Abstract. Background: Cyclin D1 overexpression exists in multiple types of cancer and is a potential chemopreventive or therapeutic target. Materials and Methods: Non-small cell lung cancer and mesothelioma cells were incubated with antisense oligonucleotides (ASO) to cyclin D1 (CD1) and evaluated for effects on cellular proliferation, apoptosis, expression of cell cycle-specific proteins, and protein phosphorylation states. Results: ASO to CD1 inhibited proliferation of non-small lung cancer cells and mesothelioma cells. ASO induced apoptosis as determined by TUNEL assay. Western blot analysis of cell lysate showed that ASO inhibited the de novo synthesis of CD1, CD3, and CDK2 in multiple cell lines. Immunoprecipitation and immunoblotting with phosphoantibodies demonstrated that CD1, CD3, and CDK2 exist in a phosphorylated state. Conclusion: The work demonstrates that non-small cell lung cancer and mesothelioma cells respond to ASO-mediated cellular growth inhibition. These findings make ASO to CD1 attractive as a potential therapeutic for mesothelioma and non-small cell lung cancer.

Complexes of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors are implicated in the various stages of cell cycle progression in mammalian cells (1). CD1, CD2, and CD3, along with CDK4 and CDK6 have been established to play a key role for the cell to enter into, and progress through, the G1 phase. Once bound to CD1, CDK4 or CDK6 phosphorylates retinoblastoma protein (pRb) (2-6). The activity of pRb is controlled by both inactivating phosphorylation and physical association with growth-promoting factors, and it must be phosphorylated for the repression and release of E2F family of transcription factors for the G1 to S transition to occur (7). CD1 has also been described as a protooncogene since cancerous cells exhibiting uncontrolled cell growth exhibit overexpression of CD1(8-9). Increased expression of CD1 has been documented in prostate cancer, squamous cell carcinoma, and colorectal cancer (10). CD1 antisense oligonucleotides (ASO), alone or in conjunction with other therapeutics/agents, have been reported to inhibit growth of cells from various cancer cells, e.g. colon (11), lung (12), pancreas (13), squamous cell carcinoma (14, 15), and hepatocellular carcinoma (16), to name a few. There is no report of any growth inhibition of mesothelioma cells by ASO to cyclin D1; hence, this work was undertaken targeting pleural mesothelioma cells along with non-small cell lung cancer cells by ASO to determine growth inhibition and to elucidate possible mechanisms of action of depleted CD1 on other cyclin proteins.

Materials and Methods

Production of ASO. CD1 ASO 5'-GGA GCT GGT GTT CCA TGG-3' was complementary to the translation start site of the CD1 cDNA, and sense oligomer 5'-CCA TGG AAC ACC AGC TCC-3' (SO) representing nucleotide 208-225, was used as control. Both sequences were synthesized at the University of Minnesota Oligonucleotide and Peptide Synthesis Facility.

Reagents. Various antibodies used included mouse anti-CD1, CD2, CD3, cyclin E, CDK2, (Invitrogen, Carlsbad, CA, USA); anti-actin (Ambion Anti, Foster City, CA, USA; Cell Signaling Technology, Danvers, MA, USA); anti-CDK4 (Invitrogen); rabbit anti-pRb, -CD1, -CD2 (Epitomics, Burlingame, CA, USA); antiphosphoserine, -phosphotyrosine, -phosphothreonine, -phosphatases (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and horse radish peroxidase-conjugated goat antimouse/rabbit antibodies (Thermo Scientific, Rockford, IL, USA). All reagents used were analytical grade.

Cell culture. Non-small lung cancer cells (H661 and H2030), mesothelioma cells (H2373, H2596, and H2461), and additional non-small cell lung cancer cells (2009, Rb negative control line)
(American Type Culture Collection) were maintained at 37°C in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and 1x concentration of an antibiotic/antimycotic reagent (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO₂.

**Effect of ASO on cell proliferation.** Live cells as determined by Trypan blue dye exclusion assay (3000 cells/well) were plated in 96-well plates (3000 cells/100 μl medium/well). On the second day, the medium was aspirated and the cells were transfected with oligofectamine 2000 with or without ASO or SO at different concentrations for 4 h in serum-free medium followed by the addition of fetal calf serum (50 μl of RMPI medium containing 30% fetal calf serum added to 100 μl of transfected cell medium). After 72 h of incubation, 10 μl of solution from Cell Counting Kit-8 (Dojindo Laboratories, Kunamoto, Japan) was added to each well and plates were re-incubated for another 2 h and read at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The reduction in optical density represented the reduction in mitochondrial succinate dehydrogenase activity, hence the reduction in surviving cell numbers.

