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Immunomodulatory activity of two new aza alkyl phospholipid antineoplastic drugs

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The present work reports the modulation of immunocompetent cell functions by two aza alkyl phospholipids (AAP), BN 52205 and BN 52211. Each compound was compared with 1-*O*-octadecyl-2-*O*-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and/or three drugs used for cancer treatment, i.e. cisplatyl (CIS), 5-fluorouracil (5-FU) and cytosine arabinoside (ARA-C). Interleukin (IL)-1 release from P388D1 cells was increased 2-fold in the presence of 5 μg/ml BN 52205 or BN 52211. However, these stimulations were lower than those obtained with ARA-C, 5-FU and CIS. Compared with ET-18-OCH_3, CIS and 5-FU, BN 52205 and BN 52211 were more efficient in increasing tumor necrosis factor production induced by lipopolysaccharide (LPS) from human monocytes. *In vitro*, all compounds exhibited similar activity in enhancing IL-6 production from human monocytes stimulated with LPS, with the exception of 5-FU and CIS that were inactive. At 20 mg/kg (i.v.), a peak of IL-6 production was reached 2 h after injection of ET-18-OCH_3 [>1280 U/ml ($n = 4$, $p < 0.001$) versus 3.5 ± 0.2 U/ml ($n = 7$)], whereas BN 52211 induced a maximum of IL-6 production after 4 h (77 ± 27 U/ml, $n = 5$, $p < 0.001$). BN 52205 induced peaks of IL-6 production after 3 and 6 h (90 ± 62 and 68 ± 35 U/ml, respectively, $p < 0.001$, $n = 4$). The proliferation of rat splenocytes was abolished in the presence of BN 52205 and BN 52211 at 10 μg/ml, corresponding to only a partial reduction of IL-2 production at the same concentration. The production of interferon-γ was stimulated 6- to 10-fold in the presence of 1–5 μg/ml BN 52205, BN 52211 and ARA-C. BN 52211 and BN 52205 were also potent enhancers of IL-3 production, whereas 5-FU and ARA-C were inhibitory. These results indicate that in addition to a direct antitumoral effect, AAP may also exhibit immunomodulatory activity both *in vitro* and *in vivo*.

Key words: Aza-alkyl-phospholipid, BN 52205, BN 52211.

Introduction

Alkyl lysophospholipids (ALP), like the reference compound ET-18-OCH_3, represent a new generation of antitumor drugs currently being tested in phase I clinical trials in cancer patients.[1] ALP have been described as a new class of biological response modifiers that inhibit the growth[2] and metastasis[3] of syngeneic experimental mouse and rat tumors.[4] In addition, ALP have been shown to lyse leukemic[5] and tumor cells,[6] indicating a direct cytotoxic effect. ALP also induce differentiation of leukemic blast cells[7] and counteract tumor cell invasion.[8]

Previous studies have indicated that the therapeutic activity of ALP may be mediated by an increase in the cytotoxic effect of macrophages.[9] ALP also play a regulatory role in cellular immunity since they enhance delayed-type hypersensitivity reactions and contact sensitivity in the mouse.[10,11] The differential action of ALP on the immune system, especially with respect to macrophage activation and suppression of lymphocyte functions, might account for their beneficial effects in autoimmune diseases and in acquired immunodeficiency syndrome.[12] Andreesen *et al.*[12] demonstrated that ALP restore the capacity of mature macrophages to respond to lipopolysaccharide (LPS) by the release of interleukin (IL)-1 and increase macrophage cytotoxicity induced by interferon (IFN)-γ and LPS. In addition, ALP have been demonstrated to inhibit IL-2 synthesis.

Two new synthetic compounds, BN 52205 and BN 52211,[13] belonging to the aza-substituted ether lipid series (aza alkyl phospholipids; AAP) were investigated in the present study. These compounds are considered as potential anticancer agents. Indeed, Morimoto *et al.*[14] demonstrated that BN 52205 and BN 52211 were endowed with cytotoxic activity against various human tumor cell lines of different histological origin. In addition to the cytotoxic effects, these authors observed that these two AAP compounds also exerted a cytotoxic effect, as assessed by the inhibition of [^{3}H]thymidine incorporation in tumor cells. Sidoti *et al.*[15] observed

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that these new AAP are characterized by multiple 'terminal points' actions. Indeed, drug action results in a G_1 blockade, in a slow-down of the transition from late S to G_2 followed by an accumulation in the G_2 phase of the cell cycle. Principe *et al.*[16] showed experimental evidence that the direct cytotoxic effect of these drugs is mediated by the cell membrane, since a positive correlation between the membrane cholesterol level and cell sensitivity to AAP was established.

