 18 h at 25 °C.

RESULTS

Concentration-dependent inhibition of macrophage activation for leishmanicidal activity by 3-ATOLE or related compounds.

CBA bone marrow-derived macrophages, when activated with IFN-γ and LPS, acquire the capacity to destroy intracellular *L. enriettii*. This effect was lost when macrophages were activated in the presence of increasing concentrations of 3-ATOLE. Inhibition of parasite killing by 3-ATOLE correlated with suppression of nitrite secretion into the extracellular medium (Fig.1).

Our group and others (5, 6, 15) have previously shown that nitrite production and parasite killing by activated macrophages could be inhibited by NGM, a guanidino methylated derivative of L-arginine, the natural substrate for NO synthase. The resemblance between the tautomeric form of 3-ATOLE and the guanidino group of L-arginine (Fig. 2) suggested that this compound also acts as a substrate analogue for NO synthase. Indeed, 3-ATINE, another

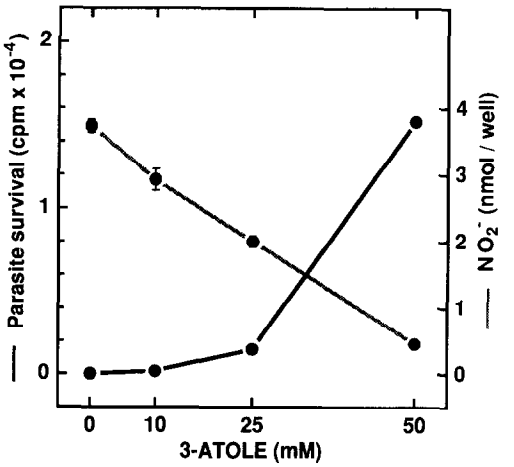

Figure 1. Inhibition of macrophage activation by increasing doses of 3-ATOLE.
CBA bone marrow-derived macrophages were incubated with IFN-γ (10 U/ml) and LPS (10 ng/ml) in the presence or absence of increasing doses of 3-ATOLE. Twenty-four h later, supernatants were harvested and assayed for the presence of nitrite and parasite survival was determined. Controls (macrophages incubated with IFN-γ alone) corresponded to 14487 ± 1240 cpm and 0.42 ± 0.04 nmol nitrite. Data are means ± S.D. and are representative of three experiments.

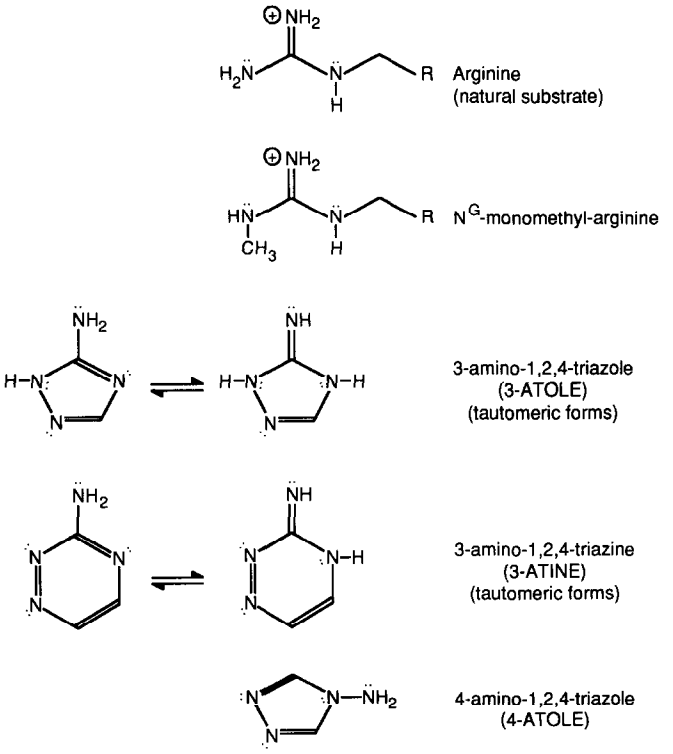

Figure 2. Structure of tested compounds.

Table I. Effect of two compounds related to 3-ATOLE on nitrite secretion by activated macrophages

Incubation medium	Compound tested	Nitrite secretion (nmol/well)
IFN-γ	-	0.06 ± 0.11
LPS	-	0.29 ± 0.02
DH Medium	-	0.00
IFN-γ + LPS	-	4.19 ± 0.22
IFN-γ + LPS	3-ATOLE 50 mM	0.60 ± 0.04
IFN-γ + LPS	3-ATINE 5mM	2.68 ± 0.11
IFN-γ + LPS	3-ATINE 10 mM	1.39 ± 0.15
IFN-γ + LPS	3-ATINE 25 mM	0.09 ± 0.15
IFN-γ + LPS	4-ATOLE 25 mM	4.20 ± 0.20
IFN-γ + LPS	4-ATOLE 50 mM	4.05 ± 0.33

Cells were incubated with IFN-γ (50 U/ml) and LPS (30 ng/ml) in the presence or absence of 3-ATOLE or related compounds. Supernatants were harvested 24 h later for nitrite determination. Results are expressed as means ± S.D.

compound whose tautomeric form resembles the guanidino group of L-arginine, inhibited nitrite production by activated macrophages, even at lower concentrations than 3-ATOLE, while 4-ATOLE, an isomer of 3-ATOLE structurally unrelated to L-arginine, was without effect (Table I, Fig. 2).

