# Anti-inflammatory Properties of the Novel Antitumor Agent Yondelis (Trabectedin): Inhibition of Macrophage Differentiation and Cytokine Production

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## Abstract

**Yondelis (Trabectedin) is a novel antitumor agent of marine origin extracted from the tunicate *Ecteinascidia turbinata*. This original compound is active against several human tumors including sarcoma and ovarian and breast adenocarcinoma, as evidenced in phase II clinical trials in advanced multitreated patients. Yondelis is a DNA minor groove binder that blocks cell cycle and interferes with inducible gene transcription in a selective manner. In this study, we investigated the immunomodulatory properties of Yondelis on leukocytes. Human blood monocytes were highly susceptible *in vitro* to its cytotoxic effect and underwent apoptosis at pharmacologically relevant concentrations (5 nmol/L), whereas lymphocytes were up to 5-fold less sensitive. Macrophages differentiated *in vitro* with macrophage colony-stimulating factor and tumor-associated macrophages (TAM), isolated from patients with ovarian cancer, were also susceptible. At subcytotoxic concentrations, Yondelis inhibited the *in vitro* differentiation of monocytes to macrophages. In tumor-treated patients, drug infusion caused a selective decrease of monocyte counts and of *ex vivo* macrophage differentiation. The *in vitro* production of two proinflammatory mediators, CCL2 and IL-6, was markedly reduced by Yondelis in monocytes, macrophages, TAM, and freshly isolated ovarian tumor cells. The chemokine CCL2 is the major determinant of monocyte recruitment at tumor sites, whereas IL-6 is a growth factor for ovarian tumors. In view of the protumor activity of TAM and of the strong association between chronic inflammation and cancer progression, the inhibitory effect of Yondelis on macrophage viability, differentiation, and cytokine production is likely to contribute to the antitumor activity of this agent in inflammation-associated human tumors.** (Cancer Res 2005; 65(7): 2964-71)

## Introduction

Yondelis (trabectedin), a tetrahydroisoquinoline alkaloid, is a natural product derived from the marine tunicate *Ecteinascidia turbinata* (1), with potent antitumor activity in *in vitro* and *in vivo* preclinical studies on several solid tumors, including ovarian and breast cancer, melanoma, and sarcoma (2–5). Of major importance, Yondelis showed antiproliferative activity on drug-resistant tumors and had synergistic activity in combination with other chemotherapeutic drugs, including cisplatin (6). Currently, phase II studies are being developed in patients with sarcoma or ovarian cancers, with clinical regression of at least 6 months in heavily pretreated patients (7).

Although the precise mode of action of Yondelis has yet to be determined, there is strong biological and biochemical evidence that it is distinct from other anticancer drugs. Yondelis forms adducts in the minor groove of DNA and binds guanine at the N2 position, whereas most alkylating agents bind guanine at position N7 or O6 in the major groove (8, 9). This binding profoundly affects DNA structure, bending it toward the major groove, as shown by nuclear magnetic resonance spectroscopy. Moreover, Yondelis induces non–p53-dependent apoptosis and produces a cell cycle block in late S and $G_2$-M phases (10).

A peculiar aspect of Yondelis is that the drug is less effective against cell lines deficient in nucleotide excision repair, which are hypersensitive to UV and other DNA-damaging agents (10–12). In this respect, Yondelis is unique and different from all other DNA-interacting compounds investigated to date.

Early studies have identified that Yondelis interferes with transcriptional activation of the CCAAT box factor NF-Y in a promoter-specific manner (13, 14). More recently, other genes and transcriptional inducers indicate that Yondelis may be a more general inhibitor of activated but not basal transcription (15, 16). NF-Y is considered a transcription factor of major importance in the differentiation of monocytes to macrophages (17). Because it is unknown whether Yondelis affects these immune cells, this study was initiated to specifically investigate the effects of Yondelis on mononuclear phagocytes.

Monocyte/macrophages are recognized as important components of innate immunity. Circulating monocytes are versatile precursors with the ability to differentiate into the various forms of specialized macrophages (17). The cytokine milieu profoundly affects the differentiation and function of tissue macrophages. Recently, a functional polarization has been defined (18, 19). Macrophages activated by bacterial products and Th1 cytokines [e.g., lipopolysaccharide and IFN-γ] are regarded as M1 or classically activated macrophages, with high bactericidal activity and cytotoxic function against tumor cells. On the other hand, macrophages activated by Th2 cytokines [e.g., interleukin (IL)-4 and IL-13] or immunosuppressors (e.g., corticosteroids, vitamin $D_3$, and IL-10) are defined as M2 macrophages, with low cytotoxic functions but high tissue-remodeling activity. Schematically, whereas M1 cells have immunostimulatory properties and defend

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the host against pathogenic infections, M2 cells attenuate the acute inflammatory reaction, potently scavenge cellular debris, and secrete a variety of growth and angiogenic factors essential to repair the injured tissues. Thus, both polarized subsets serve important physiologic functions; however, when their activation (especially that of M1 macrophages) persists over time, pathologic conditions and tissue injury may occur. Indeed, the continuous production of proinflammatory mediators by mononuclear phagocytes has been implicated in the onset and progression of several chronic inflammatory diseases (17–19).