Although AAP may represent a new class of anticancer drugs, their action on the immune system is poorly understood. The present results demonstrate the modulation of various immunocompetent cell functions *in vitro* and *in vivo* by AAP. The action of these compounds was compared with that of ET-18-OCH_3 and three drugs used in cancer treatment, i.e. cisplatyl (CIS), 5-fluorouracil (5-FU) and cytosine arabinoside (ARA-C). The effect of these compounds on IL-1 from the P388D1 cell line, and tumor necrosis factor (TNF) and IL-6 production from human monocytes, used as a model of monocyte/macro-phage activation, was analyzed. The effect of AAP on *in vivo* IL-6 production in the rat and lymphocyte functions, i.e. proliferation and IL-2 production from rat splenocytes, was also determined. Finally, the possibility that these compounds modulate human IFN-γ and mouse IL-3 production and the activity of human natural killer cells (NK) was investigated.

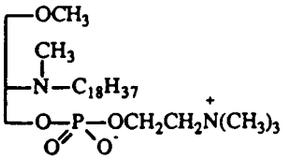
Materials and methods

Reagents

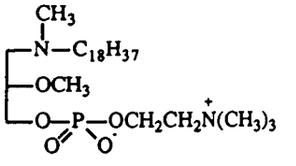
The two AAP investigated here were 3-methoxy-2-*N*, *N*-methyloctadecyl-amino-propyloxyphosphorylcholine (BN 52205) and the 1-*N*,*N*-methyloctadecyl-amino-methoxy-2-propyloxyphosphorylcholine (BN 52211) (Institut Henri Beaufour, Les Ulis, France) (Figure 1) solutions which were made extemporaneously in the medium of the experiments. LPS and Concanavalin A (Con A) (Sigma, St Louis, MO), RPMI 1640 (Flow Laboratories, McLean, VA) and fetal calf serum (FCS) (Gibco, Paisley, UK) were obtained as noted.

Assessment of IL-1 and IL-3 production from cell lines and assays

P388D1 cells (ATCC: TIB 63) (2×10^6 cells/ml) were incubated for 24 h at 37°C in RPMI 1640 containing 10% FCS in the presence or absence of defined concentrations of the various compounds.



BN 52205



BN 52211

Figure 1. Structure of BN 52205 and BN 52211.

After washing, the cells were added to 1 μg/ml LPS and incubated at 37°C for another 24 h incubation period. IL-1 production in supernatants was determined using the thymocyte assay,[17] as measured by the uptake of [^{3}H]thymidine at the end of a 72 h culture period. The results are expressed in equivalent U/ml of IL-1 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine.

EL4 cells (ATCC: TIB 181, mouse thymoma) were cultured in Dulbecco's minimal essential medium in the presence of 1% FCS at a density of 0.1×10^6 cells/200 μl/well and stimulated with 2 μg/ml Con A. After a 48 h incubation period at 37°C under a 5% CO_2 humidified atmosphere, supernatants were harvested and stored frozen at −20°C before being assayed for IL-3 activity, as determined by the proliferation of the AD3 cell line. Briefly, triplicates of 0.1×10^6 cells/ml in RPMI 1640 containing 10% FCS were mixed with defined dilutions of the supernatants to be tested or rIL-3, up to a final volume of 200 μl. After a 48 h culture period, the number of viable cells was evaluated after staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) for 6 h. Production in the various supernatants was calculated as equivalent U/ml of IL-3 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine.

Assessment of proliferation and IL-2 production from rat splenocytes and assays

Rat spleen mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients. The cells were washed, resuspended at 2×10^6 cells/ml

in RPMI 1640 containing 10% FCS with or without defined concentrations of AAP and stimulated with either 1 μg/ml Con A for 72 h for the assessment of proliferation or 15 μg/ml Con A for 24 h in the case of measurements of IL-2 production. At 66 h, for the assessment of proliferation, 50 μl of [^{3}H]thymidine (1.5 μCi) was added and the cells were harvested at the end of the incubation period using a Skatron cell harvester.

IL-2 production in supernatants was determined by the proliferation of CTLL cells (ATCC: TIB 214) over a 24 h period. At 18 h, 50 μl of [^{3}H]thymidine (1.5 μCi) was added and the cells harvested at the end of the incubation period using a Skatron cell harvester. The results are expressed as percent variations calculated over the values obtained in control cultures incubated without AAP.

Assessment of IFN-γ and NK activity

Peripheral blood mononuclear leukocytes (PBML) obtained after Ficoll gradients were washed and resuspended at 3×10^6 cells/ml/well in RPMI 1640 containing 10% FCS and stimulated with LPS (10 μg/ml) for 48 h, with or without defined concentrations of AAP. At the end of the incubation period, the supernatants were harvested and IFN-γ was quantified by commercially available IRMA kits (Centocor, Malvern, PA). This assay is specific for IFN-γ and does not detect IFN-α or IFN-β. Results are expressed in equivalent U/ml of recombinant IFN.

