Peloruside A, an Antimitotic Agent, Specifically Decreases Tumor Necrosis Factor-α Production by Lipopolysaccharide-Stimulated Murine Macrophages

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Peloruside A (peloruside) is a naturally occurring compound isolated from a New Zealand marine sponge that, like the anticancer drug paclitaxel, stabilizes microtubules and inhibits mitosis. Paclitaxel is known to induce a proinflammatory response in murine macrophages; whereas, peloruside has never been tested for its immunomodulatory effects in these cells. Although the antimitotic effects of the two drugs appear to be similar, we found that peloruside, unlike paclitaxel, does not induce murine macrophages to produce the proinflammatory mediators interleukin-12p40 (IL-12p40), tumor necrosis factor-α (TNF-α), and nitric oxide. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay confirmed that the absence of cytokine production was not caused by cytotoxicity in these nondividing cells. Additionally, there was no effect on unstimulated splenocytes; whereas, both compounds inhibited proliferation after concanalavin A (Con A) stimulation. Finally, there was a significant decrease in TNF-α and nitric oxide but not IL-12p40 when macrophages were cultured with lipopolysaccharide (LPS) and either paclitaxel or peloruside. These results suggest that peloruside may prove to be an effective anti-inflammatory treatment, since it does not induce the production of proinflammatory mediators yet can downregulate TNF-α and nitric oxide production by LPS-stimulated macrophages, as well as inhibit lymphocyte proliferation. Exp Biol Med 232:607–613, 2007

Key words: taxol; peloruside; inflammation; macrophage; microtubule-stabilizing agent

Introduction

Peloruside A (peloruside) is a novel antimitotic compound isolated from the New Zealand marine sponge *Mycale hentscheli* (1). It prevents microtubule depolymerization into free tubulin, arresting dividing cells in the G_2/M phase of the cell cycle (2). Another antimitotic agent, paclitaxel, is a naturally occurring compound originally isolated from the Pacific yew tree (3). Paclitaxel is widely used in the clinical setting as a chemotherapeutic drug (4, 5). The potent antitumor activity exhibited by paclitaxel is primarily due to its ability, like peloruside, to block mitosis in rapidly dividing cells at the G_2/M checkpoint by preventing microtubule depolymerization, ultimately causing arrested cells to apoptose (4–10).

Microtubules are fundamental structures in eukaryotic cells that have roles in cell division, cytoskeletal integrity, locomotion, and intracellular communication. Microtubule disruption has become a focus of cancer research due to the clinical effectiveness of paclitaxel. However, because paclitaxel has a limited effect in many multidrug-resistant cancers, there is a search for similar-acting compounds capable of overcoming the acquired resistance to apoptosis in some tumor cells. A recent study has shown that peloruside remains cytotoxic in multidrug-resistant cancer cell lines that are resistant to paclitaxel (11). Additionally, multiple mammalian cell lines succumb to peloruside in a way that is consistent with apoptosis (12). Little is known, however, about other possible activities of peloruside.

Paclitaxel stimulates murine macrophages through the lipopolysaccharide (LPS) receptor complex to release proinflammatory cytokines in a manner similar to bacterial lipopolysaccharide (13–16). It has been suggested that the resultant inflammatory response may enhance the cancer-killing properties of paclitaxel (17). However, the effect of this enhanced inflammatory activity *in vivo* is unclear. There are no known proinflammatory effects ascribed to peloruside, but because of its similar mechanism of microtubule stabilization to paclitaxel, we investigated whether or not it could induce a paclitaxel-like proinflammatory response.

This work was supported by Wellington Medical Research Foundation grant 2002/58 (to A.C.L.) and the Faculty of Science Small Grants, Victoria University of Wellington (to K.P.C.).

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Received May 23, 2006.
Accepted January 2, 2007.

1535-3702/07/2325-0607$15.00

Here we report that peloruside, unlike paclitaxel, does not induce murine macrophage secretion of the inflammatory mediators tumor necrosis factor-α (TNF-α), interleukin-12p40 (IL-12p40), and nitric oxide (NO). In addition, peloruside decreases the LPS-induced production of TNF-α and, to a lesser extent, NO by murine macrophages without showing cytotoxicity.

