Selective Effects of the Anticancer Drug Yondelis (ET-743) on Cell-Cycle Promoters

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ABSTRACT

Yondelis is a potent DNA-binding anticancer drug isolated from the tunicate Ecteinascidia turbinata currently undergoing phase III clinical trials. We and others have shown selective inhibition to the transcriptional induction of several genes. We tested the hypothesis that Yondelis specifically targets cell-cycle genes. Our analysis on endogenous and transfected reporter systems revealed complex patterns of transcriptional inhibition and, surprisingly, activation. Other inducible systems—the metallothionein and the CYP3A4 promoters—were little affected. We assayed whether interference of DNA binding of the common nuclear factor Y (NF-Y) activator was responsible for the observed inhibition: in vivo chromatin immunoprecipitation analysis in NIH3T3 and HCT116 cells indicates that NF-Y binding is little affected by Yondelis addition. Finally, histone acetylation was modestly affected only on Cdc2 and cyclin B2 but not on other repressed promoters. These data prove that Yondelis is not a general inhibitor of inducible genes, and its selective effects are exerted downstream from transcription factors binding and histone acetyl transferases recruitment.

Yondelis (ecteinascidin-743, ET-743) is a marine tetracydrosoquinoline alkaloid isolated from the tunicate Ecteinascidia turbinata (Pommier et al., 1996; Martinez et al., 1999). It exhibits a potent cytotoxic activity against a variety of tumor cell lines in vitro and against several rodent and human tumors and human tumor xenografts in vivo. It is under clinical investigation in Europe and the United States as a promising new class of anticancer drug (D’Incalci and Jimeno, 2003). Direct comparison of in vitro antiproliferative activities of Yondelis reveals that it is 1 to 3 orders of magnitude more potent than Taxol (paclitaxel; Bristol-Meyers Squibb, New York, NY), cisplatin, bleomycin, mitomycin C, camptothecin, or etoposide. Its primary mode of action resembles poorly understood. Yondelis binds to the minor groove of DNA with some degree of sequence specificity and subsequently forms covalent adducts by reacting with the N-2 of guanine to its carbinolamine moiety (Zewail-Foote and Hurley, 1999b). In theory, all DNA-binding drugs could interfere with crucial cellular functions, such as DNA repair, replication, and transcription. Regulation of transcription is controlled by short DNA elements positioned in the proximity of the genes—promoters and, at distance, enhancers. Promoters and enhancers that activate Pol II-transcribed mRNA genes are formed by a combinatorial puzzle of elements recognized by sequence-specific regulators (Levine and Tjian, 2003). Binding of such DNA-binding proteins is a prerequisite for recruitment of coactivators, mediators, and general transcription factors, finally leading to the establishment of a multisubunit transcription complex. Impairment of the complex interactions among activators and their DNA targets could lead to a change in the pattern of gene expression; interference at this level by alkylating drugs might help explain their profound biological functions. Many anticancer drugs seem to influence gene expression by acting on specific promoters: cisplatin on cyclin A, doxorubicin on Id2A, Taxol on interleukin-8, genistein on glucose-regulated protein 78, and HSP70 (Kurabayashi et al., 1995; Lee et al., 1997; Spit-
kovsky et al., 1997; Moos and Fitzpatrick, 1998). However, the conditions used in these studies involved long incubation times at drug concentrations in the micromolar range, 1 to 3 orders of magnitude higher than concentration levels required to show in vitro cytotoxic effects. This indicates that the transcriptional effects might be secondary to impairment of other known functions: microtubule depolymerization, protein tyrosine kinases, DNA synthesis, or DNA topoisomerase II poisoning.

