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TITLE: Therapeutic Conversion of Viability Promoting MCL1 to Death-Inducing Forms:
A Novel Strategy for Breast Cancer

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14. ABSTRACT The BCL2 family member MCL1 is expressed in breast cancer cells in its full-length, anti-apoptotic form. The goal of this project was to induce conversion of MCL1 to pro-apoptotic forms as a means of enhancing the death of these cells. The approach was identify means of inducing alternate splicing of MCL1 using antisense oligonucleotides, since splice variants are known to promote cell death rather than cell survival. We identified reagents and conditions that result in decreased expression of the antiapoptotic MCL1L protein and increased expression of proapoptotic splice variants. These splice-switching agents inhibited tumor cell growth, and did so in a more sustained fashion than siRNA directed against MCL1L. Reagents that induce splice switching of MCL1 thus have promise for further development for the treatment of breast cancer.					
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INTRODUCTION

The BCL2 family member MCL1 is expressed in breast cancer cells in its anti-apoptotic form, MCL1_L. The goal of this project was to induce conversion of MCL1 to the much shorter pro-apoptotic forms as a means of enhancing the death of these cells. The approach is to induce alternate splicing of MCL1, since splice variants are known to promote cell death rather than cell survival (1). MCL1 is encoded in 3 exons, which are normally spliced together to yield the full-length gene product¹. This full-length form of MCL1 contains the BCL2 Homology domains BH1, BH2, and BH3, which are important for the promotion of viability. Alternate splice variants of MCL1 cause cell death. For example, in MCL1_{s/ΔTM}, exon 1 is spliced to exon 3 with skipping of exon 2. This truncated variant contains BH3 (in exon 1) but not other BH domains. MCL1_{s/ΔTM} thus behaves like the death-inducing BH3-only members of this family. In this fashion, differential splicing can convert MCL1 from anti- to pro-apoptotic forms. The purpose of this grant was to devise approaches for targeting the conversion of the full-length anti-apoptotic form of MCL1 to pro-apoptotic forms, to assess this as a potential therapeutic strategy. Our approach was to use antisense oligonucleotides to attempt to induce alternate splicing of MCL1. We also monitored the effects of manipulating the expression of these MCL1 isoforms on cell viability. The induction of MCL1 splice-switching may represent a novel, feasible, and efficacious approach for targeted gene therapy for breast cancer.

RESEARCH ACCOMPLISHED

- I. Identify oligonucleotides and conditions that optimize alternative splicing, assess alternative splicing by PCR and Western blotting, and assess effects on cell growth and viability.
 - A. Conditions for the use of splice-site directed antisense oligonucleotides, and the effects of these agents on MCL1 mRNA and protein expression, have been determined.

We designed antisense phosphorothioate 2'-O-methyl oligonucleotides directed against the upstream and downstream splice sites flanking MCL1 exon 2, as described in the previous progress report. These reagents were tested in the NT2 cell line, where essentially the entire cell population can be transiently transfected using oligofectamine. Application of these antisense oligonucleotides at a concentration of 100 nM each resulted in an increase in the expression of MCL1_{s/ΔTM}, but not MCL1_L, mRNA (Fig. 1, upper two photographs). Optimization of the PCR

conditions for detection of MCL1_{s/ΔTM} indicated that the extent of the increase was at least 6-fold (Fig. 1, third photograph). Antisense oligonucleotides directed against sequence within intron 1 or intron 2, used as controls, did not produce a dramatic effect. This effect of splice site-directed antisense oligonucleotides was concentration-dependent, with extensive conversion to MCL1_{s/ΔTM} occurring at concentrations of 100-200 nM (Fig. 2A). An increase in the expression of proapoptotic forms of the MCL1 protein was accompanied by a decrease in the expression of the antiapoptotic MCL1_L protein (Fig. 2B), despite the fact that the mRNA for the latter was still in evidence. In sum, these reagents are capable of inducing a dual effect -- increasing proapoptotic, and decreasing antiapoptotic, forms of MCL1.