Materials and Methods

Animals and Reagents. C57BL/6 male mice were bred at the Wellington School of Medicine and Health Sciences, Wellington, New Zealand. All mice were used at 8–12 weeks of age. Paclitaxel, which was purified from *Taxus yannanensis,* and LPS from *Escherichia coli* were purchased from Sigma Chemical Co. (St. Louis, MO). Peloruside was generously provided by Dr. Peter Northcote (School of Chemical and Physical Sciences, Victoria University of Wellington, Wellington, New Zealand). Both paclitaxel and peloruside were dissolved in ethanol and stored as 1-m*M* stocks at –80°C.

Bone Marrow-Derived Macrophage (BMMØ) Culture. BMMØs were cultured from C57BL/6 mice as previously described (18). Briefly, long bones were flushed with sterile divalent cation–free Dulbecco's phosphate-buffered saline (Invitrogen, Auckland, New Zealand) with a 23-gauge needle. Cells were cultured in complete medium (CTCM) containing Dulbecco's modified Eagle's medium, 10% fetal calf serum, 100 U/ml penicillin plus 100 μg/ml streptomycin, 10 m*M* HEPES, 2 m*M* L-glutamine, and 5×10^{-5} *M* 2-mercaptoethanol (all from Invitrogen) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3. The cells were cultured for 24 hrs at 37°C in air containing 5% CO_2. Nonadherant progenitor cells were removed and further cultured for 7–10 days at 37°C in air with 5% CO_2 to allow the cells to differentiate into macrophages. Once a monolayer of BMMØ was established, the cells (5×10^5 cells/ml) were removed by vigorous pipetting and were resuspended in CTCM. BMMØs were then activated overnight with 40 U/ml interferon-γ (IFN-γ; BD Pharmingen, Franklin Lakes, NJ) in flat-bottom 96-well plates (Falcon, Franklin Lakes, NJ) at 37°C with air containing 5% CO_2. Paclitaxel, peloruside, and/or LPS were added to the cultures, followed by a further incubation. A portion of the supernatants was removed after 2 hrs or 8 hrs for cytokine detection by enzyme-linked immunosorbent assay (ELISA). At 72 hr after adding the drugs, the remaining supernatants were removed from the cultures for NO detection, and fresh CTCM was added to the wells for the MTT reduction assay.

MTT Colorimetric Assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that reduces to purple formazan in the presence of reducing compounds, such as nicotinamide adenine dinucleotide, reduced form (NADH), and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH; Refs. 19–21). Dose-response curves were obtained by comparing drug-treated cells to untreated controls following the addition of MTT to cultures. Similarly, the metabolic activity of nonproliferating cells, which was based on the relative presence of reducing compounds, was assayed with MTT in BMMØ cultures.

Cytokine and NO Detection. A sandwich ELISA was used to measure IL-10, IL-12p40, and TNF-α cytokines following the manufacturer's recommendations (BD Pharmingen). The accumulation of NO_2^-, an indicator of NO production in the culture medium, was assayed by the Greiss reaction (22).

Splenocyte Isolation and Culture. Splenocytes were isolated by dissociation through sterile 70-μm cell strainers (Falcon; Ref. 23). Red blood cells were lysed with red blood cell lysis buffer (Sigma Chemical Co.). Splenocytes were resuspended in CTCM and cultured at 37°C in 5% CO_2 in flat-bottom 96-well plates (10^6 cells/well; Falcon) with peloruside or paclitaxel and 3 μg/ml concanavalin A (Con A; Sigma Chemical Co.) for T-cell stimulation.

Statistical Analyses. Significance at any individual point was determined by the Student *t*-test and adjusted with Holm Sequential Bonferroni correction. Comparisons were made with each group to the control. $P \leq 0.05$ was accepted as significant.