The mechanisms of action of the Yondelis seem to be unique, and interference with transcription is most likely part of the picture (Scotto, 2002). The drug is able to inhibit the binding of several transcription factors, and in fact, only SRF/TCF and NF-Y showed any level of sensitivity to the drug (Bonfanti et al., 1999). Experiments on the HSP70 promoter indicated that Yondelis has a specific negative effect on transcription that is unique among minor groove binders (Minuzzo et al., 2000). Likewise, Jin et al. (2000) examined Yondelis activity on the induction of the human MDR1 promoter by a number of inducers—trichostatin A, UV, and butyrate—which were effectively prevented by equally low Yondelis concentrations (Jin et al., 2000). It is important to note that no inhibitory effect of the drug on the basal level of transcription was reported (Jin et al., 2000; Minuzzo et al., 2000; Friedman et al., 2002). Another gene shown to be sensitive to pharmacological doses of the drug is the type I collagen (Louneva et al., 2003). Collagen, HSP70, and MDR1 have different architectures, and a common feature, that is, the presence of a CCAAT box on which NF-Y acts. NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-VC, all necessary for DNA binding (Mantovani, 1999). NF-Y makes important contacts in the minor groove (Ronchi et al., 1995), and two unrelated cytotoxic drugs (i.e., genistein and HMM-154) inhibit in vitro binding of NF-Y to the CCAAT box by targeting the NF-YB subunit (Mantovani, 1999).

Further links between transcriptional inhibition and Yondelis action were uncovered. A study indicated that Sp protein 1-mediated activation was also inhibited by Yondelis (Friedman et al., 2002); the orphan receptor SXR, which has been identified for its transcriptional activation of the cytochrome P450 CYP3A4 gene and shown to activate the MDR1 promoter indicated that Yondelis has a specific negative effect on transcription-coupled nuclear excision repair mechanisms (Damia et al., 2001; Takebayashi et al., 2001). Finally, gene-expression analysis with microarrays technology identified selected groups of genes that are inhibited by low doses of the drug in different cell lines (Martinez et al., 2001; Marchini et al., 2005). In parallel studies, evidence that Yondelis is more active in the G1 phase of the cell cycle, eventually resulting in a G2/M block, was reported (Erba et al., 2001). To explain the antiproliferative activity of Yondelis, putative transcriptional targets might be inducible promoters: genes involved in DNA metabolism and/or genes coding for cell-cycle regulators (Gajate et al., 2002). To investigate further the mechanisms of action, we used several inducible and cell-cycle–regulated promoters and chromatin immunoprecipitation (ChIP) assays in vivo against two of the suspected targets.

### Materials and Methods

#### Drug and Cell Culture

Yondelis was prepared as a 1 mg/ml stock solution dissolved in dimethyl sulfoxide and kept at −20°C. Before use, the drug was freshly diluted in double-distilled sterile water to the desired concentrations (1, 3, 10, and 30 nM). Mouse NIH3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were cotransfected with 10 μg of cyclin E-Luc (−543 to −263; Botz et al., 1996), DHFR-Luc (Fry et al., 1997), TK-Luc (−319/+51; Sorensen and Wintersberger, 1999), E2F1-Luc (Wang et al., 1999), cdc2-CAT (cdc2-Pst; Ku et al., 1993), cyclin A-Luc (−215/+245; Henglein et al., 1994), cyclin B2-Luc (−1189/+31; Bolognesi et al., 1999; Lange-zu-Dohna et al., 2000), CYP3A4-Luc (a kind gift of M. Ingelman-Sundberg, Stockholm, Sweden), metallothionein-Luc (MT-I, −250/+66; Li et al., 1998), SV40-β-galactosidase (Minuzzo et al., 2000) plasmids, and 2 μg of a plasmid containing the hygromycin resistance gene. A total of 50 to 150 clones for each promoter were selected, pooled, and subsequently maintained. Before serum withdrawal, cells were passaged in hygromycin-free medium for 3 to 4 days, and starvation was performed for 60 to 72 h in 0.5% FCS. The drug was added concomitantly with Dulbecco’s modified Eagle’s medium containing 10% FCS, and extracts were prepared at the indicated times. MT-I-Luc was induced by the addition of 75 μM ZnCl2 after that of Yondelis 2 h earlier. In the experiments with the CYP3A4 promoter, we performed transient transfections with 2 μg of reporters cotransfected with 2 μg of RXR/SXR (a kind gift of R. Evans, Salk Institute, San Diego) that were added 36 h later. The cells were treated with proteinase K, extracted with phenol/chloroform, and precipitated with 20% ethanol. For extract preparation, cells were washed in phosphate-buffered saline, lysed (Triton 1% and glycyglycine 25 mM, pH 7.8) and controlled for protein concentrations by the Bradford reagent (Sigma Chemical, St. Louis, MO). Luciferase assays were performed with standard procedures using Luciferin (Sigma) as a substrate. CAT assays were carried out with equivalent amounts of extracts by the quantitative method. β-Galactosidase activity was measured in 60 mM Na2HPO4, 40 mM NaH2PO4, 1 mM MgCl2, 50 mM β-mercaptoethanol, and 0.66 mM mg O-nitrophenyl β-D-galactopyranoside. The reaction was stopped by adding 1 mM Na2CO3, and the A420 was measured. Results from four to six independent experiments were plotted; standard deviations were <20%. Flow cytometric cell-cycle analysis was performed according to the protocol described in Erba et al. (2001).