B. MCL1 splice-switching reagents have been shown to produce sustained inhibition of tumor cell growth.

Along with the above effects on expression of the various forms of MCL1, the splice site-direct antisense oligonucleotides inhibited tumor cell growth and viability (Fig. 3). This effect was sustained over the 3 day assay period with the 100 mM concentration (Fig. 4); here, although the tumor cell population was not completely killed, cell number did not increase. In this respect, the splice-site directed reagents provided an advantage over more standard short interfering RNA (siRNA) treatment. siRNAs reduce the expression of viability-promoting MCL1_L (Fig. 5A) but are not designed to cause conversion to death-inducing forms. Furthermore, inhibition of expression of MCL1_L was transient (Fig. 5B), and inhibition of tumor cell growth was likewise transient such that regrowth was apparent from day 1 to day 3 (Fig. 6) even at the highest siRNA concentration tested. Thus, the results with splice switching reagents are very encouraging as these were more effective than siRNAs in this *in vitro* system.

C. Expression of different forms of MCL1 has been monitored in breast cancer cells.

In addition to the above studies aimed at inducing splice-switching with antisense oligonucleotides, we further examined expression of the various forms of MCL1 in breast cancer cells. This was done in the presence or absence of a variety of agents active in these cells. We previously observed that many breast cancer lines express only low or barely detectable levels of the proapoptotic forms of the MCL1 protein, MCL1_{s/ΔTM} and MCL1_{exon1}. Further investigation has revealed that detectable expression is present in some lines, as seen in MCF-7 (Fig. 7A). Interestingly, exposure of these cells to beta-estradiol plus EGF resulted in decreased expression of MCL1_L and increased expression of MCL1_{s/ΔTM}. The proapoptotic forms were not detectable or only very faintly detectable in Western blots with MD-MBA231 and MD-MBA468 (Fig. 7A), although very low levels of the MCL1_{s/ΔTM} mRNA were present in these lines (not shown). Proapoptotic forms were readily detectable in MD-MBA436 (provided by Dr. K. Keyomarsi), which also expressed MCL1_{ATG1} (a product representing initiation at MCL1 Met 111). Proapoptotic forms were not detected in SKBR3 cells, but exposure of these cells to TPA resulted in increased expression of MCL1_L and MCL1_{s/ΔTM} (Fig. 7A). In MCF-7 cells, the chemotherapeutic agents paclitaxel and etoposide cause increased expression of MCL1_{exon1} along with decreased expression of MCL1_L (Fig. 7B). Overall, exogenous agents can affect the expression of the different forms of MCL1 in breast cancer cells, and some agents active in this disease may act, in part, through inducing changes in the expression of the various splice variants of MCL1.

II. Apply nucleic acid reagents as developed above *in vivo*.

Because splice switching *in vitro* was not achieved easily, we focused our efforts on optimizing this in cells in culture as it would not make sense to apply a non-optimized approach *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS

1. Splice switching antisense oligonucleotides, when applied at concentrations of 100-200 nM, were found to result in decreased expression of the antiapoptotic MCL1_L protein, and increased expression of proapoptotic forms of MCL1.
2. The splice switching reagents were found to cause sustained inhibition of cell growth. In this respect, they demonstrated a more pronounced effect than MCL1 siRNAs, which caused only transient inhibition of expression of MCL1_L with no effect on proapoptotic forms, and resulted in only transient inhibition of cell growth.
3. Some currently available agents were found cause decreased expression of antiapoptotic MCL1_L and increased expression of pro-apoptotic forms in breast cancer cells, suggesting that this is one action of these agents. This provides a further rationale for continued investigation of the effects of the splice switching reagents by themselves, to determine whether they have an improved profile of efficacy, potency, and potential for side effects as compared to conventional agents.

FUNDING APPLIED FOR

BC050579: Targeted Therapy of Breast Cancer by Splice-switching of Anti-apoptotic MCL1 to Death-inducing Form

BC053432: Ultrasound-activated Micro/Nanobubbles for the Delivery of siRNAs that Target Oncogenes and Survival-promoting Genes Expressed in Breast Tumors

Personnel Receiving Salary From This Grant:

Ruth W. Craig, Principle Investigator

Samuel L. Casella, Research Technician

CONCLUSIONS

The results of these studies are extremely encouraging in that the splice-switching reagents were found to induce both downregulation of antiapoptotic MCL1_L, and increased expression of short pro-apoptotic MCL1 variants. Probably because of these dual effects, the introduction of splice-switching reagents (by lipofection) resulted in inhibition of tumor cell growth that was more sustained than that obtained with MCL1 siRNAs. The approach of inducing splice switching therefore shows promise for application to breast cancers exhibiting abundant expression of MCL1_L.