Several lines of evidence suggest a strong association between chronic inflammation and increased susceptibility to cancer (20–24). It has been estimated that up to 20% of all tumors arise from conditions of persistent inflammation due to chronic infections or other causes (20). The prominent role of inflammatory mediators has been shown in several studies. In mouse models of skin carcinogenesis, deletion of selected proinflammatory cytokines reduced tumor susceptibility (25). More recently, the activation of the transcription factor nuclear factor kB (NFκB) has been implicated in the formation and progression of inflammation-associated tumors (22).

Macrophages infiltrating the tumor tissue, or tumor-associated macrophages (TAM), constitute a peculiar macrophage population. The role of TAMs in the tumor microenvironment and tumor development is complex and multifaceted, as suggested in the "macrophage balance hypothesis" (26). Unlike M1 macrophages, there is limited evidence that TAMs can be directly tumoricidal and induce tumor-reactive T cells. On the contrary, a growing body of evidence indicates that TAMs are skewed toward M2 macrophages and produce a variety of growth and angiogenic factors as well as immunosuppressive molecules [e.g., IL-10 and transforming growth factor β (19, 26–29)]. Thus, the presence of TAMs at the tumor site and the continuous expression of their products may favor, rather than antagonize, tumor progression.

In view of these findings, there is increasing interest in defining new therapies aimed at reducing the persistent activation of macrophages and their release of proinflammatory and protumor mediators. In this study we show that blood monocytes are selectively susceptible and undergo apoptosis at therapeutic concentrations of Yondelis, a novel and original antitumor agent. In addition, at subcytotoxic concentrations, this compound significantly inhibits the *in vitro* macrophage differentiation and the production of two inflammatory mediators, IL-6 and CCL2. These anti-inflammatory properties of Yondelis may be an extended mechanism of its antitumor activity.

## Materials and Methods

**Cell preparation and treatment.** Purified populations of human blood monocytes were prepared by centrifugation on Ficoll and Percoll gradients (30) as previously described. Monocytes were usually >85% CD14+ cells. Purified T lymphocytes (>95% CD3+) were obtained on Percoll gradients as previously described (30). Human thymocytes, obtained from resected thymus of pediatric patients undergoing surgery, were teased and isolated on Percoll gradient. *In vitro* differentiated macrophages were obtained by culture of monocytes ($10^6$ cells/mL) in complete medium (RPMI, Biochrom, Berlin, Germany) + 10% FCS (Hyclone, Logan, UT) with macrophage colony-stimulating factor (M-CSF, Peprotech, London, United Kingdom; 20 ng/mL) for 5 days. In some experiments, macrophages were treated with lipopolysaccharide (100 ng/mL; Sigma Aldrich, St. Louis, MO), IFN-γ (500 IU/mL), or IL-4 (20 ng/mL; Schering Plough, Kenilworth, NJ) for 24 hours. TAMs and tumor cells were isolated from the ascitic fluid of patients with diagnosed ovarian adenocarcinoma who were admitted to the Clinical of Obstetrics and Gynecology of the University of Milan Bicocca, Milan, Italy. Cells contained in the ascitic fluid were centrifuged and isolated by Ficoll and Percoll gradients as previously described (31). Purity of TAM and tumor cell preparations was usually >65 ± 10% as defined by morphology and phenotype analysis.

Cells were treated with Yondelis at the indicated concentrations and cultured for 1 to 5 days, as specified. At the end of the incubation period cells were collected, washed, and used for DNA analysis or functional assays.

**Determination of cell viability.** Cell viability was analyzed by DNA content in flow cytometry. Cells exposed to treatments were fixed with 70% ethanol, washed in PBS, and stained with propidium iodide (PI) solution containing 10 μg/mL PI in PBS and 25 μL RNase (10,000 units) overnight in the dark. PI incorporation was evaluated on at least 20,000 cells per sample using a FACS Calibur instrument (Becton Dickinson, Sunnyvale, CA), with a band-pass filter at 620 nm. Apoptosis was detected by staining with Annexin V and PI. Fluorescence-activated cell-sorting analysis was done using a band-pass filter, 530 and 620 nm for green (Annexin V) and red (PI) fluorescence, respectively, in combination with a 570-nmol/L dichroic mirror.

**Phenotype analysis.** Expression of cell membrane markers was done by immunofluorescence and analyzed by flow cytometry. Cells were incubated with anti-CD16 (clone B73.1), anti-CD68 (clone KP1, DAKO), anti-CD206/mannose receptor (clone PAM-1; ref. 30), and FITC-goat anti-mouse immunoglobulin. At least 10,000 cells were analyzed.

**Antineoplastic agents.** Yondelis was obtained from Pharmamar SA (Madrid, Spain), cisplatin from Sigma Chemical Co. (St. Louis, MI), doxorubicin from Pfizer (Milan, Italy), and taxol from Bristol-Myers Squibb (New York, NY).

**Cytokine production.** Supernatants of untreated cells or cells treated with Yondelis or other antineoplastic agents were collected after 16 hour culture and frozen. Monocytes, macrophages, and TAMs were stimulated with 100 ng/mL lipopolysaccharide to induce maximal cytokine production. Determination of cytokine CCL2, tumor necrosis factor (TNFα), and IL-6 was done by specific ELISA following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

**Tumor patients.** Patients with ovarian cancer undergoing phase II trial with Yondelis were admitted to the European Oncology Institute, Milan, Italy. Patients received Yondelis (1,300 $\mu g/m^2$) in a 3-hour infusion. Dexamethasone was also administered to reduce drug toxicity (4-10 mg). Blood withdrawal was done under informed consent following procedures approved by the ethical board. Blood samples (40 mL) were collected immediately before treatment and at the end of the infusion (+3 hours). Blood samples were immediately processed and Percoll-purified monocytes (usually $10^6$ cells) were cultured with M-CSF (20 ng/mL) for 5 days. Differentiated cells were harvested, counted, and analyzed for phenotype expression. Results are presented as absolute numbers of marker-positive cells per 10,000 cells. Significant inhibition of macrophage differentiation was considered at least 50% reduction of marker-positive cells relative to cells collected before therapy.

