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SUPPLEMENTAL MATERIAL

Molon et al., http://www.jem.org/cgi/content/full/jem.20101956/DC1

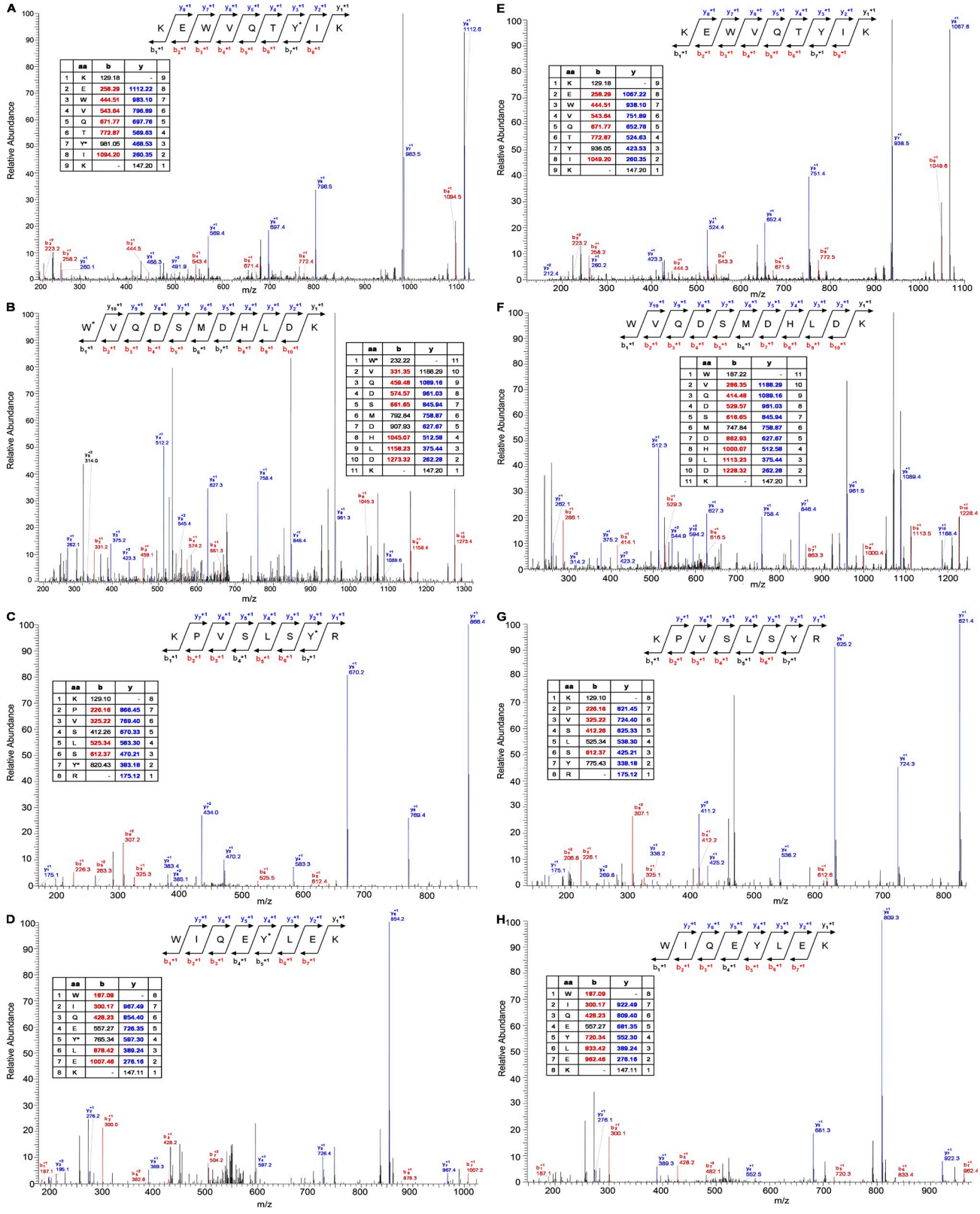


Figure S1. Fragmentation (MS/MS) spectra of nitrated peptides and of unmodified control peptides from mouse CCL2, human CCL2, and mouse CXCL12. (A and E) Mouse CCL2. (B and F) Human CCL2. (C, D, G, and H) Mouse CXCL12. In each MS/MS spectrum the fragments allow for peptide sequence identification and characterization of nitrated amino acid (* indicates +45 D modification). The fragment ions of detected b and y series (corresponding to N- and C-terminal) are in red and blue, respectively, in the inserted tables.