Results

Peloruside Does Not Induce the Release of Proinflammatory Cytokines by BMMØs. Because paclitaxel, in addition to its microtubule-stabilizing effects, can mimic LPS in thioglycolate-elicited murine macrophages and can cause the release of proinflammatory cytokines (16), we cultured BMMØs with paclitaxel or peloruside at a wide range of concentrations to assess whether peloruside has similar LPS-mimetic properties to paclitaxel. In contrast with paclitaxel, peloruside did not cause the production of IL-12p40 or NO in IFN-γ–activated BMMØs (Fig. 1a and b). Surprisingly, no TNF-α was detectable at 8 hrs in the supernatants of BMMØs when cultured with paclitaxel (Fig. 1c), but TNF-α was detected at 2 hrs in cultures containing 10 μ*M* paclitaxel (Fig. 1d). With peloruside, TNF-α was not detected at either time point (Fig. 1c and d). Additionally, the anti-inflammatory cytokine IL-10 was not detected in supernatants of either peloruside- or paclitaxel-treated cells (data not shown). These results indicate that although paclitaxel and peloruside share a similar mechanism of microtubule stabilization, peloruside does not share the LPS mimicry of paclitaxel.

Absence of Cytokine Production by Peloruside-Treated BMMØs Is Not Due to Cytotoxicity. To determine whether the absence of cytokines in supernatants of BMMØs cultured with peloruside was due to general cytotoxicity, the viability of drug-exposed BMMØs was assayed by MTT reduction. After a 72-hr incubation, neither