**In situ apoptosis detection.** The type of cell death (necrosis/apoptosis) was evaluated via TUNEL assay using the commercial ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Chemicon International, Temecula, CA, USA). The method utilizes conjugation of digoxigenin- nucleotides with free 3’-OH termini of fragmented DNA strands, and (via terminal deoxynucleotidyl transferase) the conjugant reacts with anti-digoxigenin antibodies. The bound peroxidase reacts with its substrate 3,3′-diamino benzidine (DAB) that generates chromogenic DNA.

Cells were grown in 16-well chamber slides (3000 cells/well) as described in the proliferation assay section. The cells were treated with 1 μM of ASO as well as nonspecific oligonucleotides as a control. After 72 h of incubation, media were aspirated and replaced with 200 μl of solution from Cell Counting Kit-8 (Dojindo Laboratories, Kunamoto, Japan) was added to each well and plates were re-incubated for another 2 h and read at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The reduction in optical density represented the reduction in mitochondrial succinate dehydrogenase activity, hence the reduction in surviving cell numbers.

**Immunoprecipitation.** Cells were grown as above to 100% confluence. After 24 h media were discarded and cells were washed with cold PBS. Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, containing 1x protease inhibitor mixture and 1x phosphatase inhibitor mixture; Santa Cruz Biotechnology) and left on ice for 10 min. Protein content was determined by Bio-Rad protein DC assay. Cell lysates were diluted to 1.0 mg/ml. Five micrograms of anti- CD1, CD2, CD3 and CDK2 antibodies were added to each of 1 ml of lysate from different cell lines. The tubes were kept on a rotatory shaker for 1 h in the cold room. Fifty microliters of protein A/G sepharose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 10 min and the supernatant was discarded. Theagarose gel beads were washed three times in Tris HCl buffer (10 mM, pH 7.4) containing 0.05% Triton X-100, then incubated with shaking (at 4°C for 10 min) and centrifuged at 10000 × g. Sepharose beads were resuspended in 2x Laemmli sample buffer, heated at 95°C for 5 min and processed for immunoblotting using phosphoantibodies as described above.

**Results**

The effect of ASO on cellular growth is shown in Figure 1. All the cell lines, except for H2030 and H2596, exhibited 48-95% inhibition of cellular growth upon treatment with 10 μM of ASO, while no change in growth pattern was observed after treatment with the SO (data not shown). At a lower concentration (1.0 μM), three of the cell lines showed 43-94% inhibition. The fastest growing cell line (H661) exhibited maximum inhibition (94%), while slowest growing cell line (H2373), had the minimum inhibition (43%). Cell line H2461 exhibited 52% inhibition.

ASO-treated cell lines exhibited condensed fragmented nuclei covered with membrane, while the control cells grew normally with dividing nuclei apparent at various stages.
lysate of cells treated with ASO show that ASO inhibited the
kDa), CD2 (34 kDa), CD3 (34 kDa), β-actin (42 kDa), pRb
antibodies i.e. CDK2 (33 kDa), CDK4 (34 kDa), CD1 (36
approximately in range as outlined by the vendor of the
Western blot analysis showed that all the proteins reacting
with their respective antibodies showed up as discrete bands
approximately in range as outlined by the vendor of the
antibodies (PP1) increased the intensity of bands in ASO-
straining fronts for CD1 and CD2 as compared to all other bands in all
in H661 and H2461, and to a lesser extent in H2373 cells. A similar pattern
was observed for CD3, while there was no apparent change in
in cell line H661 remained unaffected but there was
a 50% decrease in H2461 cells as well as in H2373 cells.
expression at 34 kDa, though it appeared to decrease in
ASO-treated H661 cells, was difficult to evaluate in other cell
lines. It also appeared to exist as a dimer (68 kDa) as well as
degraded. The major degraded products moved along with the
dye front (10% gel). Along with the right-sized bands, we
observed slow moving trailing bands of CDK2, CD1, and CD3
that tended to disappear upon ASO treatment selectively in
different cell lines. In the CD1 immunoblot there is complete
disappearance of this slow moving band from almost all the
cell lines; even the normal band is hardly visible. The same blot
treated with anti-CD2 shows complete disappearance of the slow
moving band in H2461 and H2373 cells; however, in
H661 cells it remained unaffected. The β-actin control on the
same blot did not show any slow moving part. The CD3
immunoblot appeared like that of CD1 except that ASO-treated
661 cells had a lesser amount of both slow moving bands as
well as the band front. ASO-treated 2373 cells had faster band
fronts for CD1 and CD2 as compared to all other bands in all
of the blots. Stripping the blot and reprobing with phosphatase
antibodies (PP1) increased the intensity of bands in ASO-
treated cell lysate as compared to control.
There was no change in the concentration pRb in cell line
H661 and H2373 while in H2461 cells a high molecular
weight, trailing, phosphorylated band was observed in
control, which disappeared upon ASO treatment. The Western
blot results of lysate from normally growing cells
immunoprecipitated with antibodies to CD1, CD2, CD3,
CDK2 and immunoblotted with anti-phospho antibodies are
shown in Figure 4. All the immunoprecipitated proteins
showed the existence of serine and threonine phosphorylation
of CD1, CD2, CD3, and CDK2 and tyrosine phosphorylation
of CD3 and CDK2. The results in the case of CD1 and CD2
tyrosine phosphorylation appear to be inconclusive because
of the presence of very close double bands.