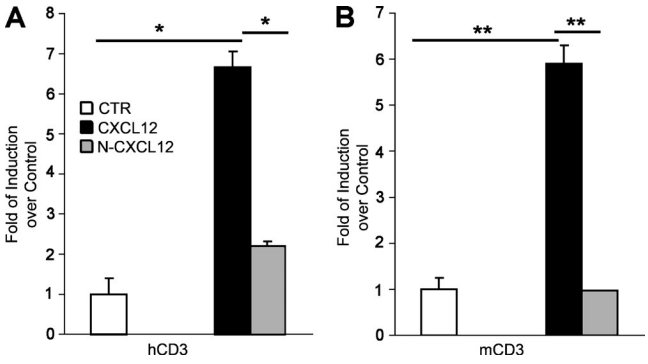


Figure S2. N-CXCL12 is not chemotactic for human and mouse T cells. PB human T lymphocytes (A) or mouse CD3+ splenocytes (B) were exposed to a gradient of untreated or RNS-treated recombinant human or mouse CXCL12 (2.5 nM) and transmigrated cells were counted. Results are expressed as fold of induction over control values. Data are representative of at least three different experiments and are expressed as mean ± SE. *, $P \leq 0.05$; **, $P \leq 0.01$.

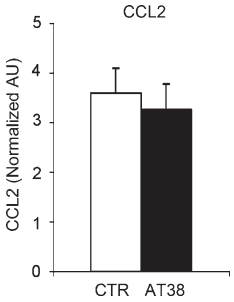


Figure S3. AT38 treatment does not induce CCL2 synthesis. Total RNA was isolated from tumor tissues of EG7-OVA tumor-bearing mice either treated or not with AT38 for 4 d. The qRT-PCR data were reported as ΔCt mean of three tumor samples. Data are expressed, on a base 2 logarithmic scale, as the fold change of mRNA abundance in control and AT38-treated tumor-bearing mice, normalized to the expression of β-actin housekeeping gene. Data are representative of at least three different experiments and are expressed as mean ± SE.

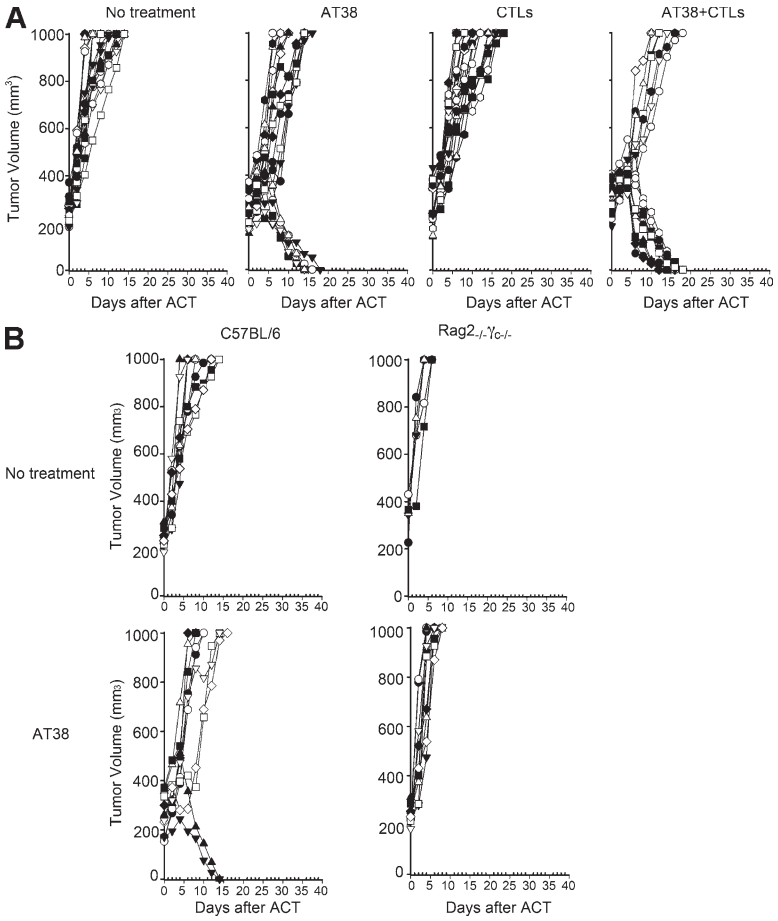


Figure S4. Tumor growth curves of mice treated with the combination of AT38 and/or OVA257–264–specific CTLs. (A) EG7-OVA tumor-bearing mice (n = 18) were either untreated or treated with AT38 for 7 d (30 mg/Kg/d; AT38 only), with adoptive transfer of 2 × 10^6 CTLs (when tumor volume was ∼300 mm^3; CTLs only), or with a combination of CTLs and AT38 (4 d before and 3 d after ACT; CTLs+AT38). Tumors were measured on blind using digital calipers. Mice were euthanized when tumor area reached 1,000 mm^3. Different symbols represent single mice. (B) C57BL/6 (n = 10) and $Rag2^{-/-}\gamma c^{-/-}$ (n = 10) mice were injected with 0.5 × 106 EG7-OVA cells. When tumor volume was ∼300 mm3, mice were either treated or not with AT38 (30 mg/Kg/d) for 7 d. Tumors were measured on blind using digital calipers. Mice were euthanized when tumor area reached 1,000 mm^3.