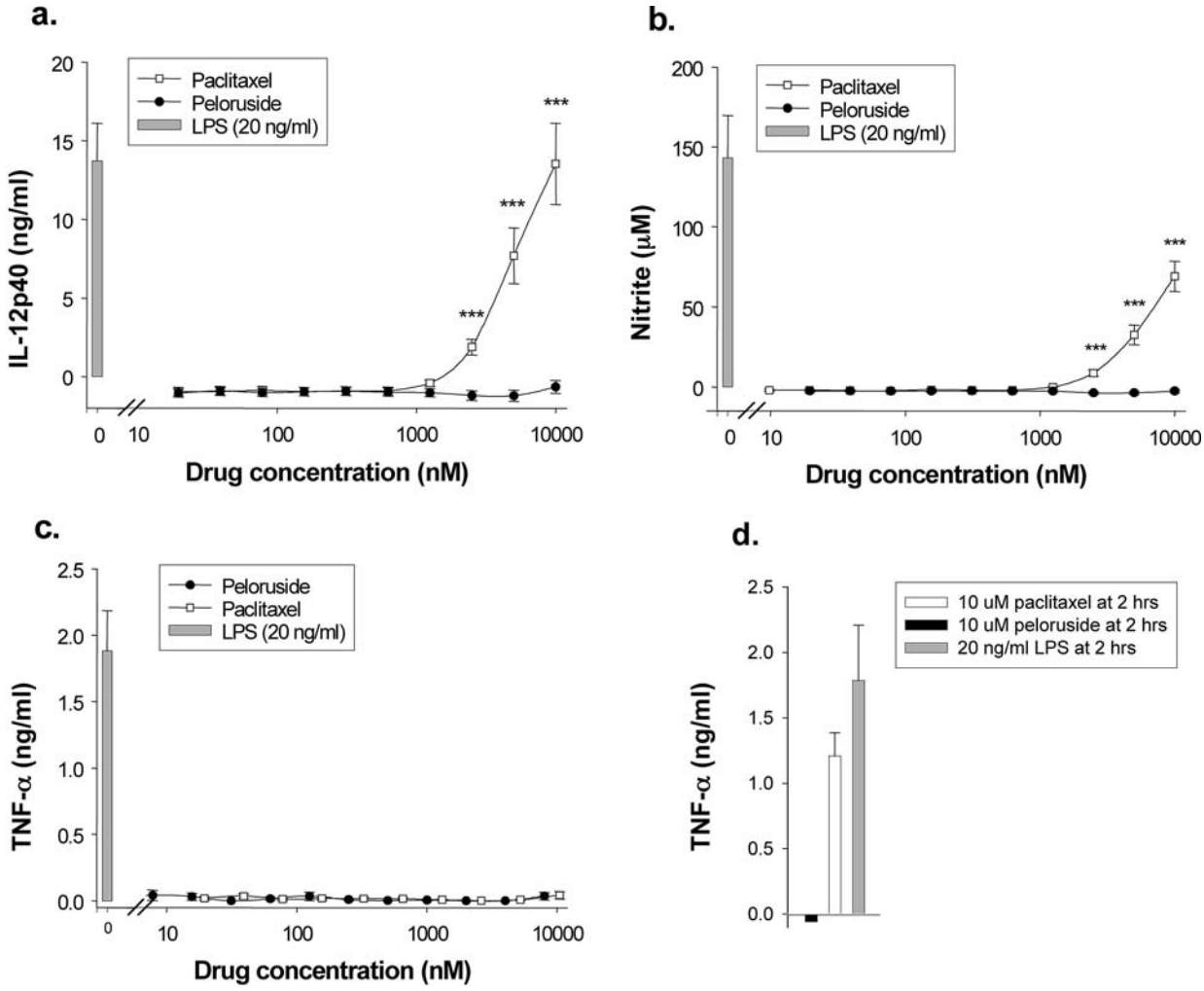

Figure 1. Effect of peloruside and paclitaxel on BMMØs. IFN-γ–activated BMMØs (5×10^4 cells/well) were cultured with either peloruside (black circles) or paclitaxel (white squares) at a range of concentrations. The response to LPS (20 ng/ml) on its own is presented for comparison. Paclitaxel but not peloruside induced the production of IL-12p40 (a), nitrite (b), and TNF-α (d) from BMMØs. No TNF-α was detected from BMMØs after 8 hrs of culture with paclitaxel or peloruside (c); however, TNF-α was present in paclitaxel-treated cultures at 2 hrs (d). NO and cytokine levels were determined as described in *Materials and Methods*. Results are representative of duplicate or triplicate wells from four to eight experiments. ***$P < 0.001$ compared with no drug.

peloruside nor paclitaxel were cytotoxic to BMMØs (Fig. 2a). Interestingly, concentrations of paclitaxel in the micromolar range produced up to a 2-fold increase in MTT reduction, indicating a large increase in reducing compounds such as NADH and NADPH within these BMMØs, thus suggesting enhanced metabolic activity.

Because identical concentrations of paclitaxel and peloruside had different effects on nonproliferating BMMØs, we determined the respective half maximal inhibitory concentration (IC_{50}) values of these compounds in a Con A–stimulated, mixed lymphocyte population of cells to ensure that the concentrations used had comparable antimitotic effects, as previously reported (Refs. 2, 24; Fig. 2b). We found that paclitaxel and peloruside were cytotoxic to Con A–stimulated splenocytes with IC_{50} values of 30 n*M* and 83 n*M*, respectively. Little to no cytotoxicity was observed in cultures of unstimulated splenocytes treated with either drug (Fig. 2c) compared with stimulated splenocytes (Fig. 2b). In addition, concentrations of paclitaxel in the micromolar range did not cause an increase in metabolism of unstimulated splenocytes (Fig. 2c) as observed in macrophages (Fig. 2a). Con A is a T-cell mitogen, and splenocytes are composed of 30% T cells and 60% B cells, and the remaining 10% are other leukocytes. As expected, complete ablation of MTT reduction did not occur in Con A–stimulated splenocytes treated with drugs at high concentrations (Fig. 2b), suggesting that the non–T-cell population was largely unaffected by the antimitotic effects of paclitaxel and peloruside.