#### Chromatin Immunoprecipitation

Exponentially growing cells, untreated and treated with 10 nM Yondelis, were washed in phosphate-buffered solution and were incubated for 10 h with the cross-linking solution containing 1% formaldehyde. The reaction was quenched with 100 mM glycine, and cross-linked material was broken by sonication into chromatin fragments having an average length of 1 kb. Immunoprecipitation was performed with protein G-Sepharose, previously blocked with 1 μg/ml sheared sperm DNA and 1 μg/ml bovine serum albumine for 4 h at 4°C. The chromatin solution was preclarified by adding protein G-Sepharose for 15 min at 4°C, aliquoted, and incubated with 25 μg of antibodies (anti-YB from our laboratory, anti-Ach3 and anti-Ach4 from Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. Immunoprecipitates were eluted, cross-links were released at 65°C, and DNA was treated with protease K, extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated. Pellets were resuspended into 30 μl of H2O and were analyzed by PCR with specific primers. The input sample was resuspended in 100 μl of H2O and diluted 1:100. The primers used in the semiquantitative PCR reactions of Fig. 5 were described by Caretti et al. (2003). The primers for the amplification of the human promoters in Fig. 6 were: hCCNB1, 5′-TGTCACCTTCTAAAGGGCACTA-3′ and 5′-AGAAGAAAGACCGCTAGCCTAGCAG-3′; hCCNB2, 5′-AGAAGGCCTCTACCTGCTTCTT-3′ and 5′-ATTCAAATCCGCGTCGGCTTG-3′; hCde2, 5′-TAGCTTCTGCTTCGCTGTGAC-3′ and 5′-TCCCTCTGACACGCGCTGA-3′; hCdc25C, 5′-GCTGAGGAAAGACGCCAA-3′ and 5′-CGCCAGC-CGCAAAAACCCATATCC-3′; and human topoisomerase IIa, 5′-CCTGCAACTTGGCTTCAG-3′ and GACCAGCAAATTCGTCAGTCT.
RT-PCR Analysis. RNA was extracted using an RNeasy kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer’s protocol, from NIH3T3 cells untreated or treated for the indicated times with Yondelis. For cDNA synthesis, 4 μg of RNA were used with the MMLV-RT kit (Invitrogen, Carlsbad, CA). Semiquantitative PCR analysis was performed. The primers for the RT-PCR were: actin, 5'-TGGGTCAAGAGATTCTATG-3' and 5'-CACGCCGGTACTACACCC-3'; hE2F1, 5'-GCAAGAAGTCTCGGAGGAGACGTC-3'; human cyclin A2, 5'-TATTCGTGGGACCTCCTGTC-3' and 5'-CCCTGGGCGTTGAGGAGAG-3'; hCCNB1, 5'-CAGTTCAGGAGCAAGCATAAT-3' and 5'-CAGGTGCTGCATAGCTGCCTTTC-3'; hCCNB2, 5'-CACTTCCTTCGGAGAGCATC-3'.

Results

Yondelis Is Not a General Inhibitor of Induced Transcription. Several promoters are affected by treatment of cells with pharmacological concentrations of Yondelis (Jin et al., 2000; Minuzzo et al., 2000; Friedman et al., 2002). In run-on assays, additional immediate-early inducible genes, such as c-Jun and c-Fos, and cell-cycle–regulated ones, such as H4 and H2B, were also affected (Minuzzo et al., 2000). All of these genes share the common feature of being rapidly inducible by different stimuli. On the other hand, Yondelis showed no sign of altering the “basal” levels of these genes. The possibility therefore exists that Yondelis might be a general inhibitor of “induced” transcription. We therefore tested two additional promoters that are rapidly inducible: the metallothionein, and the CYP3A4. The latter was chosen because it is an intended target of SXR, the orphan receptor whose function has been hypothesized to be affected by the drug (Synold et al., 2001). We stably transfected an MT-I-Luc construct into NIH3T3 cells and selected pools of colonies expressing the gene; we added increasing doses of Yondelis for 2 h before ZnCl2 addition. Cells were collected 6 h later, expressing the gene; we added increasing doses of Yondelis construct into NIH3T3 cells and selected pools of colonies drug (Synold et al., 2001). We stably transfected an MT-I-Luc