Monocyte-depleted PBML were cultured for 18 h at 2.5×10^6 cells/ml in RPMI 1640 containing 10% FCS, in the presence or in the absence of AAP. K 562 target cells (1×10^6 cells/10 ml) were labeled overnight with 200 μCi $Na_2^{51}CrO_4$ at 37°C, washed 3 times and mixed at defined effector/target cell ratios with the effector cells pretreated or not with the AAP. The cell suspensions were incubated for 4 h at 37°C under a 5% CO_2/95% air atmosphere. Upon completion of incubation, 100 μl of culture supernatants was harvested and counted in a gamma counter. The cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total radioactivity} - \text{spontaneous release}} \times 100$$

The variation between triplicates never exceeded 10% of the mean in all sets of experiments.

Assessment of TNF and IL-6 production from monocytes

Human mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients. Monocytes were separated by adherence on plastic Petri dishes for 1 h at 37°C and detached with a rubber policeman. The cells were washed, resuspended at 2×10^6 cells/ml in RPMI 1640 containing 10% FCS and stimulated with LPS (2 μg/ml) for 24 h at 37°C, in the presence or in the absence of defined concentrations of AAP or anticancer drugs. The supernatants were collected and tested for TNF production by the lysis of L 929 cells and for IL-6 production by the proliferation of the 7TD1 cell line (obtained through the courtesy of Dr J Van Snick, The Ludwig Institute, Brussels, Belgium). Results are expressed in equivalent ng/ml of TNF and U/ml IL-6 with respect to the linear portion of calibration curves performed with known amounts of the corresponding recombinant cytokine.

Assessment of IL-6 in sera

Sprague Dawley rats (250 g) were injected i.v. with AAP (50, 20 and 10 mg/kg) and blood samples were obtained after 1, 2, 3, 4, 6 and 24 h. IL-6 in sera was determined by the proliferation of the 7TD1 cell line. Briefly, triplicates of 2×10^3 cells were mixed with defined dilutions of the samples to be tested or rIL-6, up to a final volume of 200 μl. After 96 h in culture, the number of viable cells was evaluated by the colorimetric determination of β-hexosaminidase levels. Results are expressed in equivalent U/ml of recombinant IL-6 (rIL-6) with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine.

Statistical analysis

Results were analyzed for statistical significance using the F test or analysis of variance.

Results

Effect of AAP on IL-1 production by LPS-stimulated P388D1 cells

Over a 24 h incubation period. ARA-C, 5-FU and CIS (0.1–10 μg/ml) increased the LPS-induced IL-1

production. For these drugs a 7-11 times increase in IL-1 production from P388D1 was noted ($p < 0.001$, Figure 2). In contrast, ET-18-OCH_3 did not significantly modify IL-1 production by P388D1 cells over the range of concentrations from 1 ng/ml to 10 μg/ml. BN 52211 and BN 52205 at 5 μg/ml were poor inducers of IL-1 production as compared with the three drugs tested above, since they stimulated the release of this cytokine only 2-fold ($p < 0.05$, Figure 2).

Effect of AAP on TNF production from LPS-stimulated human monocytes

BN 52211 markedly increased LPS-induced TNF production at concentrations between 1 and 5 μg/ml, with a peak effect at 2.5 μg/ml (Figure 3). BN 52205 and ARA-C also significantly increased TNF production at 2.5 and 5 μg/ml, with a maximum at the latter concentration (Figure 3). These increases were, however, lower as compared with those induced by BN 52211. ET-18-OCH_3 exhibited a similar profile of activity as compared with that induced by BN 52211. Indeed, ET-18-OCH_3 increased TNF production in the concentration range from 1 to 5 μ/ml, with a maximal effect at 2.5 μg/ml. However, using this concentration of ET-18-OCH_3 the amount of TNF induced was lower (1.7 ± 0.3 ng/ml) than that observed after treatment of human monocytes with BN 52211 (4.6 ± 1.3 ng/ml). 5-FU and CIS at concentrations ranging from 0.05 to 5 μg/ml did not significantly modify TNF production.

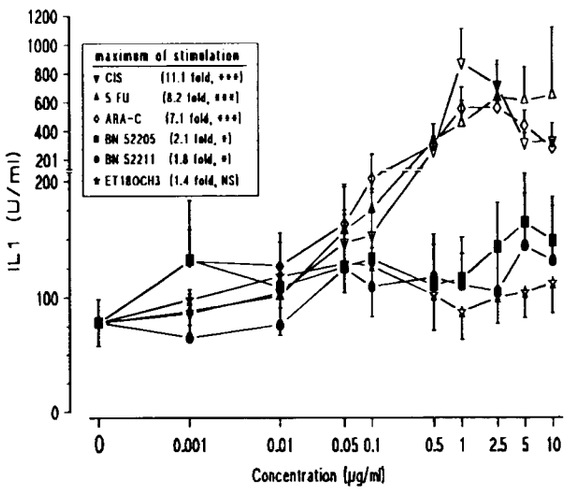


Figure 2. P388D1 were incubated at 2 × 10^6 cells/ml in 24-well plates overnight at 37°C in the presence of defined concentrations of the various compounds. After 3 washes with RPMI 1640 containing 10% FCS, the cells were mixed with 1 μg/ml LPS and incubated for another 24 h incubation period. The IL-1 production in cell-free supernatants was determined in a bioassay by the proliferation of mouse thymocytes. The results are expressed in equivalent U/ml of IL-1 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine. Mean ± SEM of five experiments. $^*p < 0.05$, $^{***}p < 0.001$.