Both Peloruside and Paclitaxel Cultured with BMMØs Decrease Levels of TNF-α and NO Production in the Presence of LPS. LPS stimulates BMMØ primarily through Toll-like receptor-4 (TLR-4), resulting in the release of proinflammatory cytokines. To determine

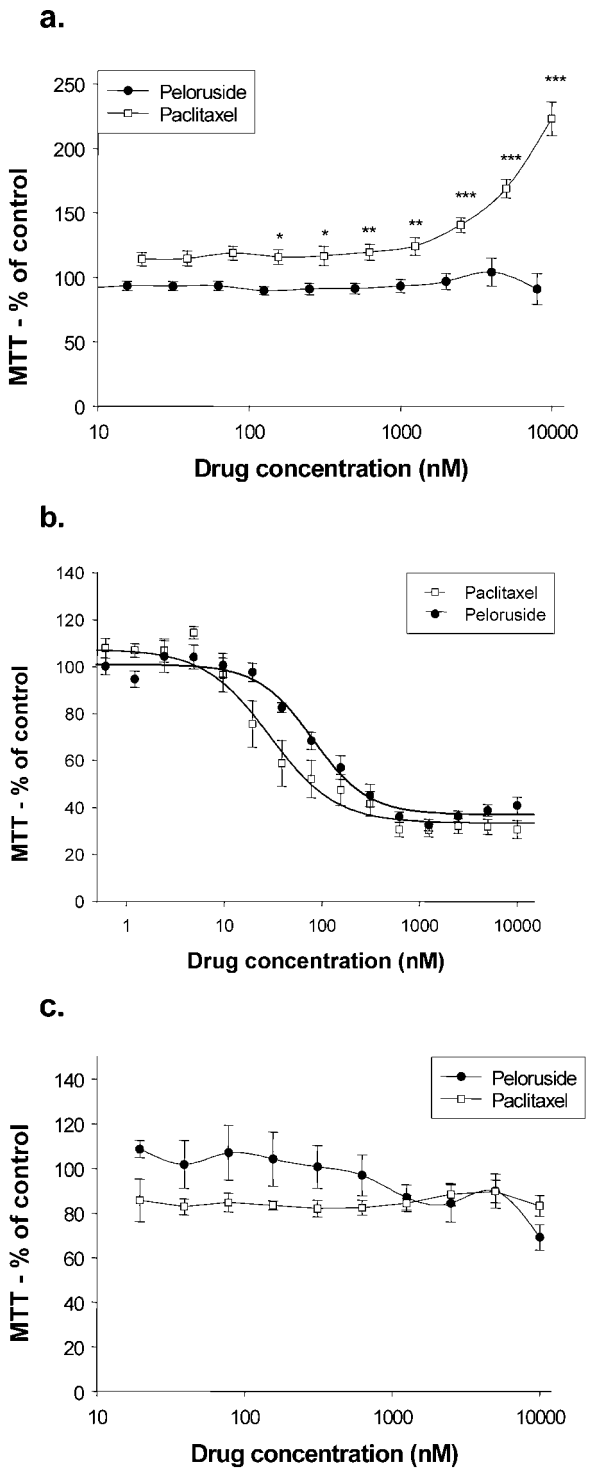

Figure 2. Effects of peloruside and paclitaxel on cell growth and metabolism. Results of MTT assays on BMMØs (a), Con A–stimulated splenocytes (b), and unstimulated splenocytes (c) cultured with peloruside (black circles) or paclitaxel (white squares) at a range of concentrations. Paclitaxel but not peloruside enhanced metabolic activity of BMMØs (a). Both peloruside and paclitaxel inhibited proliferation in stimulated splenocytes (b), with little to no cytotoxicity in unstimulated splenocytes (c). BMMØs were seeded at 5×10^4 cells/well (a), and splenocytes were seeded at 10^6/well and cultured in the presence (b) or absence (c) of the T-cell mitogen Con A (3 μg/ml). Viability at 72 hrs was assayed by MTT reduction (a–c). All values are expressed as a percentage of untreated controls. The IC_{50} values for Con A–stimulated splenocytes (b) were determined by nonlinear regression. Data points are representative of duplicate or triplicate wells from three to four experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with control.