**Real-time PCR.** Total RNA extraction was done with Trizol. cDNA was synthesized by random priming from 1 μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was done using SYBR Green dye and GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City CA) as described (32). The sequences of primer pairs specific for each gene (Invitrogen, San Diego, CA) were designed with Primer Express Software (Applied Biosystems). For each gene, mRNA was normalized to β-actin mRNA by subtracting the cycle threshold (Ct) value of β-actin mRNA from the Ct value of the gene (ΔCt). Fold difference ($2^{-\Delta\Delta Ct}$) was calculated by comparing the ΔCt with the ΔCt of unstimulated cells (32).

## Results

**Yondelis shows selective cytotoxic effect on monocytes *in vitro* and *in vivo*.** We first studied the effect of Yondelis treatment on the viability of human leukocyte subsets *in vitro*. Purified preparations of blood monocytes were highly susceptible

to the cytotoxic effect of the drug. There was a dose-dependent cytotoxicity with a lethal dose 50% ($IC_{50}$) of 5 nmol/L after 48 hours of culture (Fig. 1*A*). Purified T lymphocytes were much less susceptible, with an $IC_{50}$ of 20 nmol/L. Even more resistant were freshly isolated thymocytes ($IC_{50}$ >40 nmol/L; Fig. 1*A*). Virtually all dying monocytes stained positive for Annexin V, indicating that the drug induces apoptosis (Fig. 1*B*). Monocyte cytotoxicity was also confirmed by DNA analysis in flow cytometry (Fig. 1*C*). A partial protection from the toxic effect of Yondelis was observed when monocytes were pretreated with M-CSF. M-CSF shifted monocyte death from 55% to 30% at 5 nmol/L Yondelis, after 48 hour incubation, and from 65% to 35% at 10 nmol/L, after 24 hour treatment (Fig. 1*C*). This protective effect was no longer observed when M-CSF was added 4 hours after drug treatment (not shown).

Because Yondelis is being actively studied for the treatment of ovarian adenocarcinoma, it was of interest to compare its cytotoxic effect on monocytes with that of other compounds conventionally used in this disease: doxorubicin, cisplatin and taxol. Doxorubicin significantly reduced monocyte viability at 0.5 to 1 μmol/L, but was less potent than Yondelis (Fig. 2), whereas cisplatin and taxol were not cytotoxic at any pharmacologically relevant concentration active on tumor cells *in vitro* (marked with an asterisk). With cisplatin, significant toxicity was observed only at very high concentrations (40 μmol/L), whereas taxol was ineffective even at 300 nmol/L. These results indicate that Yondelis shows potent cytotoxic activity for monocytes compared with other antitumor agents.

We next investigated whether the *in vivo* administration of Yondelis in tumor patients could have measurable effects on monocyte viability. A phase II trial is currently under way in patients with advanced ovarian adenocarcinoma who have failed two different cycles of conventional cisplatin- and taxol-based chemotherapies. Tumor patients selected for this study were treated with Yondelis at 1,300 $\mu g/m^2$. Monocyte values were obtained from blood formula during routine clinical analysis. Of nine patients whose monocyte records were available, seven patients showed a decrease (at least 25% inhibition compared with values before infusion) in the number of monocytes, evaluated both as percentage of monocytes over total leukocytes and as absolute number of monocytes per microliter of blood. Results from three representative patients are shown in Fig. 3. Despite constant levels or transient increase in the total number of leukocytes, monocytes were decreased in numbers in the first few days after drug infusion.

**Yondelis inhibits macrophage differentiation and macrophage viability.** We next studied the effect of Yondelis on the differentiation of macrophages *in vitro*. Blood monocytes were cultured for 5 days with M-CSF and different concentrations of Yondelis from the first day of culture. A kinetic analysis showed that at 5 and 10 nmol/L significant toxicity was observed at early time points and increased over time (Fig. 4*A*). Lower concentrations (2.5 nmol/L) caused modest cytotoxicity after 24 and 48 hours and 40% to 50% of cell death after 5 days. At 0.5 and 1 nmol/L, cell viability did not significantly differ from that of untreated cells (Fig. 4*A*).

To study the phenotype of differentiating macrophages treated with Yondelis, noncytotoxic concentrations were used. Usually, an average of 65 ± 15% of input monocytes differentiate into large cells expressing typical macrophage markers after 5-day culture with M-CSF (mean ± SD of >10 experiments). Treatment with Yondelis, even at low concentrations, inhibited the expression of CD68, CD16, and CD206 (mannose receptor; Fig. 4*B*).

To confirm these findings, we tested whether the *in vivo* administration of Yondelis in tumor patients could affect the differentiating capacity of monocytes into macrophages. Tumor patients selected for these experiments were treated with Yondelis at 1,300 $\mu g/m^2$ in the same phase II study as described above. Blood samples were drawn immediately before drug administration and at the end of a 3-hour infusion. Purified monocytes were cultured for 5 days with M-CSF and then tested for phenotype analysis. Of 12 patients tested, monocytes from 6 subjects showed marked inhibition of macrophage differentiation compared with cells collected before drug infusion, evaluated as at least 50% decrease in the number of cells expressing CD68, CD16, and CD206 (Table 1). Possible association between the clinical response to drug treatment and the reduced differentiation activity *in vitro* is being investigated and will require a larger number of cases.

