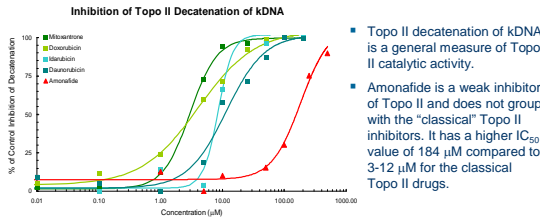


OVERVIEW

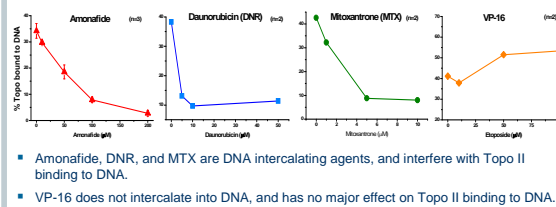
- We compared the effects of amonafide (Xanafide[®], amonafide L-malate) and the "classical" topoisomerase II (Topo II) active-drugs, daunorubicin (DNR), VP-16, mitoxantrone (MTX) on the binding of Topo II to DNA, ATP-Topo II binding, cleavable complex stabilization and DNA damage induction.
- Amonafide intercalated into DNA and inhibited the binding of Topo II to DNA. However, it was a much less potent inhibitor of kDNA decatenation.
- In contrast to MTX, amonafide competed with ATP in decatenation assays and interfered with VP-16 cleavage of pRYG DNA, a representative substrate. These results suggest that amonafide acts at a step in the Topo II catalytic cycle prior to that of the classical Topo II cleavable complex inhibitors. This hypothesis is also consistent with a decreased induction of DNA damage (e.g. DNA fragmentation) by amonafide compared to Topo II inhibitors.
- We incubated CEM leukemia cells with concentrations of either amonafide, VP-16 or DNR that induced similar degrees of mitochondrial apoptosis. Amonafide induced primarily high molecular weight DNA fragmentation (50-300 kb), while VP-16 and DNR treatment resulted in extensive cleavage of DNA into fragments less than 50 kb.
- Amonafide was also much less potent than VP-16 in inducing MLL gene cleavage in CEM cells as measured by cell surface expression of NG2, a biomarker associated with MLL gene fusions.
- The most basic level of DNA organization consists of the folding of DNA into 50-100 kb loops, which are attached at their bases to the nuclear matrix. The matrix attachment regions (MARS) of DNA contain Topo II consensus sequences and mediate the binding of DNA loops to the nuclear matrix. Thus, our results provide evidence that amonafide, by inhibiting the binding of Topo II to DNA, can induce the release of chromatin loops from the nuclear matrix. This results in DNA disorganization and apoptosis in the absence of extensive Topo II-mediated DNA cleavage.

Inhibition of Topo II Decatenation of kDNA



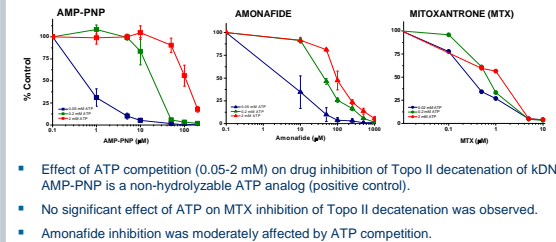
- Topo II decatenation of kDNA is a general measure of Topo II catalytic activity.
- Amonafide is a weak inhibitor of Topo II and does not group with the "classical" Topo II inhibitors. It has a higher IC₅₀ value of 184 µM compared to 3-12 µM for the classical Topo II drugs.

Comparative Effect on Topo II Binding to DNA



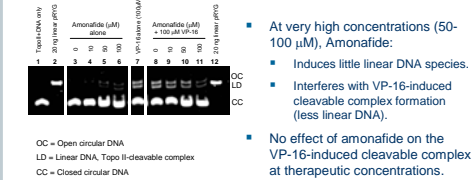
- Amonafide, DNR, and MTX are DNA intercalating agents, and interfere with Topo II binding to DNA.
- VP-16 does not intercalate into DNA, and has no major effect on Topo II binding to DNA.

Moderate ATP Competition Observed



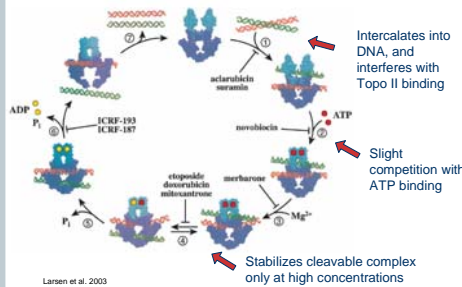
- Effect of ATP competition (0.05-2 mM) on drug inhibition of Topo II decatenation of kDNA. AMP-PNP is a non-hydrolyzable ATP analog (positive control).
- No significant effect of ATP on MTX inhibition of Topo II decatenation was observed.
- Amonafide inhibition was moderately affected by ATP competition.