Effect of Yondelis on Cell-Cycle Promoters. In another study, we found that the drug is particularly active when cells are in the G1 phase of the cell cycle (Erba et al., 2001). Because cell-cycle promoters are tightly regulated, inducible systems, we investigated whether Yondelis affects them. We selected promoters that were active in all phases of the cell cycle: G1, cyclin E, E2F1, TK, and DHFR; S, cyclin A and cdc2; and G2/M, cyclin B2. All of these promoters, except for cyclin E, are NF-Y–dependent (Fig. 2). For these experiments, we chose NIH3T3 fibroblasts stably transfected with the reporter vectors, containing either CAT or luciferase genes, together with the hygromycin resistance-containing vector. Large collections of resistant clones were pooled to minimize for the effects of single integrations on the transcriptional activities of the various promoters. The different lines of NIH3T3 were induced to enter G0 by serum withdrawal and restimulated with the addition of medium containing serum. First, we verified the cell-cycle–dependent expression of the reporter genes. Each of the cell-cycle–
regulated promoters decreases its function when cells are arrested in G0 (data not shown), followed by up-regulation after serum restimulation, with a timing that is dependent on the promoter used: G1/S promoters come up after 12 to 14 h, and S/G2 promoters are not fully active before 20 h (Fig. 3). These results are entirely consistent with the expected timing of cell-cycle progression observed in the NIH3T3 system (see below), and the magnitude of restimulation difference ~3/10-fold is within the limits observed previously (Ku et al., 1993; Henglein et al., 1994; Botz et al., 1996; Fry et al., 1997; Sorensen and Wintersberger, 1999; Wang et al., 1999; Lange-zu Dohna et al., 2000). Next, we tested the transcriptional effect of Yondelis on promoter inducibility. We found that the addition of the drug during the starvation period leads to a high rate of cell death and ineffective restimulation, even at concentrations in the low nanomolar range. The reason for this behavior was unclear and worthy of future investigations on the kinetics of this effect. If anything, this observation reinstates the unique property of Yondelis, acting in conditions different from DNA synthesis. We thus decided to add the drug together with the 10% FCS-containing medium upon restimulation. First, we checked by FACS analysis the BrdU incorporation profiles of cycling, starved, and restimulated NIH3T3 cells harvested at different time points. Table 1 shows the percentage of cells in G1, S, and G2/M stages of control NIH3T3 and cyclin A-3T3 cells. As expected, in the absence of Yondelis, NIH3T3 and cyclin A-3T3 are mostly in G1 at 8 h, in S at 16 h, and again in G1 at 24 h. At 1 nM Yondelis, there is an increase in the percentage of cells still in S at 16 h, with most cells still in G2/M at 24 h. At 3 nM, the picture is identical at 16 h, and a further increase of cells still in S at 24 h is also observed. At 10 nM, the cells are essentially blocked, and no DNA synthesis is scored. This profile is representative of all other stably transfected clones described hereafter.

We then assayed the transcriptional activity of the promoters. Figure 4 illustrates the mean values obtained in several such experiments at different time points and drug concentrations. The three S/G2 promoters contain important CCAAT boxes and heavily depend on NF-Y: the activity of the cyclin B2–3T3 cells is inhibited already at 1 nM Yondelis, whereas cyclin A-3T3 and cdc2–3T3 showed inhibition at the highest 10 nM concentration. The latter finding was expected, because at 10 nM, cells never make it past G1. On the other hand, the decrease in the levels of luciferase activity in cyclin B2–3T3 cells, which is quite prominent at 3 nM Yondelis, is effective despite the fact that most cells are actually in S phase at 24 h and a third of them are in G2/M phase (Table 1). We conclude that at low concentrations, the drug is specifically hitting cyclin B2.