The cytotoxic effect of Yondelis on already differentiated macrophages or *ex vivo* isolated macrophages was also evaluated.

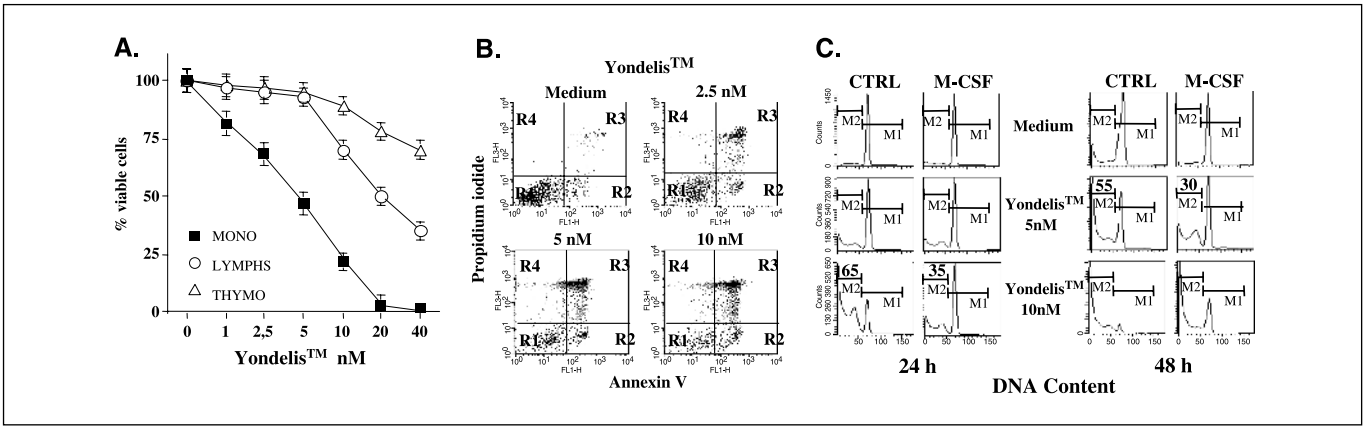


**Figure 1.** Human blood monocytes are highly susceptible to the cytotoxic effect of the antineoplastic agent Yondelis. *A,* purified preparations of blood monocytes, lymphocytes, and thymocytes were cultured with different concentrations of Yondelis for 48 hours. Cell viability was assessed by DNA analysis and PI staining in flow cytometry. Results are mean of five different experiments; *bars,* SE. *B,* apoptosis of monocytes treated with Yondelis for 48 hours. Cells were stained with Annexin V and PI and analyzed by flow cytometry. Representative of three experiments. *C,* pretreatment with M-CSF partially protects monocytes from the proapoptotic effect of Yondelis. Monocytes were treated with M-CSF (20 ng/mL) and with the indicated concentrations of the drug. One representative experiment of DNA analysis out of five done.

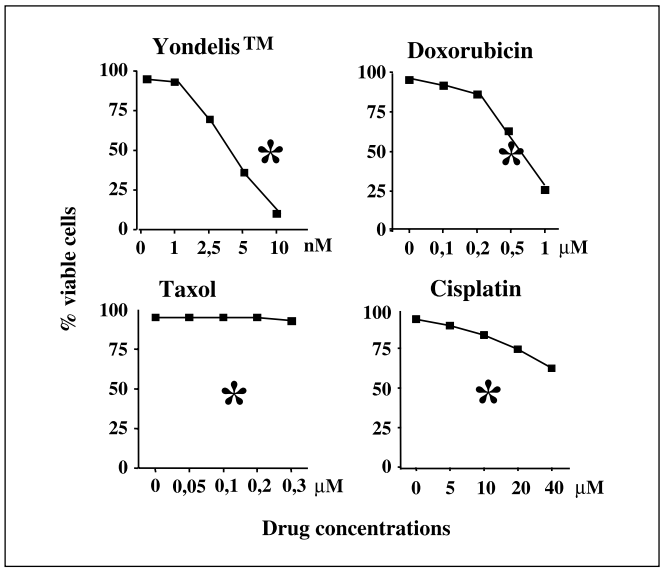


**Figure 2.** Comparison of Yondelis with other antineoplastic agents on monocyte viability. Monocytes were incubated for 48 hours with the indicated concentrations of Yondelis, doxorubicin, taxol and cisplatin. Viability was assessed by PI staining and analyzed by flow cytometry. Representative of three experiments. *, $IC_{50}$ for each drug on tumor cells *in vitro*.

Figure 4*C* (*left*) shows a representative experiment comparing the susceptibility of monocytes and macrophages from the same donor. Macrophages received Yondelis in the last 48 hours of the 5-day culture with M-CSF and underwent significant cell death, although to a lesser extent compared with freshly isolated monocytes. Polarized subsets of macrophages activated by lipopolysaccharide and IFN-γ (M1) or by IL-4 (M2) were also susceptible to drug treatment (Fig. 4*C*, *middle*). TAMs isolated from the ascites of nontreated patients with ovarian adenocarcinoma were also tested. TAMs were significantly susceptible *in vitro* to Yondelis. Results from three different patients show 40% to 60% reduction of cell viability at 10 nmol/L (Fig. 4*C*, *right*).