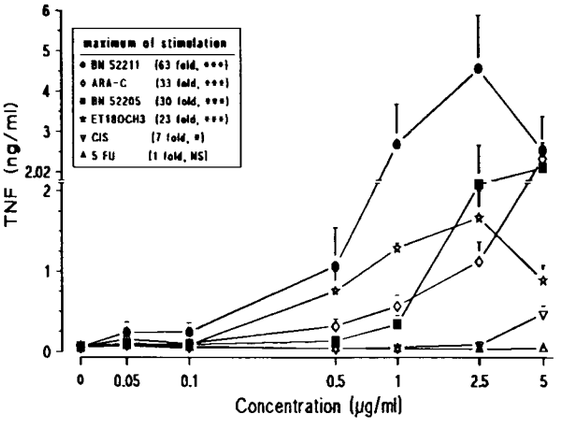


Figure 3. Human monocytes (2 × 10^6 cells/ml) were incubated in 24-well plates overnight at 37°C in the presence of LPS (2 μg/ml) and defined concentrations of AAP or anticancer drugs. The cell-free supernatants were collected and stored at −20°C prior to TNF activity determination in a bioassay. The results are expressed in equivalent ng/ml of TNF with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine. Mean ± SEM of five experiments. $^*p < 0.05$, $^{***}p < 0.001$.

Effect of AAP on *in vitro* and *in vivo* IL-6 production

ARA-C, BN 52211 and BN 52205 increased the *in vitro* LPS-induced IL-6 activity from human monocytes in a dose range from 1 to 5 μg/ml and with a peak effect at 2.5 μg/ml ($p < 0.01$, Figure 4). ET-18-OCH_3 also increased IL-6 activity with a maximum observed at 1 μg/ml. The enhancement of IL-6 production observed with this drug was half that obtained with the three first compounds. 5-FU and CIS did not alter LPS-induced IL-6 production from human monocytes.

Injection (i.v.) of 50 mg/kg ET-18-OCH_3 was highly toxic, all rats treated with this compound died within 2 h (Figure 5C). The level of IL-6 in rat serum was markedly increased by 73- and 366-fold when the rats were treated with ET-18-OCH_3 at 10 and 20 mg/kg, respectively

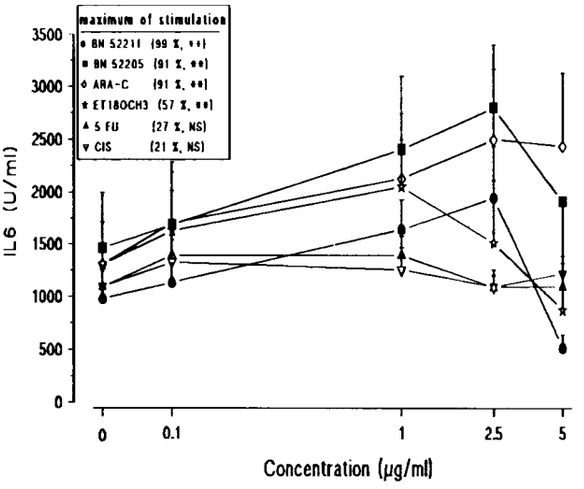


Figure 4. Human monocytes (2×10^6 cells/ml) were incubated in 24-well plates overnight at 37°C in the presence of LPS (2 µg/ml) and defined concentrations of AAP or anticancer drugs. The cell-free supernatants were collected and stored at −20°C prior to IL-6 activity determination in a bioassay. The results are expressed in equivalent U/ml of IL-6 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine. Mean ± SEM of six to eight experiments. $^{**}p < 0.01$.

(Figure 5A and B), with a peak effect observed 2 h after injection. A similar profile was noted with BN 52211 (10 mg/kg), although the maximum increase obtained after 2 h was of lower intensity (10-fold increase). When the rats were treated with BN 52211 at 20 and 50 mg/kg, significant 21- and 29-fold increases, respectively, of IL-6 production were observed, with a peak effect observed 4 h after injection (Figure 5B and C). BN 52205 administered at 10 mg/kg did not modify the IL-6 level in rat sera, whereas 20 mg/kg induced significant 23- and 18-fold increases in IL-6 production after 3 and 6 h, respectively (Figure 5B). A similar profile was observed when the rats were treated with 50 mg/kg BN 52205. Indeed, IL-6 production was increased by 22- and 35-fold after 3 and 6 h, respectively, whereas a 11-fold increase was observed after 4 h.