CHARACTERIZATION OF FUROXAN-BASED IMMUNE MODULATORS

Background and rationale

A few years ago we described, for the first time, the necessity to inhibit both arginase 1 (ARG1) and nitric oxide synthase 2 (NOS2) enzyme activity in order to achieve a complete blockade of the immunosuppressive action exerted by myeloid-derived suppressor cells (MDSCs) on antigen-activated T lymphocytes (Bronte et al., 2003). The findings were based on the use of inhibitors specific for these enzymes and were thereafter reproduced by several groups including our own (De Santo et al., 2005; Gallina et al., 2006). ARG and NOS were also found to be responsible for the suppression of T cell–mediated immune responses induced by tumor-associated macrophages (TAMs; Kusmartsev and Gabrilovich, 2005). More recently, hypoxia-inducible factor 1α was shown to drive the program leading to ARG1 and NOS2 up-regulation in mouse TAMs (Corzo et al., 2010; Doedens et al., 2010), thus providing a mechanistic basis for the concomitant expression of these enzymes.

In human prostate cancers, the neoplastic, but not the normal prostate epithelium, overexpressed ARG2 and NOS2 and addition of enzyme-specific inhibitors to in vitro prostate organ cultures was sufficient to decrease the nitrotyrosine content (and hence peroxynitrite production), both within the tumor and in tumor infiltrating lymphocytes (TILs). Only the combination of inhibitors was effective in reducing intratumoral nitrotyrosine staining and this directly correlated with the ability to restore TIL responsiveness to various stimuli (Bronte et al., 2005). In untreated cultures, in fact, prostate TILs did not react either to antigens or signals bypassing TCR binding, indicating a profound state of immune dormancy/anergy (Bronte et al., 2005).

ARG and NOS can act either separately or in synergy to suppress T lymphocytes activation, the choice being dictated by the particular tumor and the genetic background of the host (Marigo et al., 2008; Grohmann and Bronte, 2010). L-arginine depletion by ARG can cause a direct block of T cell proliferation (Rodríguez and Ochoa, 2008); however, ARG activation could have an important supplementary effect, i.e., the possible production of superoxide (O_2^-) from the NOS reductase domain, a phenomenon known as the uncoupled reaction. Biochemical data supported that idea that under low L-arginine environmental concentration, NOS function shifted from a prevalent NO production to superoxide production (Xia and Zweier, 1997; Xia et al., 1998). To demonstrate that this was true also for MDSCs, we infected an immortalized MDSC line with a vaccinia virus encoding mouse *Arg1*, in the presence of IFN-γ to up-regulate NOS2 activity. ARG1 expression led to a NOS-dependent increased rate of superoxide generation in MDSCs, which was inhibited by DPI, a flavin-binding site competitor blocking NOS reductase domain (Bronte et al., 2003).

The mechanism by which ARG can affect production of reactive nitrogen species (RNS), such as peroxynitrite, is not completely understood but it is most likely dependent on the above-mentioned uncoupling of NOS enzyme activity. Partial uncoupling of the NOS activity is caused by either L-arginine or tetrahydrobiopterin (BH4) shortage. Under limited supply of either molecule, NOS uncoupling causes the NOS enzymes to produce superoxide, the other precursor of peroxynitrite, in place of NO. Thus, partial uncoupling will cause NOS proteins to act like peroxynitrite synthases. Interestingly, peroxynitrite is known to oxidize BH4, and consequently partial uncoupling may initiate a vicious cycle, propagating the partial uncoupling over time (Xia and Zweier, 1997; Xia et al., 1998; Pall, 2007).

Under conditions of limited availability of L-arginine, not only NOS2 but also NOS1 (also called nNOS) and NOS3 (also called eNOS) produce O_2^- (Andrew and Mayer, 1999). Interestingly, L-NMMA, a common inhibitor of all NOS isoforms, has been shown either to enhance or inhibit the uncoupled reaction and, hence, superoxide and peroxynitrite production (Gori et al., 2001), and this might explain why NOS inhibitors might not be effective alone in restoring immune functions in tumor environment.

On the whole, mouse and human studies have clearly recommended that drugs aimed at reverting T lymphocyte unresponsiveness in tumor-bearing hosts should target both ARG and NOS. We thus studied different NO-donating drugs that had been already shown to inhibit inducible NOS through various mechanisms (Griscavage et al., 1995; Mariotto et al., 1995), looking for those compounds also active on ARG. After testing various NO-donating structures, different isomers of NO-donating aspirin (nitroaspirin) were able to revert in vitro the immune suppression in the spleen of tumor-bearing hosts, with the compound NCX4016 being the most effective in vivo (De Santo et al., 2005). Immune suppression by MDSCs could be relieved either by nitroaspirin, the combination of ARG and NOS inhibitors, or peroxynitrite scavengers, whereas simple NO donors (SNAP) or nitroaspirin analogues, deprived of the NO-donating group (NCX 4050), were ineffective (De Santo et al., 2005).