**Yondelis inhibits the production of inflammatory mediators.** Production of selected inflammatory mediators was tested in monocytes/macrophages, TAMs, and freshly isolated tumor cells, upon *in vitro* treatment with Yondelis. Cells were treated for 16 hours and supernatants collected for ELISA measurements; under these treatment conditions, cell viability was usually >85% for all drug concentrations tested.

Yondelis dose-dependently reduced the production of the inflammatory chemokine CCL2. Mean inhibition at 5 nmol/L was 65% ($n$ = 7) for lipopolysaccharide-stimulated monocytes and 60% for *in vitro* differentiated macrophages ($n$ = 5; Fig. 5). IL-6 production was also reduced, with an overall inhibition at 5 nmol/L of 54% ($n$ = 4) and 59% ($n$ = 4) in monocytes and macrophages, respectively (Fig. 5). By contrast, and quite surprisingly, the production of TNFα by monocytes/macrophages was never inhibited (Fig. 5), suggesting that this compound interferes only with selected genes. This result further indicates that under these conditions cells were not damaged by the treatment.

Next, TAM and freshly isolated tumor cells from ovarian adenocarcinoma were tested. Yondelis dose-dependently reduced the constitutive release of CCL2 by TAM at 5 nmol/L, 55% inhibition ($n$ = 4) and of IL-6, 38% inhibition ($n$ = 4; Fig. 6*A*). In lipopolysaccharide-stimulated TAM there was a 50% and 35% decrease for CCL2 and IL-6, respectively ($n$ = 3), whereas TNF production was not reduced. In freshly isolated tumor cells, the constitutive release of CCL2 and IL-6 by *in vitro* treatment with Yondelis was also potently reduced (65% and 68% inhibition, respectively; $n$ = 2; Fig. 6*A*).

To verify whether the inhibitory effect of Yondelis on cytokine production was at the transcriptional level, we analyzed mRNA of CCL2 transcripts from lipopolysaccharide-stimulated macrophages by real-time PCR. As shown in Fig. 6*B*, a consistent reduction of CCL2 transcripts was observed after drug treatment. This figure also shows that TNF mRNA was unaffected, in line with the results obtained in ELISA.

## Discussion

In this study we evaluated the immunomodulatory properties of the antitumor agent Yondelis on mononuclear phagocytes. Whereas the lymphocyte lineage was much less sensitive, blood-circulating monocytes were selectively susceptible to the drug and underwent apoptosis at therapeutic concentrations ($C_{max}$ ± SD at the dose of 1,300 μg/m$^2$ was reported to be 7.1 ± 2.2 nmol/L; $n$ = 6;

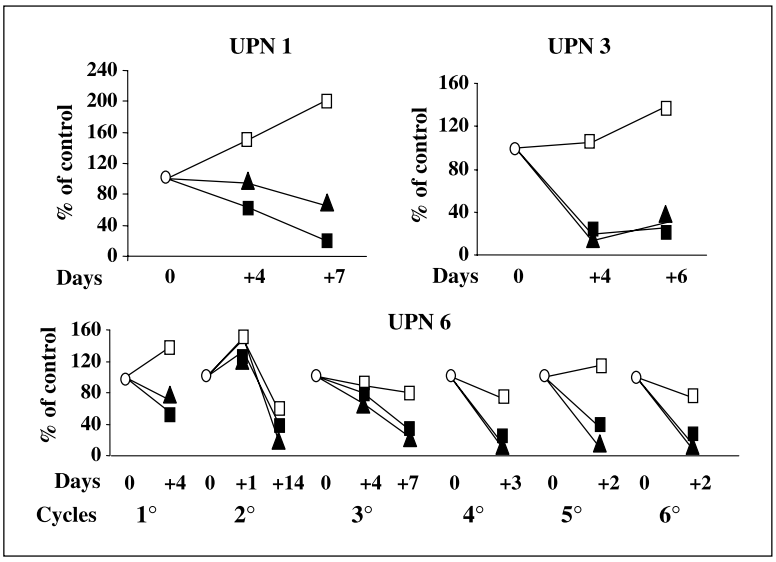


**Figure 3.** *In vivo* infusion of Yondelis in tumor patients induces transient monocytopenia. Monocyte counts were calculated either as absolute number per microliter of blood and as percent over total leukocytes. Results from three different patients. ■, monocytes (absolute number); ▲, % monocyte (% WBC); □, % total WBC.