Interferes with VP-16-Induced Cleavable Complex Formation

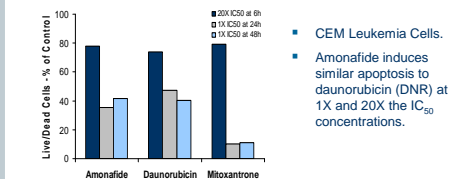


- At very high concentrations (50-100 µM), Amonafide:
 - Induces little linear DNA species.
 - Interferes with VP-16-induced cleavable complex formation (less linear DNA).
- No effect of amonafide on the VP-16-induced cleavable complex at therapeutic concentrations.

Topoisomerase II Catalytic Cycle Amonafide is a Multi-Step Topo II Inhibitor

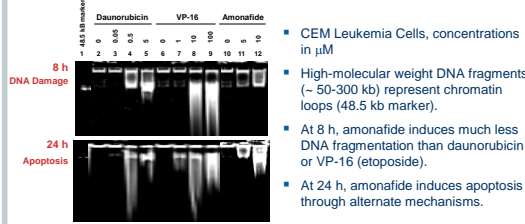


Similar Mitochondrial Apoptosis Observed



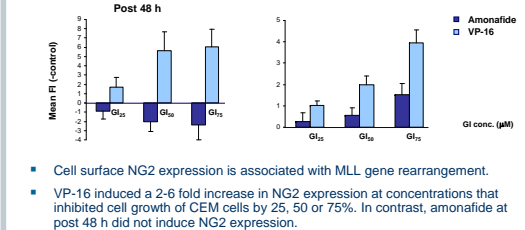
- CEM Leukemia Cells.
- Amonafide induces similar apoptosis to daunorubicin (DNR) at 1X and 20X the IC₅₀ concentrations.

Amonafide is Not a Potent Inducer of DNA Fragmentation But Does Induce Apoptosis



- CEM Leukemia Cells, concentrations in µM
- High-molecular weight DNA fragments (~50-300 kb) represent chromatin loops (48.5 kb marker).
- At 8 h, amonafide induces much less DNA fragmentation than daunorubicin or VP-16 (etoposide).
- At 24 h, amonafide induces apoptosis through alternate mechanisms.

Amonafide Induces Less NG2 Expression



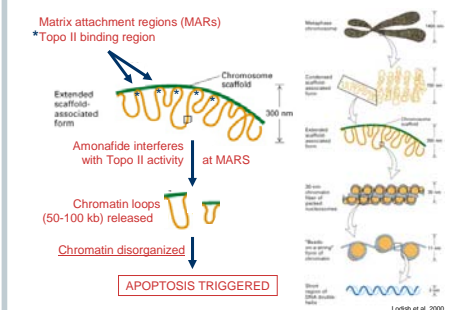
- Cell surface NG2 expression is associated with MLL gene rearrangement.
- VP-16 induced a 2-6 fold increase in NG2 expression at concentrations that inhibited cell growth of CEM cells by 25, 50 or 75%. In contrast, amonafide at post 48 h did not induce NG2 expression.

Assessment in Atypical MDR Cells

DRUG	CCRF-CEM	CEM/VM-1	Fold-Change	CEM/VM-1-5	Fold-Change
Amonafide	7.0	27	3.9	28	3.0
Daunorubicin (DNR)	0.09	1.7	18.9	3	33.3
VP-16	6	67	11.2	>>500	>100

- CEM/VM-1 and CEM/VM-1-5 are atypical MDR cell lines of CCRF-CEM; they have mutations in the ATP binding domain of Topo II with impaired ATP utilization and catalytic activity. VM-1-5 cells also overexpress P-glycoprotein (Pgp).
- Amonafide activity was only slightly decreased by cellular changes that induce a high degree of resistance to DNR and VP-16.

Proposed Mechanism: Amonafide Effect on Chromatin Disorganization



CONCLUSIONS

- The primary effect of Amonafide on the Topo II catalytic cycle is inhibition of Topo II binding to DNA.
- In contrast, VP-16 (etoposide), mitoxantrone (MTX), and daunorubicin (DNR) stabilize Topo II-DNA cleavable complex formation.
- Amonafide induces apoptosis in CEM leukemia cells without producing extensive DNA damage.
- Amonafide induces apoptosis through chromatin disorganization.
- Results suggest that Amonafide is much less potent in inducing MLL gene cleavage than etoposide.
- P-glycoprotein overexpression does not interfere with the anti-tumor activity of Amonafide.