REFERENCES

1. Bingle CD, Craig RW, Hanks BM et al. Exon skipping in MCL1 results in a BCL2 homology domain 3 (BH3)-only gene product that promotes cell death. J Biol Chem 2000; 275: 22136-22146.

Fig. 1. Increase in Expression of MCL1_{s/ΔTM} Induced by Splice Site-directed MCL1 Oligonucleotides

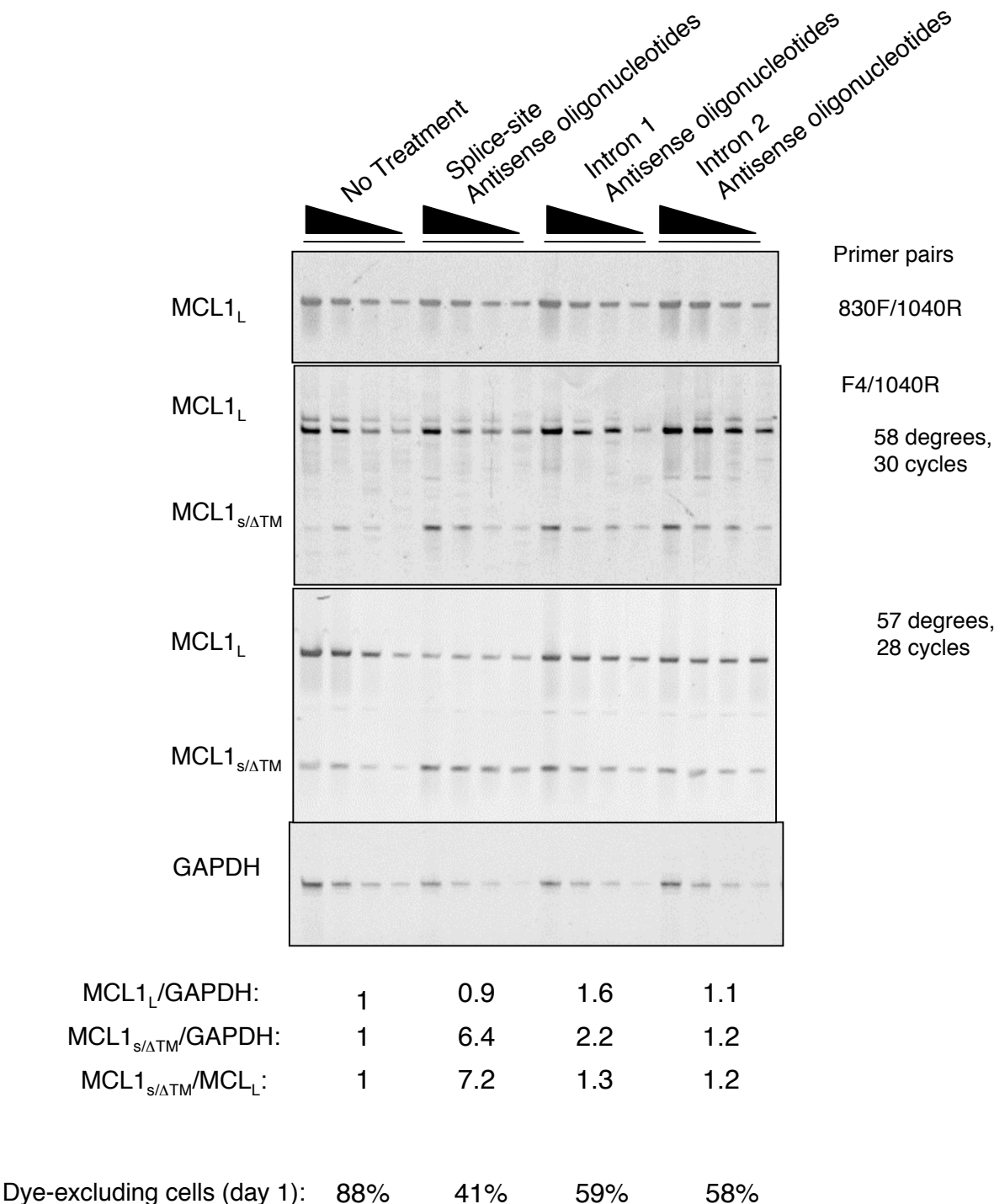
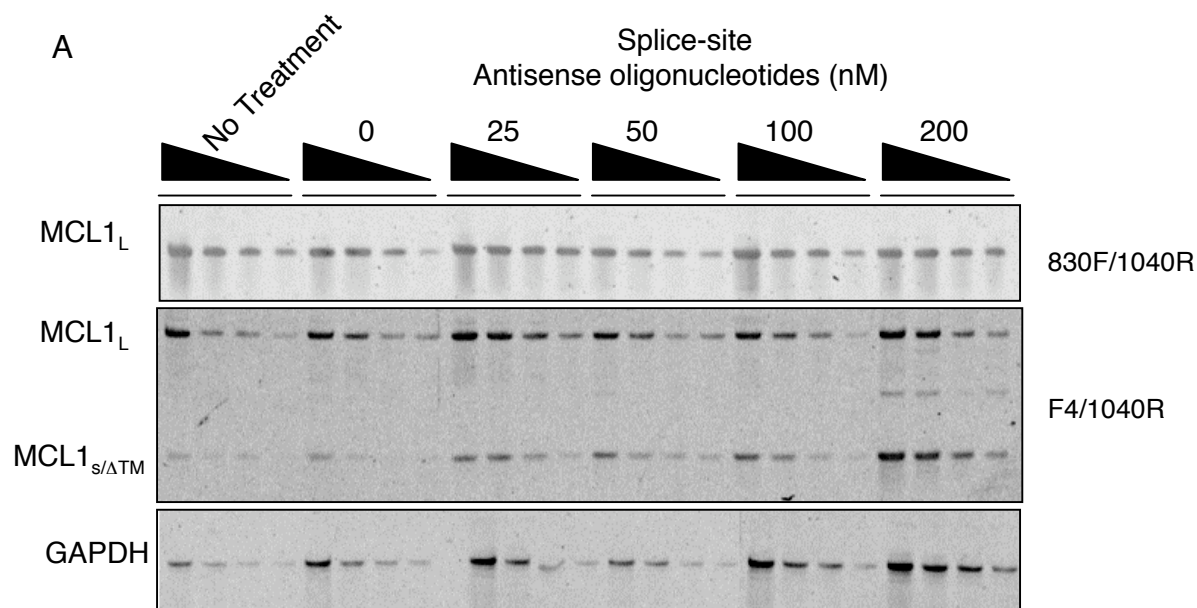