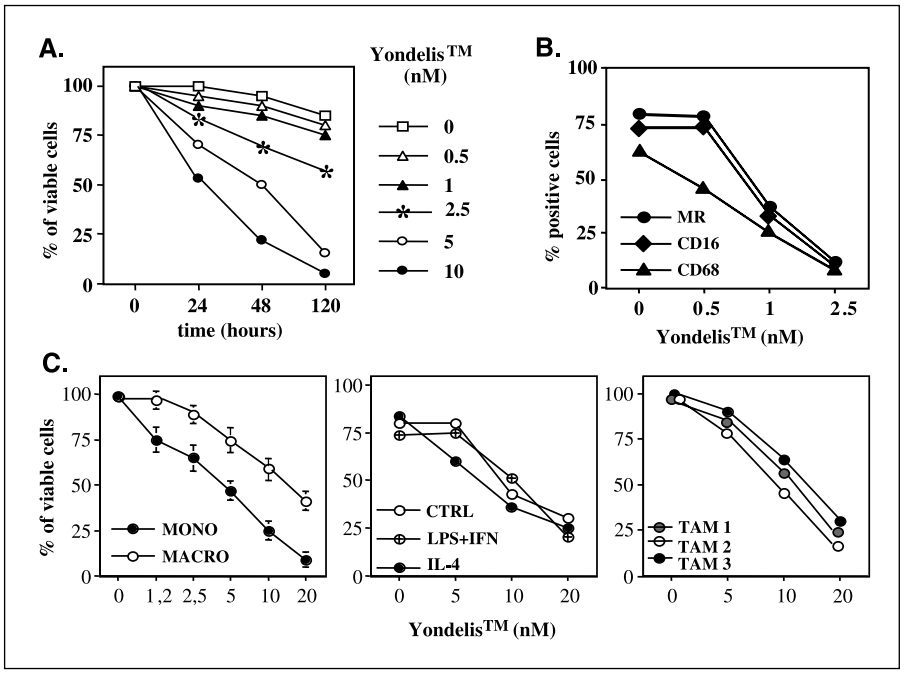


**Figure 4.** *A,* Yondelis inhibits the M-CSF–driven differentiation of macrophages and macrophage viability. Cells were treated with M-CSF (20 ng/mL) and different drug concentrations. Samples were collected at the indicated times. Viability was assessed by PI staining and analyzed by flow cytometry. *B,* phenotype analysis of macrophages obtained after 5-day culture with M-CSF (20 ng/mL) for 5 days in the presence of subcytotoxic concentrations of Yondelis added at day 1. Representative of five experiments. *C,* Yondelis is cytotoxic for already differentiated macrophages and TAMs. *Left,* comparison of susceptibility between monocytes and macrophages obtained from the same donor. For macrophages, Yondelis was added at day 3 of culture and incubated for 48 hours. *Middle, in vitro* differentiated macrophages were stimulated with lipopolysaccharide (100 ng/mL) + IFN-γ (500 IU/mL) and IL-4 (20 ng/mL) in the presence or absence of Yondelis for 48 hours. *Right,* enriched preparations of TAM isolated from the ascites of three different patients with ovarian cancer were treated *in vitro* with Yondelis for 48 hours.

refs. 33, 34). Already differentiated macrophages and freshly isolated TAMs were also susceptible at slightly higher dose (5-10 nmol/L per 48 hours), still within the effective therapeutic range. These *in vitro* findings were confirmed in patients with ovarian cancer receiving Yondelis in a phase II trial: in most of the patients a significant monocytopenia was observed in the first few days after drug infusion.

Of interest, other chemotherapeutic agents, including cisplatin and taxol did not show, *in vitro*, appreciable cytotoxicity on mononuclear phagocytes, whereas doxorubicin did affect monocyte viability but less potently than Yondelis. Thus, the cytotoxic effect of Yondelis on monocytes is a peculiar characteristic of this compound, not generally shared by other antitumor drugs.

The differentiation of macrophages *in vitro*, induced by M-CSF, was inhibited at subcytotoxic concentrations of the drug. We have confirmed these results with monocytes from tumor-bearing patients undergoing Yondelis therapy. In 6 of 12 patients tested, monocytes collected after 3 hours of drug infusion (1,300 $\mu g/m^2$) showed >50% inhibition of macrophage differentiation, compared with monocytes collected just before therapy. These results indicate that a brief *in vivo* exposure to Yondelis is sufficient to provide a measurable effect on monocytes. This is also supported by *in vitro* results that short exposure (1 hour) to the drug was sufficient to affect cell viability after 48 hours (not shown).

It should be noted that even in the presence of M-CSF, monocytes never underwent cell cycle progression, as checked by DNA analysis with flow cytometry (not shown). The toxic effect of Yondelis on monocytes is, therefore, independent of cell cycle and provides the unique opportunity to study the biological effects of this drug on nonreplicating cells.

A major finding of this work was the inhibitory activity of Yondelis on the production of inflammatory cytokines. Among various macrophage-derived products, we have tested CCL2, IL-6, and TNF. CCL2 is a chemoattractant for monocytes and lymphocytes and is produced both by mononuclear phagocytes and by tumor cells. Tumor-derived CCL2 attracts circulating monocytes at the tumor site (26, 29). In ovarian and pancreatic carcinomas, serum levels of CCL2 significantly correlate with the macrophage content of tumors (35–37); thus, CCL2 is a major determinant of the monocyte recruitment within the tumor tissue. Yondelis significantly reduced CCL2 release by lipopolysaccharide-activated monocytes, macrophages, and TAM. Most importantly, Yondelis strongly inhibited the constitutive release of CCL2 by unstimulated TAM and freshly isolated ovarian tumor cells at concentrations as low as 2.5 nmol/L. Lower levels of CCL2 produced in the tumor microenvironment are likely to effectively reduce macrophage accumulation at the tumor site. Interestingly, doxorubicin and taxol did not inhibit CCL2 production, whereas cisplatin showed a modest inhibition only at high concentrations (10 μmol/L, not shown).