Effect of 3-ATOLE and related compounds on cytosolic NO synthase activity.

When macrophages were incubated with IFN-γ and LPS for 7 h, NO synthase activity was detectable in isolated macrophage cytosolic fractions, as previously described (13). Significantly less enzyme activity was present in extracts of macrophages activated for 7 h in the presence of 3-ATOLE (Table II). Moreover, 3-ATOLE and 3-ATINE, like the well-characterized NO synthase inhibitor NGM, directly inhibited the activity of the cytosolic enzyme, while 4-ATOLE was without effect (Tables II and III).

Table II. Effect of 3-ATOLE on cytosolic NO synthase activity

		NO synthase activity (nmol/10^6 cells)	
Cell culture	Cytosolic fraction	Experiment I	Experiment II
DH Medium	PBS	0.05	0
IFN-γ + LPS	PBS	1.50	4.90
+ 3-ATOLE	PBS	0.50	1.00
IFN-γ + LPS	3-ATOLE 10 mM	0.90	4.90
IFN-γ + LPS	3-ATOLE 25 mM	0.40	2.40
IFN-γ + LPS	3-ATOLE 50 mM	0.10	1.10
IFN-γ + LPS	NGM 0.2 mM	0.60	n.d.

Cells were cultured for 7 h with IFN-γ (50 U/ml for Exp.I and 10 U/ml for Exp. II) + LPS (30 ng/ml) in the presence or absence of 3-ATOLE (50 mM). Enzyme activity was determined in the cytosolic fractions in the presence or absence of 3-ATOLE or NGM, as described in Materials and Methods. n.d. : not determined.

TABLE III. Effect of two compounds related to 3-ATOLE on cytosolic NO synthase activity

	NO synthase activity (nmol/10^6 cells)	
Compound tested	Experiment I	Experiment II
-	6.60	1.92
3-ATOLE	1.80	0.25
3-ATINE	3.80	0.92
4-ATOLE	7.30	2.00

Cells were cultured for 7 h with IFN-γ (50 U/ml) and LPS (30 ng/ml), and enzyme activity was determined in the cytosolic fractions in the presence or absence of 50 mM of the indicated compounds, as described in Materials and Methods.

DISCUSSION

Our results show that 3-ATOLE inhibited, in a concentration-dependent manner, IFN-γ + LPS-induced leishmanicidal activity and NO_2^- production in murine bone marrow-derived macrophages. Experiments performed on cytosolic extracts of activated macrophages showed that NO synthase activity was impaired by 3-ATOLE, whether the drug was added during the 7 h activation of the cells with IFN-γ and LPS or directly to the cytosolic extracts, once the enzyme activity was already expressed. These results suggest that 3-ATOLE, in addition to its known inhibitory effects on catalases and peroxidases (1, 2) and its stimulatory effects on NADPH oxidase activity of myeloperoxidase (16, 17), is an inhibitor of the activity of cytosolic NO synthase. Although the mechanism of action of 3-ATOLE on NO synthase activity is unknown, structural analysis of the compound suggests that substrate homology might be responsible for the observed effects. Indeed, the tautomeric form of 3-ATOLE resembles the guanidino group of L-arginine, the natural substrate for NO synthase. 3-ATOLE inhibited NO synthase activity in cytosolic extracts of activated macrophages with an IC_{50} (concentration of compound inhibiting 50 % of the enzyme activity) of approximately 20-25 mM (compared to 0.2-0.3 mM for NGM, a well known substrate analogue of NO synthase). The poor efficiency of this inhibitor might be explained by an unfavourable equilibrium between active and inactive tautomers and/or by an altered pK_a or conformation of the guanidino-like group within the aromatic ring. It should be noted that the amino group of 3-ATOLE is deactivated by the triazole ring and has no quenching effect on the Griess reagent, even at a concentration of 50 mM. A related compound, 3-ATINE, also inhibited NO synthase activity in macrophage extracts, although with a higher IC_{50} (approximately 40- 50 mM) than 3-ATOLE; when added to cells cultured in the presence of IFN-γ and LPS, 3-ATINE was, however, a better inhibitor of nitrite secretion than 3-ATOLE, probably because of facilitated penetration into cells due to its more lipophilic nature. Its poor efficiency as an inhibitor of the cytosolic extracts might be due to the same reasons postulated for 3-ATOLE above. 4-ATOLE, an isomer of 3-ATOLE lacking the guanidino-like group, had no effect on cytosolic NO synthase activity and nitrite secretion by activated macrophages. Finally, we were unable to reverse 3-ATOLE-induced inhibition by an equimolar concentration of L-arginine (data not shown). The reasons for this lack of reversal are unknown; fixation of the compound to the

enzyme active site might stabilize the "active" tautomeric form which would remain tightly bound to the enzyme or modify its active site, preventing the binding of L-arginine. Further studies are needed to answer these questions.

In conclusion, our results show that 3-ATOLE inhibits macrophage leishmanicidal activity and nitrite secretion through a process involving inhibition of NO synthase activity. The resemblance between the tautomeric form of 3-ATOLE and the guanidino group of L-arginine, the natural substrate for NO synthase, might be responsible for the observed inhibition.

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