Effect of AAP on proliferation of Con A-stimulated rat splenocytes

As measured after 72 h, ET-18-OCH_3 inhibited Con A-induced proliferation of rat splenocytes in a concentration-dependent fashion between 1 and 10 µg/ml (28–97%, Figure 6). The same profile of

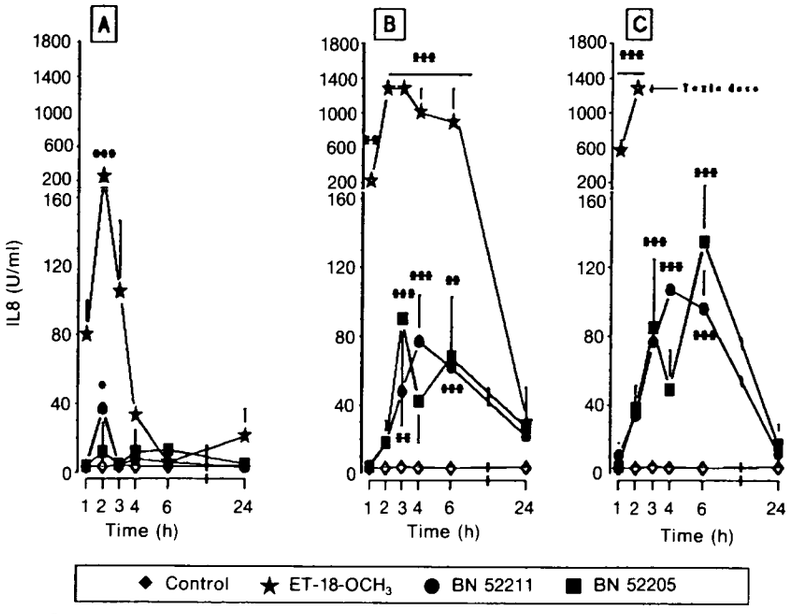


Figure 5. Sprague-Dawley rats (250 g) were injected i.v. with the various compounds at 10 mg/kg (A), 20 mg/kg (B) and 50 mg/kg (C); blood samples were then obtained after defined time intervals. IL-6 in sera was determined in a bioassay. Briefly, triplicates of 2×10^3 7TD1 cells were mixed with 1/2 to 1/256 dilutions of the samples to be tested or rIL-6 and the final volume was adjusted to 200 µl with fresh medium. After 96 h in culture, the number of viable cells was evaluated by the colorimetric determination of β-hexosaminidase. The results are expressed in equivalent U/ml of IL-6 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine. Mean ± SEM of three to six experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

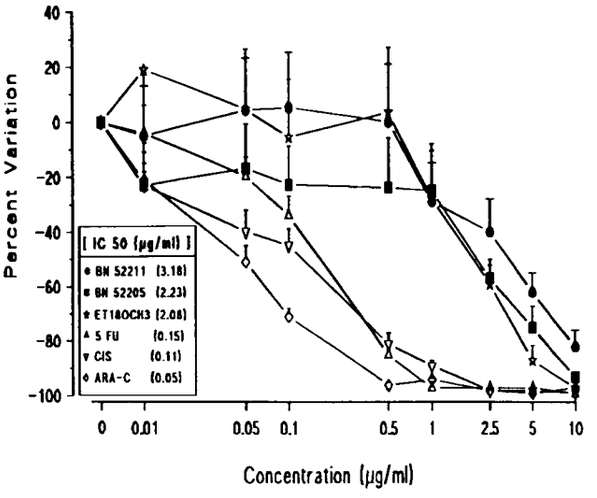


Figure 6. Rat spleen mononuclear cells (2 × 10^6 cells/ml) were incubated in 96-well plates at 37°C in the presence of Con A (1 µg/ml) and with or without defined concentrations of the various compounds. After 66 h, 50 µl of [^{3}H]thymidine (1.5 µCi) was added and after 6 h cells were collected using a Skatron cell harvester. The results are expressed as percent variation calculated over the 100% values obtained in control cultures incubated without the various compounds. IC_{50} indicates the concentration inducing 50% inhibition. Mean ± SEM of eight experiments.

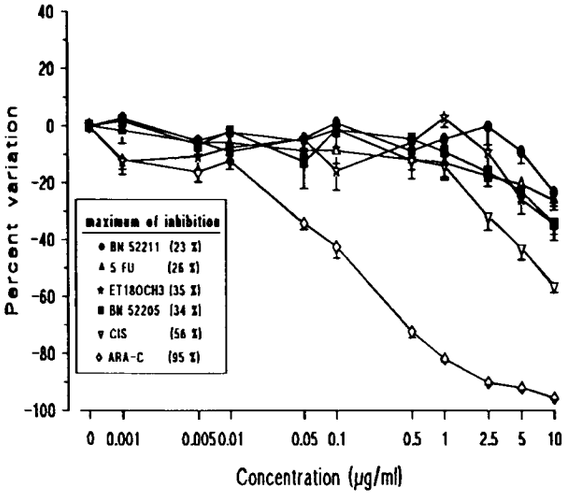


Figure 7. Rat spleen mononuclear cells (2 × 10^6 cells/ml) were incubated in 96-well plates at 37°C in the presence of Con A (15 µg/ml) and with or without defined concentrations of the various compounds. After 24 h, the cell supernatants were collected and stored at −20°C prior to IL-2 activity, as assessed by the proliferation of the CTLL cell line. The results are expressed as percent variation calculated over the 100% values obtained in control cultures incubated without the various compounds. Mean ± SEM of seven experiments.