Several lines of evidence indicate that in the tumor stroma a symbiotic relationship between cancer cells and TAM takes place, whereby cancer cells attract TAM and sustain their survival, and TAM respond to tumor-derived molecules by producing important growth factors and matrix enzymes that stimulate tumor proliferation, angiogenesis, and invasion of the surrounding tissues (26–28, 38). The relationship of TAM infiltration and prognosis in tumor patients has been investigated in several studies (39–41). As pointed out in a recent review (27), 10 of 15 studies concluded that the greater the macrophage infiltration, the worse the prognosis, whereas the opposite was found in three studies and no clear association in the remaining two. Therefore, the presence of high numbers of TAM at the tumor site is more frequently associated with poor prognosis. In this study, we found that TAM viability was significantly reduced *in vitro*. The toxic effect of Yondelis on TAM, as well as the inhibition of chemoattractant release, is likely to bear particular relevance for its antitumor activity.

Another cytokine whose production was reduced by Yondelis was IL-6. IL-6 and related members of its cytokine family are strongly involved in immune and inflammatory response by regulating a variety of biological processes including cell division, differentiation, and survival (42). Moreover, IL-6 is a cofactor for the production of CCL2 (43). Several studies have showed that IL-6 can directly act as a growth and survival factor in several tumors,

**Table 1.** Effect of *in vivo* treatment with Yondelis on the *in vitro* differentiation of macrophages in tumor patients

| Patients | Absolute numbers of marker-positive macrophages per 10,000 cells | | |
|---|---|---|---|
| | Before Yondelis | After Yondelis | % Inhibition |
| UPN 1 | | | |
| CD206 | 4,350 | 550 | 88 |
| CD16 | 3,110 | 1,248 | 60 |
| CD68 | 2,703 | 1,473 | 45 |
| UPN 2 | | | |
| CD206 | 2,810 | 595 | 79 |
| CD16 | 2,705 | 1,105 | 60 |
| CD68 | 3,500 | 1,060 | 70 |
| UPN 3 | | | |
| CD206 | 3,590 | 474 | 87 |
| CD68 | 3,260 | 632 | 80 |
| UPN 4 | | | |
| CD16 | 5,130 | 3,050 | 41 |
| CD68 | 5,550 | 2,460 | 55 |
| UPN 5 | | | |
| CD16 | 1,575 | 594 | 63 |
| CD68 | 1,620 | 815 | 50 |
| UPN 6 | | | |
| CD16 | 2,750 | 480 | 83 |
| CD68 | 2,320 | 505 | 79 |

NOTE: Blood samples were collected immediately before and at the end of a 3-hour infusion with Yondelis (1,300 $\mu g/m^2$). Purified monocytes were cultured with M-CSF (20 ng/mL) for 5 days and then analyzed for phenotype expression. Shown are the absolute numbers of marker positive cells for a total of 10,000 cells analyzed. % Inhibition of macrophage differentiation refers to cells collected before infusion.

in particular, in multiple myeloma and ovarian cancer (44, 45). In addition, IL-6 and related cytokines are involved in tumor-induced cachexia and in metastasis-induced osteolysis (42). *In vitro* treatment with Yondelis dose-dependently reduced IL-6 production by lipopolysaccharide-stimulated monocytes/macrophages. The lipopolysaccharide-induced as well as the constitutive release by TAM and freshly isolated tumor cells was also inhibited. In view of its ability to sustain tumor cell growth and survival, the drug-mediated reduction of IL-6 may be an important antitumor mechanism.

Quite surprisingly, Yondelis had no significant effect on TNF production, another important inflammatory cytokine. The release of TNF by lipopolysaccharide-stimulated monocytes/macrophages and TAM was completely unaffected by Yondelis, even at high drug concentrations. The effect on the constitutive production by unstimulated TAM and tumor cells was difficult to evaluate because both cell types released very low amounts of TNF in the absence of stimuli (not shown). The reason why TNF transcription is intact after drug treatment is not clear at the moment. It was already known that Yondelis binds DNA with some sequence specificity. Our results extend this observation by indicating that the compound interferes only with the transcription of selected cytokine genes. TNF transcription in myeloid cells stimulated by lipopolysaccharide is primarily activated by NFκB (46), although other factors could be involved. CCL2 and IL-6 transcription is also under the control of NFκB but can be activated by other transcription factors, such as activator protein (AP-1), SP-1, Smad3 for CCL2 (47) and AP-1, cFOS, and CCAAT/NF-IL-6 for IL-6 (48). Of these, AP-1 activates also TNF. The search for transcription factors common to CCL2 and IL-6 and not to TNF has been unsuccessful, up to now, and will require further study.

TNF has been strongly implicated as a promoter of carcinogenesis in mouse tumor models (25). Moreover, TNF activates NFκB, which, in addition to inducing cytokine production, is important to controlling cell proliferation and survival. Recently, activation of NFκB has been implicated in the neoplastic transformation of precancerous cells (22, 24). In ovarian cancer, TNF has been shown to increase tumor cell growth and angiogenesis and to promote the adhesion of ascitic cancer cells to the peritoneal wall (49). Therefore, the inability of Yondelis to interfere with TNF production should be considered. TNF antagonists are currently being tested in the clinic in patients with autoimmune diseases and, more recently, tumors (50). Combination therapies with chemotherapeutic drugs and TNF antagonists could be considered.