whether paclitaxel or peloruside could alter LPS-induced proinflammatory cytokine production, both compounds were added to cultures of LPS-stimulated BMMØs. When cultured with either paclitaxel or peloruside under these conditions, decreased levels of TNF-α and, to a lesser extent, NO were observed compared with a control with LPS alone (Fig. 3a and b). The observed effect of decreased TNF-α and NO production seemed to be limited by the concentrations of LPS. When BMMØs were cultured with LPS concentrations higher than 20 ng/ml, the anti-inflammatory effects of the two drugs were completely negated at all concentrations up to 10 μ*M* (data not shown). Neither peloruside nor paclitaxel had any obvious effect on IL-12p40 production (Fig. 3c). Finally, similar to our previous experiments with paclitaxel-treated BMMØ (Fig. 2a), LPS-stimulated BMMØs treated with high concentrations of paclitaxel showed an enhanced metabolic activity above the levels seen with LPS alone (Fig. 3d).

Discussion

Our results show that peloruside, in contrast to paclitaxel, does not activate murine BMMØs to release the proinflammatory mediators TNF-α, IL-12p40, and NO. The MTT reduction assay confirms that neither peloruside nor paclitaxel is cytotoxic to BMMØs and, moreover, paclitaxel causes an increase in metabolism in the 1–10 μ*M* concentration range, possibly due to the induction of the proinflammatory machinery. When BMMØs were cultured with LPS and either drug at low micromolar concentrations, there was a decrease in the production of TNF-α and NO but not IL-12p40. An interesting observation from our data was that more than 20 ng/ml LPS completely overshadowed the TNF-α–reducing effects of paclitaxel and peloruside. Taken together, these data indicate that peloruside does not induce the production of proinflammatory mediators and specifically reduces TNF-α and NO production by BMMØs.

Peloruside is known to be cytotoxic to proliferating cells by binding to a site on microtubules distinct from paclitaxel and preventing microtubule depolymerization (2, 11, 25, 26). Until now, however, there have been no investigations into the effects of peloruside on nonproliferating cells, such as macrophages. The immunomodulatory effects and cytotoxic nature of paclitaxel, on the other hand, are well characterized. Its ability to cause apoptosis in dividing cells by binding to microtubules and preventing depolymerization, similar to peloruside, has made paclitaxel one of the most successful treatments for cancer to date (4).

In addition to its tubulin-binding properties, paclitaxel also stimulates the murine TLR-4 complex in a manner nearly indistinguishable from LPS (16). This TLR-4–