A dichotomy is observed in the G1/S promoters (Fig. 4, top). TK and DHFR are clearly inhibited, whereas E2F and cyclin E are 4/5-fold increased. TK-3T3 cells show a dose-dependent

![Fig. 2. Scheme of the promoters used in this study. The constructs used are detailed under Materials and Methods and were described before. Known transcription factors binding sites are indicated.](image-url)
decrease at 16 h and are still inhibited at later time points (24 h) when most cells have long past the G1/S phase. At the latter time point, inhibition is observed at 1 nM. The DHFR promoter is also similarly affected at 1 nM and then severely inhibited at 3 nM, even at later time points. Cyclin E-3T3 cells increase their activities at 3 and 10 nM by 12 to 16 h. E2F1–3T3 show an increase only at high (10 nM) concentrations.

**Promoter Occupancy after Yondelis Treatment.** One simple explanation for the inhibition of promoter activity observed above is that the DNA-binding drug displaces or prevents the binding of crucial activators such as the NF-Y trimer. To investigate this hypothesis, we used the ChIP assay. Cells were fixed with formaldehyde, chromatin-derived by sonication, and immunoprecipitated with an anti-NF-Y–specific antibody; the resulting DNA was tested in PCRs with oligonucleotides amplifying the specific targets. Figure 5 shows the results of these experiments with NIH3T3 cells treated or untreated with high (10 nM) doses of Yondelis. It is clear that the G2/M promoters tested, all requiring NF-Y, do not show a significant reduction in factor association after 4 h of Yondelis. Likewise, DHFR and cyclin A were bound by NF-Y in control and Yondelis-treated cells. Finally, the binding of NF-Y to the induced E2F1 promoter was actually increased upon Yondelis addition (Fig. 5, top), a fact that correlates well with the increased promoter function (Minuzzo et al., 2000). Likewise, DHFR and cyclin A were bound by NF-Y in control and Yondelis-treated cells.

Together, these results indicate that the effect of Yondelis on the transcription of cell-cycle promoters is not caused by NF-Y-CCAAT competition and that histone acetylation is modestly affected only on a subset of repressed promoters.

**Discussion**

Our analysis on the transcriptional inhibition mechanisms of the antiproliferative marine compound Yondelis allowed us to reach the following conclusions: 1) not all inducible systems are hit by Yondelis; 2) cell-cycle promoters are selectively affected negatively or, surprisingly, positively; 3) the drug is unable to displace a key factor, NF-Y, from its promoter location through competitive inhibition of DNA binding; and 4) local histone acetylation is largely unaffected.

Yondelis has recently attracted considerable interest by molecular oncologists. At first glance, it might be cataloged as yet another DNA-alkylating agent. However, by all criteria used so far, it is unlike any of the compounds of the sort described previously. From a structural standpoint, NMR studies of DNA-Yondelis adducts show binding in the minor groove; Yondelis bends DNA toward the major groove, and it is clearly distinct from distamycins and CC-1065, minor groove binders binding DNA in the minor groove, and cisplatin, a major groove binder that bends into the major groove (Moore et al., 1997; Zewail-Foote and Hurley, 1999a). Furthermore, the mode of alkylation is unique (Zewail-Foote and Hurley, 1999b). As a consequence of DNA binding, Yondelis shows sequence selectivity, that, although not more pronounced than other compounds, is nonetheless different (Pommier et al., 1996). Treatment of nuclear excision repair cells with Yondelis generates a somewhat paradoxical effect,
because xeroderma pigmentosum group B-, Cockayne syndrome group B-, and excision repair cross complementation 1-defective cell lines are more resistant rather than more sensitive to the drug (Damia et al., 2001; Takebayashi et al., 2001). Yondelis shows a remarkable activity against cells that are in G_{1} phase, which is unprecedented for this class of compounds (Erba et al., 2001); other DNA-alkylating agents, in fact, are typically active on cells undergoing DNA replication when their target, DNA, is most vulnerable. Cells whose DNA has been severely damaged by adduct formation or UV or γ irradiation undergo a G_{1}/S arrest, a process requiring p53. Treatment of various cells with Yondelis indeed increases p53 protein levels, but p53^{−/−} mouse embryo fibroblasts are as sensitive as their p53^{+/−} counterparts as p53^{−/−} colon carcinoma A2780 cells and an isogenic line expressing a dominant-negative p53 (Damia et al., 2001). Thus, p53 is not required for the cell-cycle arrest mediated by Yondelis.