Fig. 2. Splice-site Directed Antisense Oligonucleotides Increase Expression of Proapoptotic MCL1 Splice Variants and Decrease Expression of the Antiapoptotic MCL1_L Protein



B

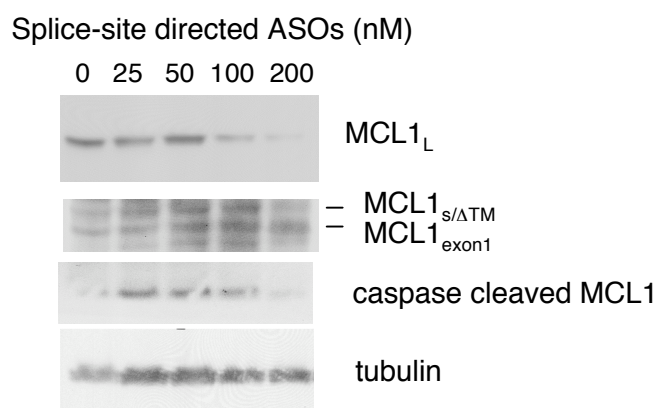


Fig. 3. Splice-site Directed Oligonucleotides Promote Cell Death

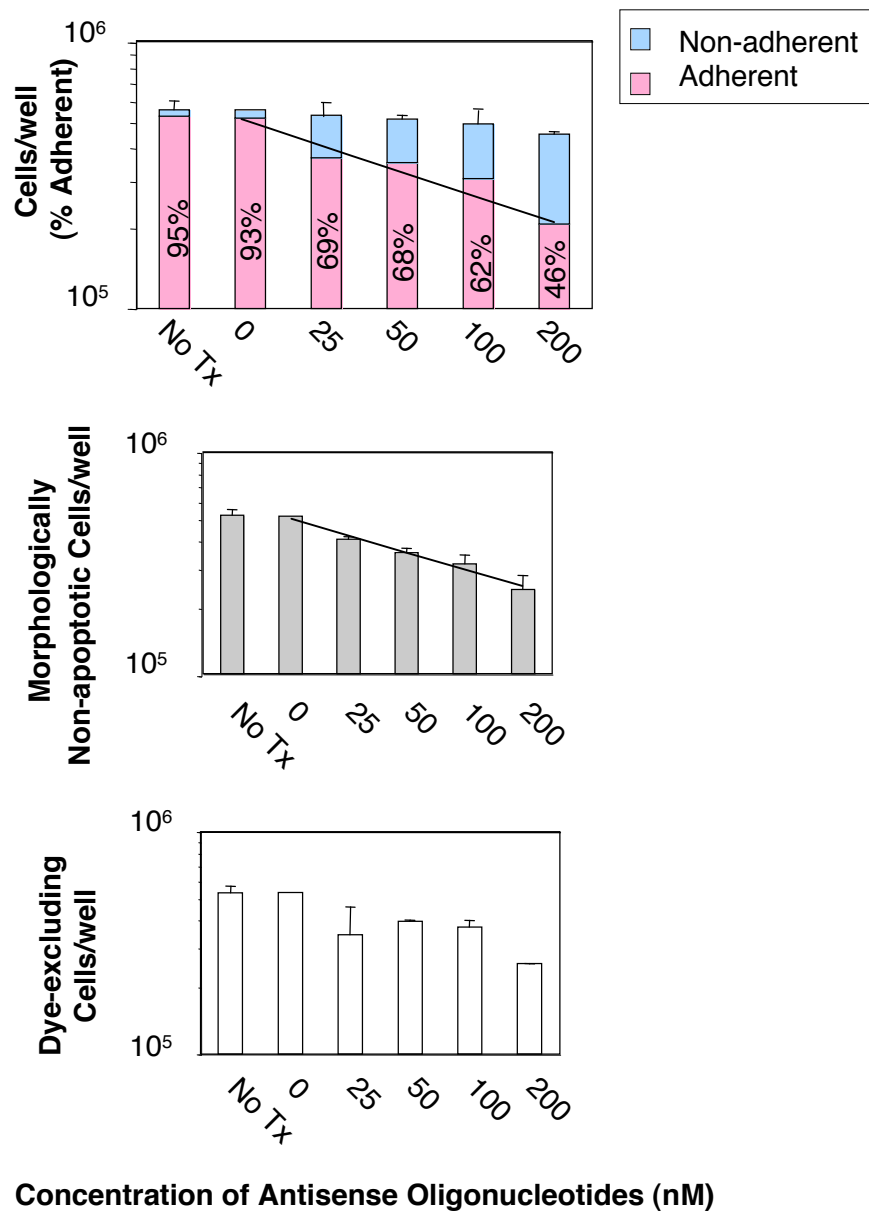


Fig. 4. Splice-site Directed MCL1 Oligonucleotides Produce Sustained Inhibition of Tumor Cell Growth