activity was observed with BN 52205 and BN 52211 in this concentration range (Figure 6). As compared with these compounds, the inhibitions of Con A-induced proliferation observed with ARA-C, CIS and 5 FU were already maximum (97%) at 1 µg/ml. Classification of the different compounds was made according to the concentration (expressed in µg/ml) corresponding to the half maximal effect (Figure 6). For the three drugs used for cancer therapy (5-FU, CIS and ARA-C), concentrations in the range 0.05–0.15 µg/ml induced a 50% inhibition of cell proliferation. In contrast, concentrations of ET-18-OCH_3, BN 52205 or BN 52211 that were 20–30 times higher were required to induce 50% inhibition of proliferation of rat splenocytes.

Effect of AAP on IL-2 production by Con A-stimulated rat splenocytes

Rat splenocytes were stimulated by Con A and incubated with or without the various compounds for 24 h prior to IL-2 determination in supernatants. ARA-C dramatically inhibited Con A-induced IL-2 production in a concentration-dependent fashion in the range between 0.01 and 10 µg/ml (10–95%, Figure 7). The half maximal effect was reached at 0.12 µg/ml. In the case of CIS, a concentration-dependent effect was also observed between 1 and 10 µg/ml. At the highest concentration, ET-18-OCH_3 and BN 52205 decreased IL-2 production by about 35%, whereas BN 52211 and 5-FU were almost inefficient (−24%) (Figure 7).

Effect of AAP on IFN-γ production from LPS-stimulated PBML

Enhancement of LPS-induced IFN production by ARA-C was observed at the lowest concentration investigated, i.e. 0.1 µg/ml; the maximal effect being noted at 2.5 µg/ml (Figure 8). BN 52211, BN 52205 and ET-18-OCH_3 significantly increased IFN-γ production at concentrations above 0.5 µg/ml. In contrast, 5-FU and CIS did not enhance LPS-induced IFN-γ production in the concentration range between 0.1 and 5 µg/ml (Figure 8). The classification of the potency of the various compounds is indicated in the legend of Figure 8.

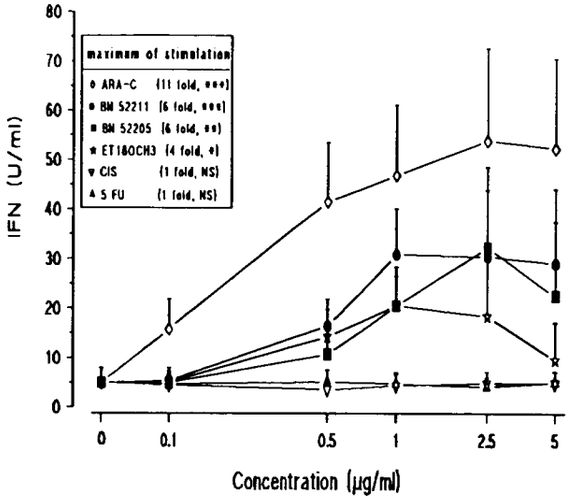


Figure 8. PBML (3×10^6 cells/ml/well) were incubated in 24-well plates for 48 h at 37°C in the presence of LPS (10 µg/ml) and with or without defined concentrations of the various compounds. At the end of the incubation period, the supernatants were harvested and IFN-γ was quantitated by an IRMA kit. Results are expressed in equivalent U/ml of IFN. Mean ± SEM of six experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

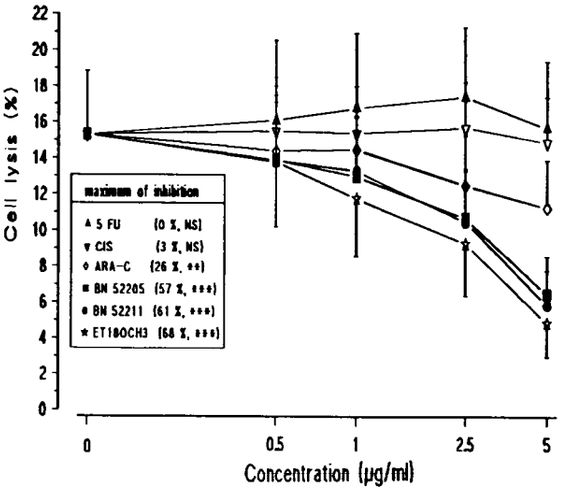


Figure 9. PBML depleted in monocytes (2.5×10^6 cells/ml) were incubated for 18 h at 37°C in the presence or in the absence of defined concentrations of the various compounds. In parallel, the K562 target cells were labeled overnight with 200 µCi $Na_2^{51}CrO_4$ at 37°C. After washing, target cells were mixed with the effector cells for 4 h at different effector/target cell ratios. At the end of the incubation period, 100 µl of culture supernatant was harvested and counted in a gamma counter. The cytotoxicity was calculated as described in Materials and methods. In this figure, only the effector/target cell ratio 6.25/1 is represented for legibility. Mean ± SEM of six experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

Effect of AAP on NK cell activity

NK activity was decreased by about 60% when the effector cells were preincubated in the presence of 5 µg/ml ET-18-OCH_3, BN 52211 or BN 52205 for 18 h prior to addition to target cells (Figure 9). ARA-C was less efficient in inhibiting *in vitro* NK activity as compared with the lipid analog compounds. 5-FU and CIS did not modify NK cytotoxic activity against K 562 cells.