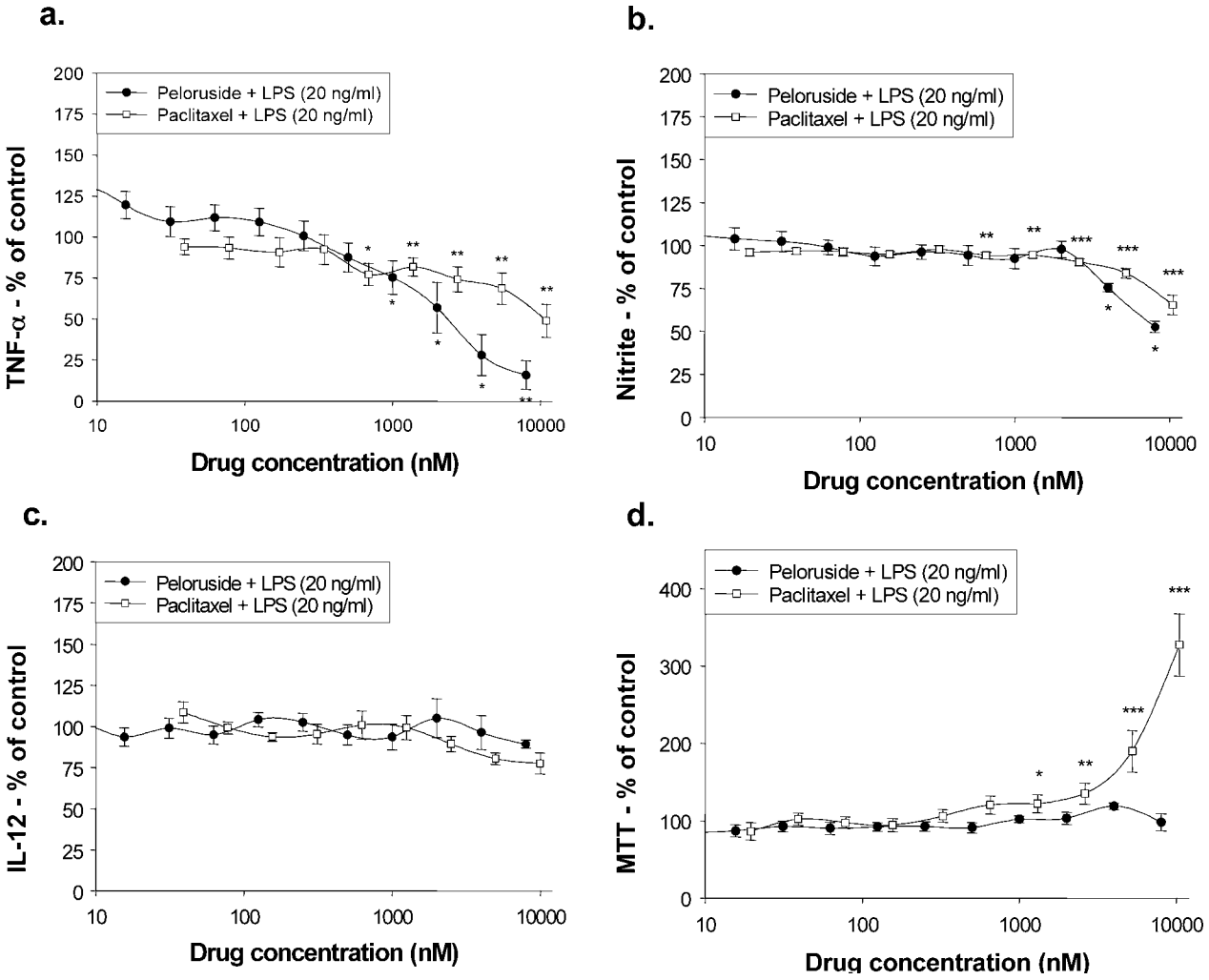

Figure 3. Effect of peloruside and paclitaxel in LPS-stimulated BMMØs. BMMØs were cultured with 20 ng/ml LPS and either peloruside (black circles) or paclitaxel (white squares) at a range of concentrations. Both compounds caused a significant decrease in levels of TNF-α (a) and nitrite (b) at higher concentrations. IL-12p40 was unaffected by either drug (c). Paclitaxel also enhanced metabolic activity (d). Cytokine levels were determined by ELISA after 8 hrs of incubation. NO (b) was determined by Greiss reaction after 72 hrs. The MTT assay (d) was performed as described in *Materials and Methods* after 72 hrs. All values are expressed as a percentage of drug-free LPS-stimulated control BMMØs. Results are representative of duplicate or triplicate wells from three to five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with LPS stimulation on its own.

stimulating activity is unique to paclitaxel, as other microtubule-stabilizing compounds, such as docetaxel and epothilone B, do not induce an inflammatory response in murine macrophages (27–29). Concentrations of paclitaxel in the low micromolar range cause murine BMMØs to release inflammatory cytokines and NO (13–15). In our experiments we found TNF-α in BMMØ supernatants after 2 hrs but not 8 hrs of exposure to paclitaxel. Other studies have reported higher levels of paclitaxel-induced TNF-α production for more prolonged periods (16, 30). These discrepancies may be due to the use of thioglycolate to obtain macrophages in the aforementioned studies (16, 23). Thioglycolate is known to induce an inflammatory phenotype (31). The enhanced metabolic activity of BMMØs in response to paclitaxel has, to our knowledge, never been shown before, although it is not surprising that the energy requirements of an inflammatory response would increase the production of NADH and NADPH, thus enhancing MTT dye reduction (21).