In the same manuscript, we also showed that whereas NO donation was important for the feedback inhibition of NOS2, the aspirin portion (with the attached spacer) was active on ARG1 (De Santo et al., 2005). NO donation was known to regulate negatively NOS2 activity and expression (Griscavage et al., 1995; Mariotto et al., 1995). We speculated that the salicylic portion of the molecule could affect ARG by inhibiting STAT6-mediated signals triggered by Th2 cytokines (like IL-4 and IL-13) required to up-regulate ARG1 expression (Perez-G et al., 2002; Gallina et al., 2006). Moreover, nitroaspirin, but not aspirin, was shown to inhibit the catalytic subunit of NADPH oxidase and superoxide production induced by LPS, TNF, and IL-1α in pulmonary artery vascular smooth muscle cells and endothelial cells (Muzaffar et al., 2004). As expected from all this evidence, in vivo administration of nitroaspirin resulted in decreased intratumoral staining with anti-nitrotyrosine and anti-NOS2 antibodies, together with a reduced intratumoral ARG activity (De Santo et al., 2005).

Nitroaspirin was thus the ideal candidate to block peroxynitrite production within tumor environment. Unfortunately, nitroaspirin did not enhance the therapeutic activity of adoptively transferred, tumor-specific CTLs on established tumors (unpublished data), indicating a low in vivo efficacy and promoting a search for novel, more powerful moieties. We thus started to combine different NO-donating groups with chemical modifications of the aspirin portion, a process of oriented drug design assisted by an in vitro screening strategy which led to the identification of AT38.

Screening pipeline

To evaluate our compounds, we designed a screening process similar to that previously used to characterize nitroaspirin (De Santo et al., 2005). We made use of two assays by which we could rapidly test different but related aspects of the immune dysfunctions induced by tumors in T lymphocytes. The first assay was performed to evaluate whether novel compounds could restore T cell ability to proliferate in the presence of MDSCs. Splenocytes from tumor-free BALB/c mice or splenocytes isolated from BALB/c mice bearing a colon carcinoma (C26-GM) were cultured in 96-well flat-bottom plates and stimulated with anti-CD3 and anti-CD28 antibodies, with or without NO-donating drugs. After 2 d of incubation, cultures were pulsed with 1 μCi/well ^{3}HTdR for 18 h, and ^{3}HTdR incorporation was measured by scintillation counting. Data were expressed as cpm (mean) of triplicate wells. The putative drugs had to show no effect on control cultures from tumor-free mice (BALB/c splenocytes) and restore, in a dose-dependent fashion, T lymphocyte responsiveness inhibited in the spleen of C26-GM tumor-bearing mice.

Compounds positive in first screening were subsequently tested for their efficacy to restore T cell cytolytic activity against allogeneic target cells. Splenocytes isolated from the spleens of either tumor-free or tumor-bearing mice (C26-GM) were stimulated with γ-irradiated allogeneic C57BL/6 splenocytes in 96-well flat-bottom plates, with or without NO-donating drugs, added at scalar dilutions at the beginning of the culture. The percentage of $CD11b^{+}/Gr\text{-}1^{+}$ MDSCs present in the spleen of these mice varied from 20 to 30%. After 5 d of incubation, cultures were tested for ability to kill either allogeneic (MBL-2) or syngeneic (CT26) target cells in a 5-h ^{51}Cr-release assay. The ^{51}Cr-labeled target cells (2,000 cells per well) were incubated with effectors cells at various effectors to target cell ratios in 96-well microplates. After a 5-h incubation at 37°C, supernatants were harvested, and radioactivity was counted in a microplate scintillation counter. The percentage of ^{51}Cr released was calculated as: $[(R \text{ experimental} - R \text{ spontaneous})/(R \text{ maximal} - R \text{ spontaneous})] \times 100$.

To be defined as effective, the tested compounds should recover the function of tumor-bearing T lymphocytes without affecting the function of control cultures set up with lymphocytes from tumor-free mice (this was considered a toxic action of the screened molecules). Compounds that scored positive in the first two screening assays were further tested for the capability to reduce the ARG1 and NOS2 protein levels in vitro. Only after this stage, the prospective molecule was administered in vivo (summarized in the flowchart below). This pathway was completed only for AT38, and it will be discussed in detail here and in the main text (for the in vivo data).

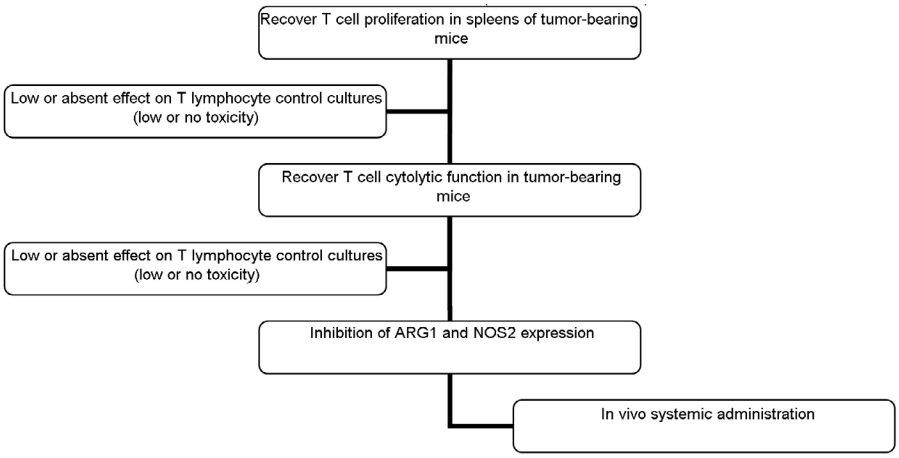
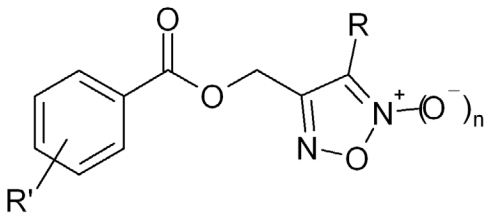