Finally, by performing single-strand breaks and DNA-protein cross-link assays in SW620 cells, we found no gross abnormalities. Although this is certainly no proof that Yondelis causes none of these phenomena, it is an indication that DNA adducts might be relatively few, compared with other DNA-alkylating agents, and possibly targeted to specific spots within the genome. In keeping with this, gene expression-profiling analysis yielded none of the common p53 targets among the genes activated or inhibited by Yondelis (Martinez et al., 2001; Marchini et al., 2005). It is therefore possible that DNA alterations might be too subtle for the p53-mediated DNA-damaged checkpoint to intervene.

Perhaps the most striking feature is that, unlike other drugs that require long incubation times and high concentrations to generate transcriptional effects, Yondelis’ promoter-specific inhibition is rapid and occurs at low, achievable pharmacological concentrations (Scotto, 2002). Some of the transcriptional data might be the results of cell-cycle perturbation, hence an indirect effect of the drug. However, the degree of inhibition of the cyclin B2 promoter did not parallel the retardation in the progression through the cycle, as indicated by FACS analysis, because promoter function was down-regulated at doses at which cells have progressed through S phase; thus, the idle status of this promoter is not caused by a block. This concept is further strengthened by the data obtained with the DHFR and TK promoters, which are still inhibited after 24 h at low Yondelis concentrations (1–3 nM) that allow cell-cycle progression well into the G_{2}/M phase. Therefore, the effects of Yondelis on specific promoters are not primarily caused by an inhibition of cell-cycle progression.

We were intrigued by the peculiar preference of Yondelis for inducible systems (Jin et al., 2000; Minuzzo et al., 2000; Synold et al., 2001; Friedman et al., 2002). In particular, we tested whether the CCAAT-binding factor NF-Y and/or the orphan receptor SXR was implicated. Our data are at odds with the interpretation that the orphan receptor SXR is the pharmacological target of the drug, as suggested by Synold et al. (2001). On the CYP3A4 promoter, which is the prototypical SXR target, no effect of Yondelis was observed, even at high doses. Yondelis might interfere with the activity of SXR on promoters of other cytochromes, which are important for xenobiotic-mediated activation of subsets of cytochrome P450 genes. This might be particularly relevant for the liver toxicity of this compound (Donald et al., 2002). However, one should note that expression of SXR is restricted to liver,

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**Fig. 4.** Transcriptional profile of cell-cycle promoters. The indicated cell lines were serum-starved for 60/72 h, restimulated, and concomitantly added with the indicated concentrations of Yondelis. At the different time points indicated, extracts were prepared, and average enzymatic activities were plotted against the activity of the reporter gene in the absence of Yondelis treatment. The experiments were repeated three to six times, and mean values indicated. Standard deviations were less than 20% of the values.
kidney, and small intestine (Blumberg et al., 1998), whereas Yondelis is active in a wide variety of cells, many of which show no expression of SXR.

Likewise, NF-Y is also unlikely to be an intended target, despite the fact that several CCAAT-containing promoters are inhibited, including HSP40, HSP70, MDR1, collagen (Jin et al., 2000; Minuzzo et al., 2000; Friedman et al., 2002; Louneva et al., 2003), cyclin B1/B2, cyclin A, TK, and DHFR, and some of the CCAAT-less, including metallothionein and SV40, are not. In fact, several findings are inconsistent with this interpretation: 1) the E2F1 promoter, which heavily depends on NF-Y binding (28), is up-regulated (Figs. 4 and 6); 2) the NF-Y–dependent CYP3A4 and cdc2 are little affected; and 3) the CCAAT-less cyclin E is increased, similarly to E2F1. Most important is that in ChIP assays, Yondelis shows no sign of displacing the trimer from its promoter location, even at high doses. In general, our data are in agreement with those of Friedman et al. (2002), showing that the activation mediated by different factors (TATA-binding protein, Sp protein 1, and CCAAT transcription factor) was impaired by Yondelis in artificial galactosidase 4-based assays; these findings strongly suggest a postrecruitment mechanism.