Effect of AAP on IL-3 production by Con A-stimulated EL4 cells

Most of the drugs used in cancer treatment not only induce cancer cell death but are also toxic for hematopoietic progenitors. Therefore, the effect of the two new compounds on IL-3 production was investigated. Between 0.01 and 10 µg/ml, ARA-C and 5-FU significantly decreased IL-3 production (Figure 10). A bell-shaped effect was noted with ET-18-OCH_3, with a significant increase of IL-3 production observed at 1 µg/ml (4.3 ± 1.9 U/ml, $p < 0.001$), followed by a decrease of 5 and 10 µg/ml (0.6 ± 0.1 U/ml, $p < 0.001$, Figure 10). IL-3 production was enhanced by BN 52211 and BN 52205 in the range of concentrations between 1 and 5 µg/ml. Moreover, after treatment with

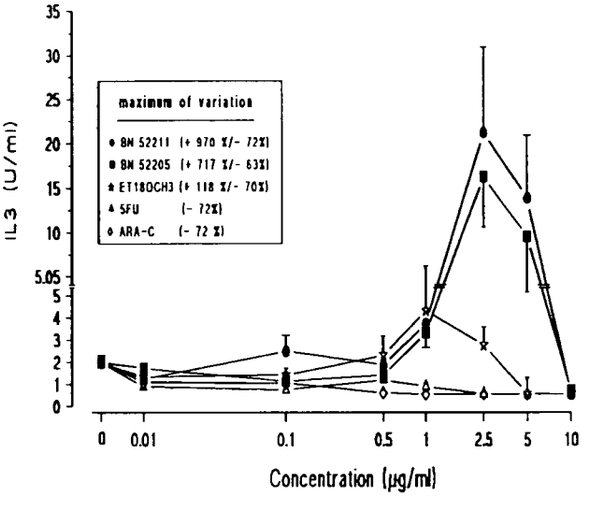


Figure 10. EL4 cells (5×10^6 cells/ml) were incubated in 96-well plates for 48 h in the presence of Con A (2 µg/ml) and defined concentrations of the various compounds. The cell supernatants were collected and stored frozen at −20°C prior to IL-3 as assessed by the proliferation of AD3 cells. Results are expressed in equivalent U/ml of IL-3 with respect to the linear portion of calibration curves performed with known amounts of the recombinant cytokine. Mean ± SEM of three experiments.

2.5 μg/ml BN 52211 (21.3 ± 9.7 U/ml, $p < 0.001$) or BN 52205 (16.3 ± 5.7 U/ml, $p < 0.001$) the IL-3 production was dramatically increased as compared with control values (2 ± 0.3 U/ml).

Discussion

Bausert *et al.*[18] demonstrated that i.p. injection of 2-lysophosphatidylcholine (2-LPC) or ALP induced temporary ascites containing 70% macrophages. When reinjected i.v. after the excision of a primary syngenic 3 LL tumor, these cells significantly protected mice from metastasis, suggesting that the antineoplastic activity of ALP *in vivo* might be partially mediated by cytotoxic macrophages.[19] Andreesen and Giese[12] also provided evidence for a novel function of ether lipids, i.e. the differential manipulation of the immune response *in vitro*. Indeed, these authors demonstrated that ALP render monocytes/macrophages responsive to subsequent stimulation. Under their experimental conditions only ALP-primed macrophages release high amounts of a lymphocyte-activating factor activity (IL-1) when stimulated with LPS.

IL-1, TNF and IL-6 are typical examples of multifunctional cytokines involved in the regulation of the immune response, hematopoiesis and inflammation. Their functions widely overlap but each of them exhibits specific properties in various aspects of inflammatory processes. IL-1 is the most potent inflammatory cytokine that induces prostaglandin synthesis. TNF was initially described as a product of endotoxin-activated macrophages causing hemorrhagic necrosis of tumors *in vivo*.[20] TNF is cytotoxic for tumor cells either alone or in combination with anticancer drugs.[21] As with IL-1 and TNF, monocyte-derived IL-6 possesses multiple biologic activities that affect a broad range of cell types including those directly involved in the immune responses. Indeed, Mule *et al.*[22] demonstrated that treatment of tumor-bearing mice with a combination of subtherapeutic doses of rTNF and rIL-6 resulted in the eradication of the tumor in a significant proportion of animals, a phenomena not observed when the animals were treated with these cytokines alone.