Table I. NO-donor aspirins (AT27, AT28, and AT33), corresponding des-NO-aspirins (AT89, AT90, and AT125) and related derivatives (AT44–AT107)

Compound	*n*	R	R'	Activity dose range[a]		Effective dose[b]		Toxic dose[c]	
				Proliferation	Cytotoxicity	Proliferation	Cytotoxicity	Proliferation	Cytotoxicity
				μm	*μm*	*μm*	*μm*	*μm*	*μm*
AT27	1	$CONH_2$	o-$OCOCH_3$	100–25	100–12	50	75	200	200
AT28	1	CN	o-$OCOCH_3$	33–16.5		16.5		33	
AT33[e]	1	C_6H_5	o-$OCOCH_3$	-	-	-	-	-	-
AT89	0	$CONH_2$	o-$OCOCH_3$	-		-		-	
AT90	0	CN	o-$OCOCH_3$	-[d]		-		-	
AT125[e]	0	C_6H_5	o-$OCOCH_3$	-		-		200	
AT44	1	$CONH_2$	H	76–38	-	76	-	-	-
AT38	1	$CONH_2$	o-OH	71.6–18	50–25	25	50	200	200
AT47	1	$CONH_2$	m-OH	71.6–35.8	50–25	71.6	50	-[d]	100
AT45	1	$CONH_2$	p-OH	71.6–9	50–25	9	50	-[d]	100
AT104	1	$CONH_2$	p-CH_3	144	100–50	144	100	289	200
AT117[e]	1	$CONH_2$	o-OCH_3	100	25	100	25	100	50
AT116[e]	1	$CONH_2$	m-OCH_3	-		-		200	
AT105	1	$CONH_2$	p-OCH_3	136.5–68.2	100–50	68.2	100	-[d]	200
AT101	1	$CONH_2$	p-F	285–142	100–25	142	100	285	200
AT102	1	$CONH_2$	p-NO_2	-[d]		-[d]		-[d]	
AT122[e]	1	$CONH_2$	o-CN	200	100–25	200	50	200	200
AT123[e]	1	$CONH_2$	m-CN	-		-		-	
AT103	1	$CONH_2$	p-CN	139	100–50	139	100	278	200
AT124	1	$CONH_2$	p-CF_3	-[d]		-[d]		-[d]	
AT106	1	$CONH_2$	p-NH_2	-[d]		-[d]		144	
AT107	1	$CONH_2$	p-Cl	-[d]		-[d]		269	

[a]Activity dose range represented the range of scalar dilutions of tested compound able to give a significant recover in either T cell proliferation (proliferation) or T cell cytolytic activity. In some cases the range was so limited that a single concentration is indicated.
[b]Effective dose was the lower dose able to rescue completely either T cell proliferation (proliferation) or T cell cytolytic activity.
[c]Toxic dose was the lower concentration of drug affecting either T cell proliferation (proliferation) or T cell cytolytic activity in control cultures lacking MDSCs. A significant reduction of 20% in either proliferation of cytolytic activity was considered as threshold.
[d]- indicates that no activity was detected at any concentration. These compounds were thus tested only in proliferation assays.
[e]Unpublished compounds. See chemical characterization.

A new class of NO-donor aspirins containing the furoxan system (1,2,5-oxadiazole 2-oxide) as NO donor moiety (AT27, AT28,and AT33) and the related furazans (des-NO aspirins, AT89, AT90, and AT125), unable to release NO, were recently developed (Cena et al., 2003; Table I). Table I shows that phenyl-substituted compound AT33 was inactive, whereas the cyano-substituted compound AT28 was a quite potent regenerator of lymphocyte T proliferation when used in the 33–16.5-μM concentration range but displayed high toxicity on T cells. The best results were obtained working with amide AT27, and consequently this product was selected for additional studies. Structurally related des-NO aspirins AT89, AT90, and AT125 were ineffective (Table I).



AT38 identification

When incubated in human serum, AT27, AT28, and AT33 were rapidly transformed into the corresponding salicylates without any intermediate formation of aspirin (ASA). In particular, AT27 was immediately transformed into AT38 ([3-(aminocarbonyl)furoxan-4-yl]methyl salicylate), which in turn was metabolized into salicylic acid (SA) and 4-hydroxymethyl-furoxan-3-carboxamide (CAS1609; Scheme 1 and Fig. 1 A).