Our most surprising finding is that some G1/S promoters (cyclin E and E2F1) are up-regulated, whereas the promoters of genes required for DNA metabolism (TK and DHFR) are down-regulated. Most likely, the activation of the regulators precedes that of the regulated genes. It is extremely difficult to perform precise kinetic analysis in the NIH3T3 system so that the exact activation timing is determined. In this respect, Yondelis could represent a valuable tool to discriminate the controllers from the controlled genes. The mechanism of this activation is unclear, but our finding that treated cells show an increase in vivo binding of NF-Y supports the idea that perturbation in the normal physiology of factor binding are responsible. ChIP analysis, in fact, revealed that NF-Y is not present on the E2F1 promoter before activation and disappears after the gene has been switched in late S phase (Caretti et al., 2003). Yondelis might therefore alter the NF-Y-CCAAT turnover, either directly, by binding to specific sequences nearby, or indirectly, through yet-to-be-identified modifications of the factor.

Transcription is the result of an immensely complicated array of local interactions; the initial step is generally believed to be the binding of transcription factors (TFs) to their cognate cis-acting promoter elements, followed by recruitment of an array of cofactors, chromatin remodelers, general transcription factors, and enzymatic activities. Yondelis might target any of these steps. The peculiar susceptibility of cells in G1 to Yondelis is mirrored by the differential activity on G1/S promoters shown here. It should be remembered that the cellular DNA of G1 cells is believed to be largely compacted in chromatin structures, hence potentially refractory to the DNA-binding properties of the drug. Indeed, using in vitro nucleosome reconstitution systems, we showed that Yondelis has little influence on preformed nucleosomes, even at high micromolar concentrations (Bonfanti et al., 1999). Genes whose promoters are active in G1 might represent spots of an “open” chromatin configuration, allowing access of the drug to DNA. Indeed, there is now firm evidence that cell-cycle promoters are in an open configuration in phases in which they are transcriptionally inactive (Takahashi et al., 2000; Wells et al., 2000). The same is true for genes that are quickly inducible upon external stimuli, such as HSP70 and MDR1 (M. Pitarque-Martí, R. Mantovani, unpublished data), with mechanisms involving the recruitment of histone acetyl transferases (HATs) and rearrangements of chromatin structures occurring only after the stimulus has been given. We recently showed that a Yondelis analog, ET-637, lacking the C-ring does not impair its transcriptional inhibitory capacity or its antiproliferative effect (Erba et al., 2005). This analog showed normal DNA-binding capacity but lost the minor groove-protruding moiety. From these data, the interference of factor binding was therefore still a possibility. It is clear.

Fig. 5. ChIP analysis of NF-Y binding to cell-cycle promoters. NIH3T3 cells were treated for 4 h with 10 nM concentrations of Yondelis and chromatin immuno precipitation with control and anti-NF-YB (left) or anti-acetyl-H3 and acetyl-H4 (right) antibodies performed. The indicated promoters were PCR-amplified.

Fig. 6. Analysis of cell-cycle gene in HCT116 cells. A, RT-PCR analysis of mRNA derived from untreated and treated (4 and 8 h at 10 nM Yondelis) human HCT116 cells. B, ChIP analysis according to the scheme as in A with the indicated antibodies. The semiquantitative PCR amplifications are depicted for the different promoters.
from our data that direct competition between NF-Y and the drugs is not effective and it is not the cause of promoter repression. The results shown here do not rule out that factors other than NF-Y might be prevented from access. However, because HAT recruitment and histone acetylation usually require a complete build-up at the DNA-binding level, we consider this possibility unlikely.

Previous work suggested that inhibition of MDR1 expression was not linked to histone deacetylation (Jin et al., 2000). We fully confirm and extend these data to other promoters. Although the G/M promoters’ overall acetylation does drop, this decrease is nowhere as dramatic as the one observed after treatment of NIH3T3 with alkylating agents such as adriamycin (Imbriano et al., 2005). Overall, our ChIP analysis suggests that the build up of DNA-binding TFs required for HAT recruitment and histone-tail acetylation is not severely affected. Other steps might; in particular, our attention should now turn to the numerous events that follow TF and HAT recruitment. The ChIP assays used here should enable us to systematically search for alterations in the capacity of the selected classes of Yondelis-sensitive promoters to recruit the vast array of complexes required for transcription to ensue.

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