Until now, the known immunomodulatory activities of peloruside were limited to preventing the production of IFN-γ by α-CD3–stimulated splenocytes through a cytotoxic mechanism (32). Our findings confirm that peloruside is cytotoxic to proliferating splenocytes from Con A stimulation with an IC_{50} value of 83 n*M*. Paclitaxel also has been shown to be immunosuppressive on the basis of its cytotoxicity to proliferating B cells (33). However, there is conflicting evidence as to whether or not it is cytotoxic to proliferating T cells. In the studies by Lee et al. (33), paclitaxel-induced cytotoxicity was not seen in Con A–stimulated splenocytes, contrary to what had been described previously (24). In the present study, we confirm the

cytotoxicity of paclitaxel in Con A–stimulated splenocytes with an IC_{50} value of 30 n*M*. This value is slightly greater than what others have seen with paclitaxel in cancer cell lines (11, 34). These studies support the idea that antiproliferative compounds may be of benefit to inflammatory autoimmune disorders by targeting the dividing populations of autoreactive T cells that mediate many of these diseases.

Since paclitaxel by itself induces an inflammatory response in BMMØs, it was important to determine whether it synergizes with LPS to enhance inflammation. Surprisingly, both paclitaxel and peloruside had no effect on IL-12p40 compared with control BMMØs cultured with only LPS, but both drugs reduced the levels of TNF-α and NO without causing cytotoxicity. The reduction of NO was significant but was not nearly as marked as the reduction of TNF-α. The mechanism behind the reduced NO production may relate to the reduction of TNF-α, as the production of nitric oxide synthase-2 (NOS-2), the enzyme primarily responsible for NO synthesis in macrophages, is a downstream effect of TNF-α (35). This reduction is interesting, since paclitaxel alone has been shown to induce TNF-α and NOS-2 transcription in macrophages (36, 37). To our knowledge, however, our study is the first to look at the effects of LPS + paclitaxel in IFN-γ–stimulated macrophages. Although it is difficult to speculate at this time, one mechanism by which paclitaxel and peloruside may specifically affect TNF-α production is through microtubule-mediated secretion of TNF-α by BMMØs when stimulated by LPS. Previous studies have shown that the microtubule–depolymerizing compound, colchicine, inhibits macrophage production of TNF-α, GM-CSF, and IL-6 in response to LPS (38). In contrast to our results, no change was found in NO and IL-1β levels. It was concluded that the LPS response had a microtubule-dependent and microtubule-independent set of pathways. Another study found that both paclitaxel and docetaxel, a paclitaxel derivative compound that does not stimulate the TLR-4 receptor complex, also decrease TNF-α when administered to cancer patients (39). Taken together, this evidence leads us to believe that microtubule stabilization may be responsible for the decrease in LPS-induced production of TNF-α. It has been established that TNF-α is a rate-limiting step in the pro-inflammatory process, and neutralization of TNF-α by monoclonal antibodies causes a marked amelioration in several autoimmune diseases (40). It is important to point out that neutralizing TNF-α also causes the reduction of GM-CSF and IL-6 (41) in a manner similar to what has been observed with microtubule-depolymerizing compounds. Our findings raise the possibility that microtubule-stabilizing compounds, such as peloruside, also may be able to impact on inflammation by preventing secretion of TNF-α.

As we have shown in the present study, peloruside is able to induce cell death of proliferating cells without having the proinflammatory side effects on BMMØs of paclitaxel. Moreover, peloruside inhibits the production of a key proinflammatory cytokine, TNF-α, when added to an inflammatory environment. These combined results warrant further investigation into the use of peloruside as a potential treatment for inflammatory autoimmune disorders or other hyperproliferative diseases.

We thank Dr. Shirley Pledger for her statistical advice, as well as Dr. Thomas Gaitanos for his helpful hints and technical advice. We also extend our gratitude toward Dr. Peter Northcote and the Natural Products Group at the Victoria University of Wellington.

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