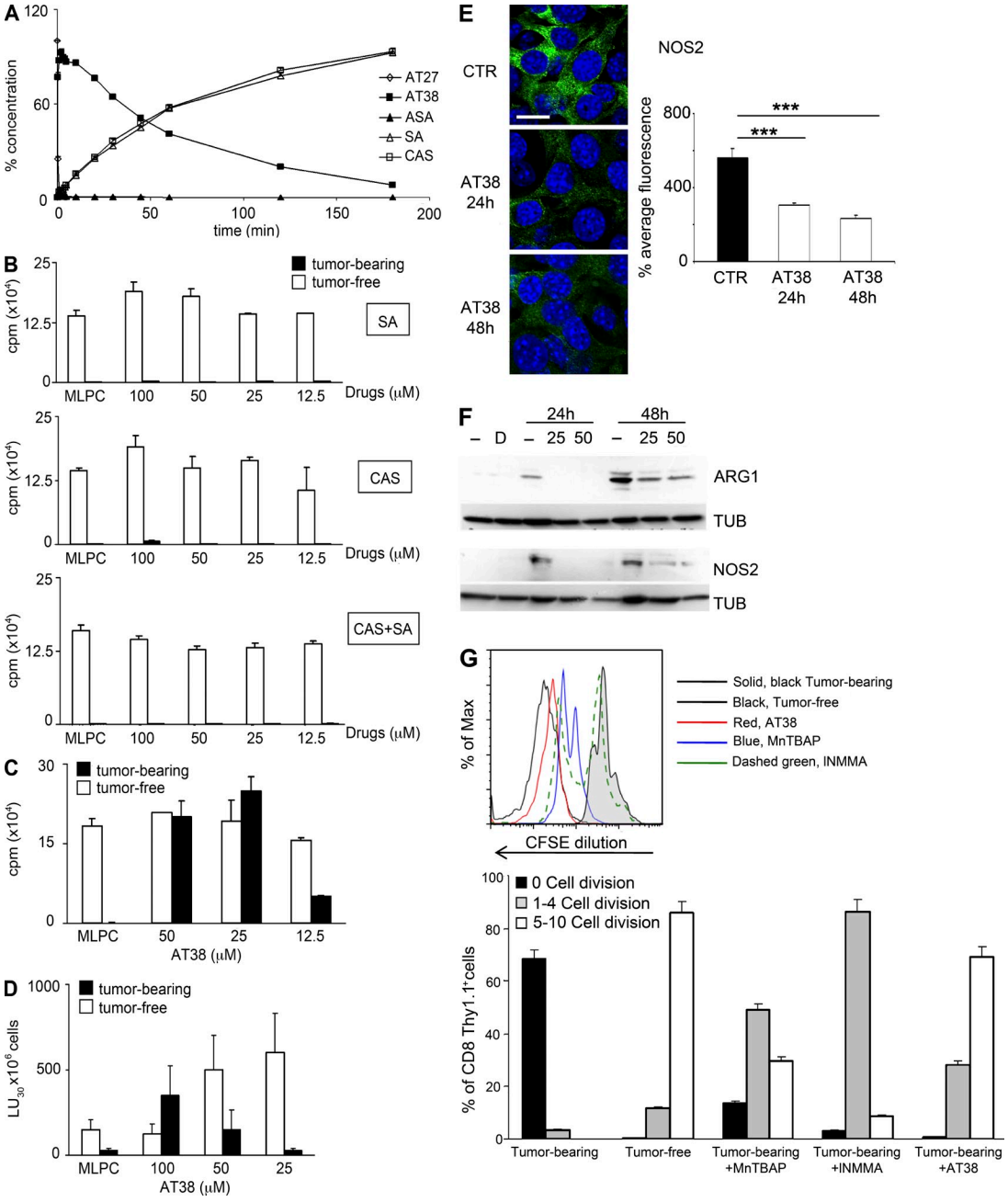
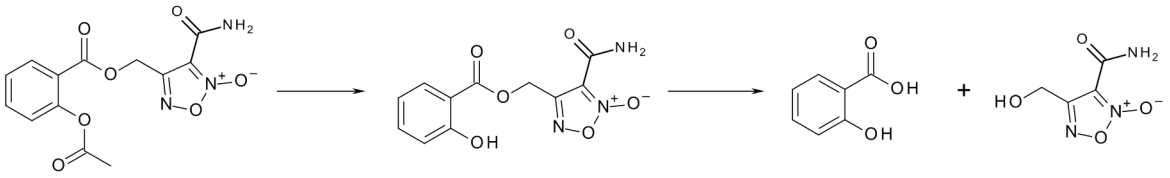