Because tumor patients undergoing Yondelis therapy are also treated with corticosteroids to prevent drug-induced hepatotoxicity, it was of interest to evaluate whether the *in vitro* treatment

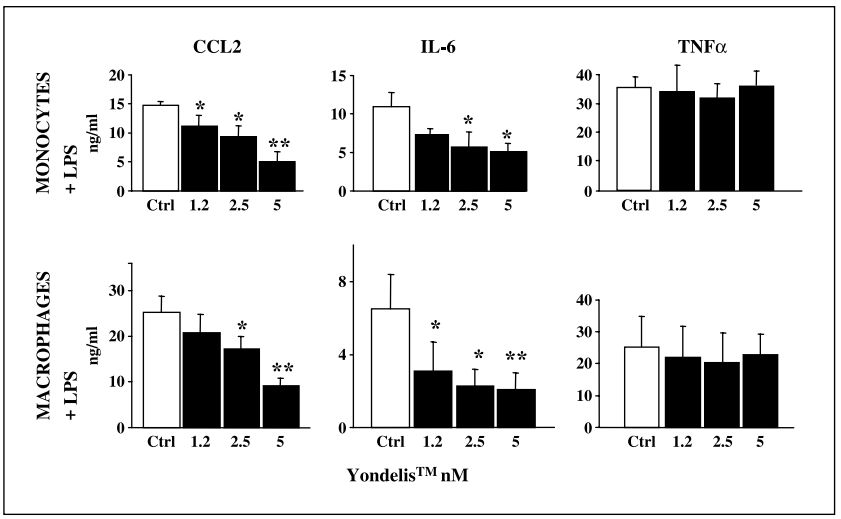


**Figure 5.** Yondelis inhibits the production of CCL2 and IL-6, but not TNF, by monocytes and macrophages. Blood monocytes and *in vitro* differentiated macrophages were stimulated with lipopolysaccharide (100 ng/mL). Treatment with Yondelis preceded 1-hour lipopolysaccharide stimulation. After 16 hours' incubation, cell supernatants were harvested and tested in ELISA. *Columns,* mean of four to seven experiments; *bars,* SE. *, $P < 0.05$ (significantly different, Student's *t* test); **, $P < 0.01$ (significantly different).

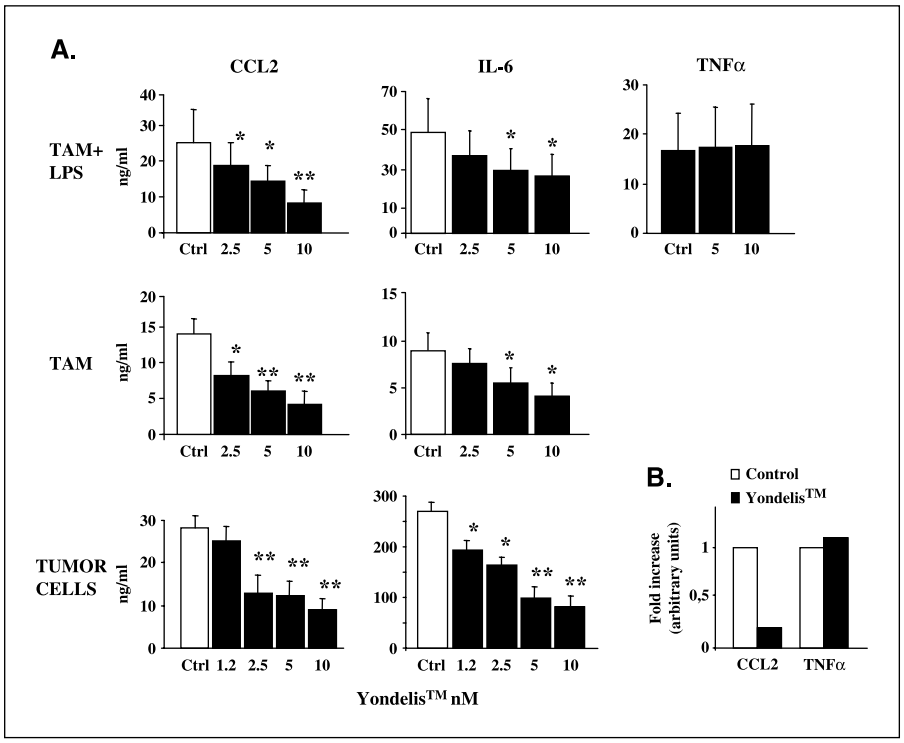


**Figure 6.** Yondelis inhibits CCL2 and IL-6 production in TAM and tumor cells from patients with ovarian cancer. *A,* freshly isolated ovarian tumor cells and TAM were incubated with Yondelis for 16 hours. Where indicated, TAMs were stimulated with lipopolysaccharide (100 ng/mL). Cell supernatants were harvested and tested in ELISA. Mean of four experiments for TAM and two for tumor cells; *bars,* SE. *, $P < 0.05$ (significantly different, Student's *t* test); **, $P < 0.01$ (significantly different). *B,* real-time PCR of CCL2 and TNF transcripts in lipopolysaccharide-stimulated monocytes exposed to 5 nmol/L Yondelis for 6 hours.

of monocytes with the combination Yondelis + dexamethasone affected TNF production. As expected, dexamethasone inhibited TNF production by 50%, and the drug combination resulted in a similar inhibition (not shown). The association of decreased TNF levels with the corticosteroid-induced protection from hepatotoxicity remains to be investigated.

In conclusion, the antineoplastic agent Yondelis showed a selective cytotoxic effect on monocytes and macrophages including TAM. At subcytotoxic concentrations, it significantly reduced the production of two proinflammatory cytokines, IL-6 and CCL2, which have a major role in host-tumor relationship and in cancer progression. In view of the protumoral role of TAM and the enlarging link between inflammation and cancer, these properties of Yondelis may contribute to its antitumor activity. This compound can be seen as the prototype of a new class of anticancer agents possessing high anti-inflammatory activity as well as selectivity for macrophages.

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