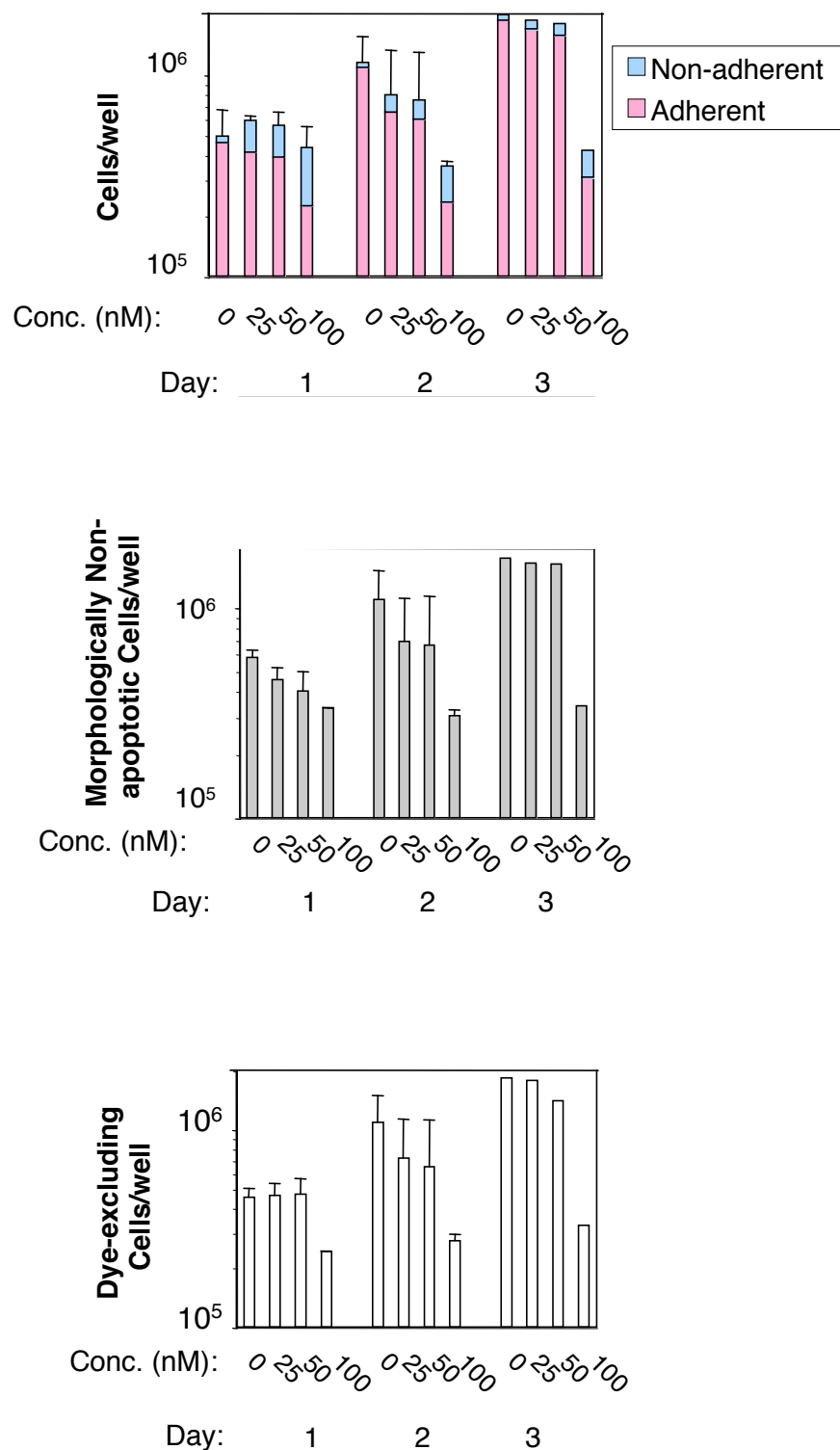
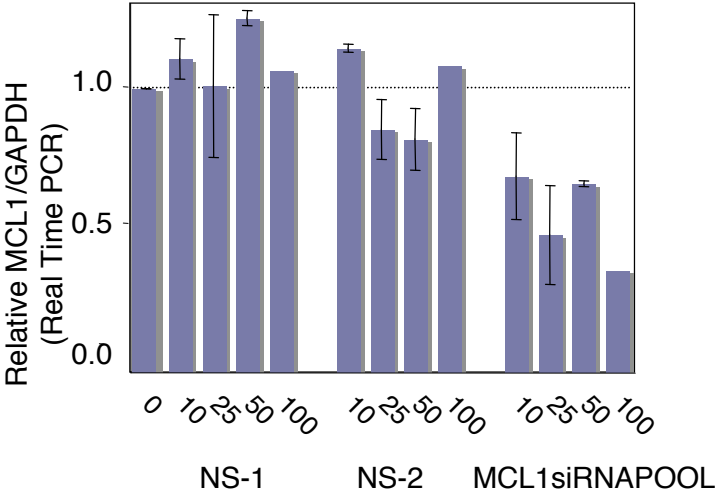