Figure 1. AT38 is a novel drug affecting ARG and NOS activity. (A) Kinetics of AT27 decomposition. AT27 was added to serum-containing medium, and AT38, acetylsalicylic acid (ASA), salicylic acid (SA), and CAS 1609 (CAS) byproducts were measured over time by HPLC. (B) Splenocytes from either tumor-free or C26GM tumor–bearing mice were stimulated with anti-CD3 and anti-CD28 mAbs, with or without different concentrations of SA, CAS1609, or a combination of both. After 3 d of incubation, cultures were pulsed with ^{3}HTdR for 18 h, and ^{3}HTdR incorporation was measured by scintillation counting. Data are expressed as mean ± SD, $n = 3$ different experiments. (C) As in B, splenocytes were stimulated and proliferation evaluated in the presence of different concentrations of the NO-donating drug AT38. (D) Splenocytes derived from either tumor-free or C26-GM tumor-bearing mice were stimulated for 5 d with allogeneic cells before being tested in a 5-h ^{51}Cr-release assay. Data are expressed as $L.U._{30}$ in 10^6 cells recovered in allo-stimulated lymphocyte cultures. Mean ± SD is shown. $n = 3$ independent cultures/group. (E) Confocal images of C26-GM colon carcinoma cells incubated or not with 50 µM AT38 for either 24 or 48 h and then immunostained for NOS2 protein (green fluorescence). The graph represents the mean of fluorescence (means ± SE, $n = 10$ ROIs). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test (***, $P < 0.001$). Bar, 20 µm. (F) MSC-2 cells were cultured for 4 d in the presence of either 100 ng/ml IL-4 or 25 ng/ml IFN-γ. Cells were then treated or not with 25 or 50 µM

Scheme 1



Both SA and CAS1609 were found to be unable to restore T lymphocyte functionality (Fig. 1 B). In contrast, salicylate AT38 could recover T lymphocyte proliferation and cytotoxic activity at doses lower than AT27 with similar toxicity (Table I).

Shift of the hydroxyl group to meta and para position gave rise AT47 and AT45, respectively, which were found to be as active as AT38 but slightly more toxic. Similarly, introduction at the phenyl ring of a number of substituents at orto, meta, para position for the hydroxyl group (Viola et al., 2010) afforded less active and more toxic compounds. Selected examples are reported in Table I.

AT38 was selected as a lead to submit to additional studies. It was able to interfere with MDSC-dependent blockade of T cell proliferation at doses 10-fold lower than nitroaspirin (Fig. 1 C, 25 instead of 250 μM; compare with De Santo et al., 2005). Slightly higher doses were required to restore the cytolytic activity of CTLs cultured in the presence of MDSCs (Fig. 1 D). When tested for its ability to interfere with L-arginine metabolizing enzymes, it effectively inhibited NOS2 expression in colon carcinoma C26GM (Fig. 1 E), as well as ARG1 and NOS2 expression induced in an immortalized MDSC line by exposure to either IL-4 or IFN-γ, respectively (Fig. 1 F). AT38 was also compared with L-NMMA and MnTBAP, a NOS inhibitor and peroxynitrite scavenger, respectively, for the ability to rescue T lymphocyte proliferation in a CFSE dilution assay. This assay allowed us to quantify accurately the number of cell cycles performed by the activated, antigen-specific $CD8^+$ T cells. In the tumor-conditioned spleen, AT38 was more effective than other inhibitors in restoring the number of cell divisions induced by the antigen peptide stimulation in TCR transgenic $CD8^+$ T lymphocytes (Fig. 1 G), whereas L-NMMA and MnTBAP supported only a limited number of cell cycles in T cells. AT38 was thus selected for further in vivo studies shown in the main text of the manuscript.

Chemical characterization of AT33, AT125, AT116, AT117, AT122 and AT123.

AT33 and AT125 were synthesized according to the procedure previously described (Cena et al., 2003).

(*3-Phenylfuroxan-4-yl*)*methyl 2-*(*acetyloxy*)*benzoate* (*AT33*): Yield 43%. Colorless oil. ^{1}H-NMR ($CDCl_3$): δ, 7.82 (*d*, 1H, C_6H_4); 7.59 (*t*, 2H, C_6H_4); 7.51 (*d*, 3H, C_6H_5); 7.26 (*t*, 2H, C_6H_5); 7.12 (*d*, 1H, C_6H_4); 5.47 (*s*, 2H, -OCH_2-Fx); 2.31 (s, 3H, -COCH_3). ^{13}C-NMR ($CDCl_3$): δ, 169.6; 163.2; 152.2; 151.0; 134.7; 131.6; 130.9; 129.4; 127.6; 127.4; 126.2; 124.0; 122.1; 121.8; 114.4; 57.4; 29.7; 20.9. Anal. Calc. For $C_{18}H_{14}N_2O_6$.

(*4-Phenylfurazan-3-yl*)*methyl 2-*(*acetyloxy*)*benzoate* (*AT125*): Yield 85%. White solid, m.p: 97–98.8°C. ^{1}H-NMR ($CDCl_3$): δ, 7.85 (*d*, 1H, C_6H_4); 7.73 (*t*, 2H, C_6H_4); 7.61–7.49 (*m*, 5H, C_6H_5); 7.26 (*t*, 1H, C_6H_4); 7.12 (*d*, 1H, C_6H_4); 5.59 (*s*, 2H, -OCH_2-Fx); 2.27 (s, 3H, -COCH_3). ^{13}C-NMR ($CDCl_3$): δ, 169.6; 163.4; 154.0; 151.0; 148.9; 134.6; 131.8; 130.9; 129.5; 128.2; 126.1; 125.0; 124.0; 121.9; 55.5; 20.9. Anal. Calc. For $C_{18}H_{14}N_2O_5$.