In the present study, two new AAP compounds, i.e. BN 52205 and BN 52211, are shown to increase 2-fold the *in vitro* LPS-induced IL-1 production by monocytes/macrophages. However, this effect was moderate compared with that of CIS, 5-FU and ARA-C, which are able to increase IL-1 production by 7- to 11-fold. In contrast, the most potent enhancer of LPS-induced TNF production was BN 52211, whereas BN 52205 and ARA-C were less active. BN 52211 was as efficient as BN 52205 and ARA-C in inducing a 2-fold increase of IL-6 production by LPS-stimulated human monocytes. Given the fact that the effects of AAP on LPS-induced TNF and IL-6 production were investigated on the same cells and that the potentiation of TNF was markedly higher than that of IL-6, it is tempting to conclude that these two cytokines possess different regulation pathways.

Injected i.v. at 20 mg/kg, BN 52211 and BN 52205 induced IL-6 in rat serum, although to a lower extent as compared with ET-18-OCH_3. For ET-18-OCH_3 (10 and 20 mg/kg), the IL-6 level in rat serum increased after 1 h for up to 4 or 6 h and with a maximal effect observed about 2 h after injection. A significant increase was also obtained with BN 52211 and BN 52205; however, in contrast to ET-18-OCH_3, the maximal effect was observed 4 h after injection for BN 52211, and 3 and 6 h after injection for BN 52205. These results may indicate a difference in the biodisponibility of these compounds and/or that the production of IL-6 evoked by BN 52205 might originate from a different cell pool.

The second part of this work was devoted to analyzing the effect of AAP on lymphocyte functions. ET-18-OCH_3 has already been shown to suppress human lymphocyte response *in vitro* by selective killing of lymphoblasts.[23] Andreesen and Giese[12] observed also that ET-18-OCH_3 inhibited IL-2 production in the concentration range between 1 and 8 μg/ml. In the present study, we demonstrate that various compounds at 10 μg/ml suppressed the activation of lymphocytes induced by Con A. 5-FU, CIS and ARA-C abolished cell proliferation at concentrations above 0.1 μg/ml. In contrast, BN 52211, BN 52205 and ET-18-OCH_3 exhibited a significant effect only at concentrations above 1 μg/ml. In the concentration range between 2.5 and 10 μg/ml, ET-18-OCH_3, BN 52205 and CIS decreased Con A-induced IL-2 production in a dose-dependent manner, whereas BN 52211 and 5-FU were inefficient. Only ARA-C inhibited IL-2 production at concentrations similar to those acting on cell proliferation. These results suggest that the inhibition of cell proliferation induced by AAP is independent of their effect on IL-2 production.

IFN-γ is an antiviral and anti-proliferative cytokine exhibiting potent immunoregulatory effects on a variety of cell types. These effects include activation of macrophages, and differentiation and activation of various cell types of the

immune system such as the NK cell.[24] BN 52211 and BN 52205, like ET-18-OCH_3, are presently shown to significantly enhance IFN production. 5-FU and CIS had no effect on IFN production by LPS-stimulated human leukocytes. Despite this potentiating effect of AAP on IFN production, inhibition rather than enhancement of NK activity was noted in the presence of these compounds, suggesting that these two processes are unrelated. However, whether these compounds acted on target or effector cells remains to be investigated, although the experimental conditions used in the present study rather favors an action of AAP on the effector ones. With respect to the effect of ALP on NK cell function, discrepancies appear in the literature since Berdel *et al.*[1] reported an inhibition, whereas in studies of the Biological Response Modifiers Program of the NCI in the US,[25] ET-18-OCH_3 demonstrated no effect.

The present data demonstrate that AAP increased the production of IL-1, IL-6, TNF and IFN-γ, suggesting that these compounds may regulate the immune response, inflammatory reaction and hematopoiesis *in vivo*. The latter process is regulated by a variety of growth and differentiation factors,[26] and most drugs used in chemotherapy are toxic for progenitor cells. IL-3 supports the proliferation of murine or human multipotent hematopoietic progenitors, either alone or in combination with IL-6.[27] Interestingly, BN 52211 and BN 52205 markedly increased IL-3 production by Con A-stimulated EL4 cells, indicating that these compounds might not affect hematopoiesis *in vivo*.

The present results indicate that besides direct antitumoral effects, AAP might also exhibit immunomodulatory activity both *in vitro* and *in vivo*. The newly synthesized compounds BN 52211 and BN 52205 may represent a new therapeutic approach since they induce macrophage activation as detected by IL-1, TNF and IL-6 production. Despite suppression of lymphocyte proliferation and a slight inhibition of IL-2 production, AAP increase IFN-γ production, a cytokine endowed with antiviral and antitumor effects. A possible synergy between AAP and drugs used for chemotherapy should be investigated since this may validate the use of the former compounds as adjuvants in anticancer therapy.

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