Fig. 5. MCL1 siRNA Produces only Transient Suppression of MCL1 Expression

A



B

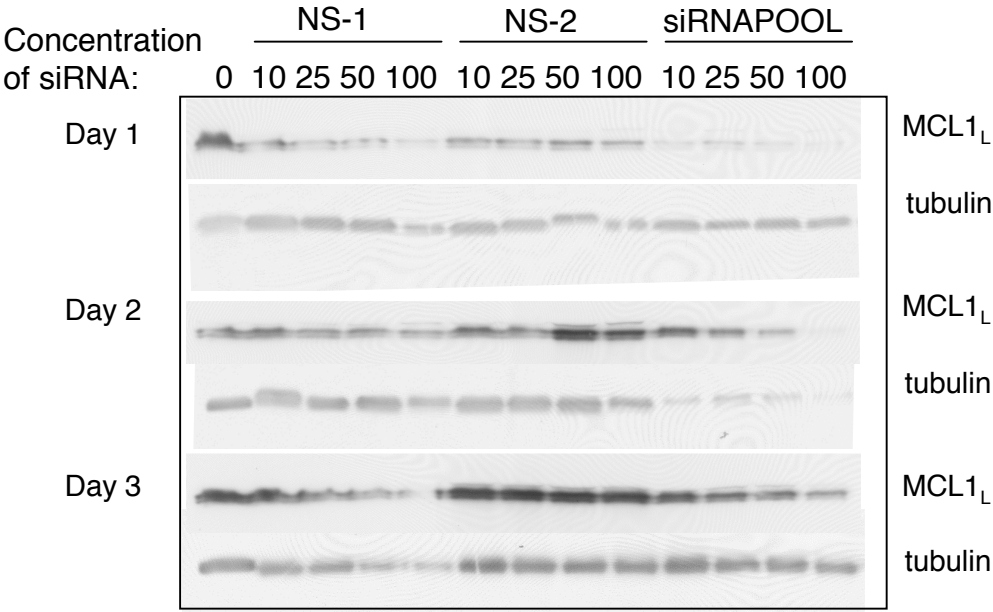


Fig. 6. MCL1 siRNA allows Tumor Cell Outgrowth

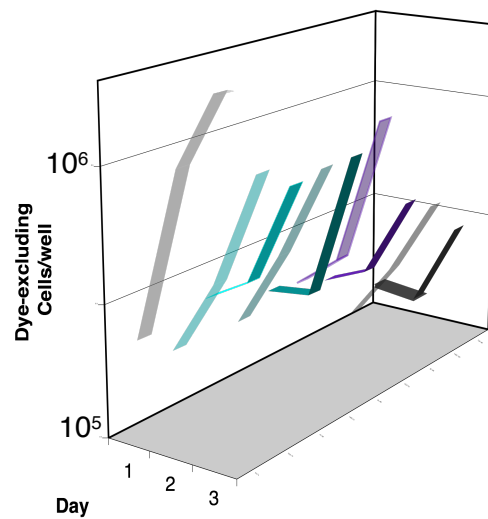
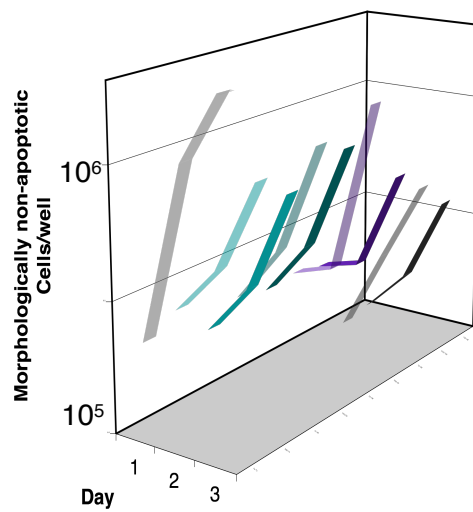
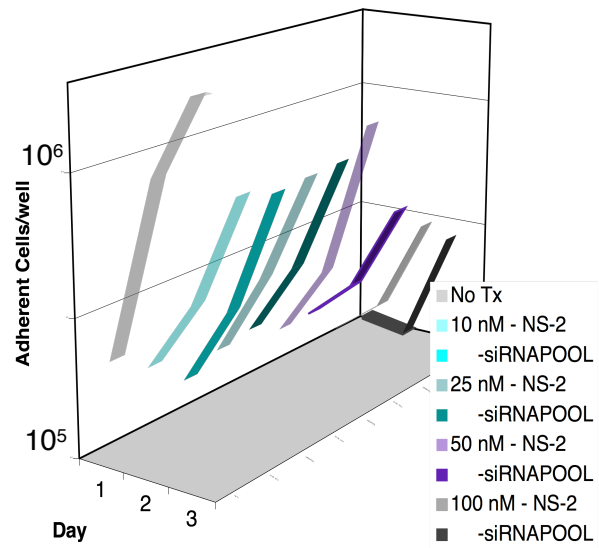


Fig. 7. Agents Used in Breast Cancer Decrease Expression of MCL1_L and Increase Expression of Proapoptotic Forms