AT116, AT117, AT122, and AT123 were synthesized according to the procedure previously described (Viola et al., 2010).

[*3-*(*Aminocarbonyl*)*furoxan-4-yl*]*methyl 3-methoxybenzoate* (*AT116*): Yield 87%. White solid, m.p. 134–135°C (iPrOH). ^{1}H-NMR (DMSO-d_6): δ, 8.52 (s_{br}, 1H, -$CONH_2$); 7.84 (s_{br}, 1H, -$CONH_2$); 7.61–7.58 (*m*,1H. Ph); 7.50–7.45 (*m*, 2H, Ph); 7.30–7.26 (*m*, 1H, Ph); 5.62 (*s*, 2H, -OCH_2-Fx); 3.82 (*s*, 3H, -OCH_3). ^{13}C-NMR (DMSO-d_6): δ, 165.6; 160.2; 156.5; 155.6; 130.9; 122.5; 120.6; 114.9; 111.3; 58.5; 56.2. Anal. Calc. For $C_{12}H_{11}N_3O_6$.

[*3-*(*Aminocarbonyl*)*furoxan-4-yl*]*methyl 2-methoxybenzoate* (*AT117*): Yield 87%. White solid, m.p. 115–117°C (iPrOH). ^{1}H-NMR (DMSO-d_6): δ, 8.52 (s_{br}, 1H, -$CONH_2$); 7.84 (s_{br}, 1H, -$CONH_2$); 7.76–7.73 (*dd*, J_1= 1.8 Hz, J_2= 7.5 Hz, 1H, Ph); 7.62–7.56 (*m*, 1H, Ph); 7.17 (*d*, J= 8.4 Hz, 1H, Ph); 7.07–7.01 (*m*, 1H, Ph); 5.55 (*s*, 2H, - OCH_2-Fx); 3.83 (*s*, 3H, -OCH_3). ^{13}C-NMR (DMSO-d_6): δ,165.1; 159.6; 156.5; 155.7; 135.2; 132.0; 120.9; 119.4; 113.5; 111.3; 58.0; 56.7. Anal. Calc. For $C_{12}H_{11}N_3O_6$.

[*3-*(*Aminocarbonyl*)*furoxan-4-yl*]*methyl 2-cyanobenzoate* (*AT122*): Yield 82%. White solid, m.p. 146–147°C (iPrOH). ^{1}H-NMR (DMSO-d_6): δ, 8.51 (s_{br}, 1H, -$CONH_2$); 8.20–8.16 (*m*, 1H, Ph); 8.07–8.03 (*m*, 1H, Ph); 7.92–7.86 (*m*, 2H, Ph); 7.82

AT38 for 24 and 48h. Immunoblot were performed on whole cell extracts were obtained from 2 × 10^5 cells. TUB, tubulin control. (G) CFSE-labeled $CD8^+$ $Thy1.1^+$ T lymphocytes were admixed with splenocytes from either tumor-free or tumor-bearing mice and stimulated with the $HA_{512-520}$ peptide for 3 d. 500 μM L-NMMA, 500 μM MnTBAP, or 25 μM AT38 were added once at the beginning of the cultures. CFSE dilution was evaluated among gated $CD8^+$/$Thy1.1^+$ (histograms in top panel) and number of cell divisions calculated with Flow-Jo Software (bars in bottom panel). Data are expressed as mean ± SD, *n* = 3 different experiments.

(s_{br}, 1H, -$CONH_2$); 5.69 (s, 2H, - OCH_2-Fx). ^{13}C-NMR (DMSO-d_6): δ, 163.6; 156.4; 155.2; 136.2; 134.8: 134.3; 132.1; 131.5; 118.0; 112.7; 111.3; 59.0. Anal. Calc. For $C_{12}H_8N_4O_5$.

[*3-(Aminocarbonyl)furoxan-4-yl]methyl 3-cyanobenzoate* (*AT123*): Yield 97%. White solid, m.p. 179–180°C (iPrOH). ^{1}H-NMR (DMSO-d_6): δ, 8.50 (s_{br}, 1H, -$CONH_2$); 8.38–8.37 (*m*, 1H, Ph); 8.31–8.28 (*m*, 1H, Ph); 8.20–8.16 (*m*, 1H, Ph); 7.81–7.79 (*m*, 1H, Ph); 7.77 (s_{br}, 1H, -$CONH_2$); 5.64 (s, 2H, - OCH_2-Fx). ^{13}C-NMR (DMSO-d_6): δ, 163.7; 155.8; 154.6; 137.4; 134.1; 133.2; 130.6; 130.3; 118.0; 112.5; 110.8; 58.2. Anal. Calc. For $C_{12}H_8N_4O_5$.

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