Discussion
Various inhibitors of CDKs and their complexes have been
reported to inhibit cellular growth and have been implicated
as cancer therapeutics or chemopreventive agents, (17-35)
and several are in clinical trials (36). ASO to CD1 alone or in
conjunction with other therapeutics/agents inhibits growth of
various cell lines (10-16). We observed that CD1 ASO have
varied effects on cellular proliferation inhibition of non-small
lung cancer cells as well as pleural mesothelioma cells. The
apparent reduction in cell number following treatment with
ASO has been attributed, at least partially, to programmed
cell death as determined by in situ peroxidase apoptosis
detection. Targeting CD1 not only depleted CD1 but also
affected the expression of CD2, CD3, CDK2. Slow moving
bands that appeared in blots of CD1, CD3, and CDK2 in
normal growing cells almost disappeared or decreased in
intensity in ASO-treated cells. Ruling out the low likelihood
of any other posttranslational modifications because of the
intracellular site of these proteins and absence of other
consensus sequences such as NXG/S, the stripped blot was
reprobed with antiphosphatase antibodies. The result showed
elevation of phosphatase level in the ASO-treated cells,
thereby implicating the role of phosphatases in
dephosphorylation of these proteins leading to inhibiting the
cell division, and hence, the cell number. CD1 splice variant
CD1b does not have the T286 phosphorylation site required
for nuclear transportation (37). Phosphorylation of CD1
[T286] has been implicated in its proteosomal degradation
(38), while degradation of CD3 has been reported to be
independent of phosphorylation at T283 (39). However in
our experiment it is obvious that overexpression of protein
phosphatases during the CD1 down-regulation may lead to
dephosphorylation of phosphorylated CD1 and CD3 and
CD2. This effect may be responsible for blocking the cell
cycle from entering into the S phase from the G1 phase.
Since in our experiment CD1 synthesis was blocked at the
transcriptional level, we cannot label it as proteosomal
degradation that has been targeted, as reported earlier (33-
34). Blocking CD1 synthesis or enhancing its proteosomal
degradation both prevent cell entry into S phase, where
dephosphorylation may play an important role. Cyclin D1
has been reported to initiate the phosphorylation of Rb that
disrupts the Rb-mediated transcriptional repression leading
the cell to enter into the S stage (37). Phosphorylation of
cyclins, as those of CDKs, may be an essential requirement
for this phosphorylation in a co-operative chain reaction.
We utilized the NetPhosK server (http://www.cbs.dtu.dk/
services/NetPhos/) to identify various predicted phosphorylation
sites that have been noted in cyclins and CDKs (40), and the data
has been tabulated as below (Table 1). Cell lysate
immunoprecipitated with anti-CD1, -CD2, -CD3, and -CDK2
and immunoblotted with phosphoantibodies showed serine and
Figure 1. Effect of cyclin D1 antisense oligonucleotide (ASO) on cancer cells.

Figure 2. Microphotograph of mesothelioma and non-small lung cancer cells treated with cyclin D1 ASO. Cells were grown in 16 well chamber slides and treated with 1 μM of ASO (A, B, C) as well as non-specific oligonucleotides as a control (D, E, F), respectively, for 72 hrs [Light microscopy].
Figure 3. Western blot analysis of lysate of cells (661, 2461, 2373) treated with cyclin D1 antisense oligodeoxynucleotides (A) and control (B). Cellular protein (25 μg) resolved by SDS-PAGE (10% gel), blotted onto PVDF membrane and immunoblotted with various antibodies. CD1, cyclin D1, CD2, cyclin D2, CD3, cyclin D3, CE, cyclin E, CDK4, cyclin-dependent kinase 4, pRb, retinoblastoma protein, PP1, phosphatase antibodies, kDA, kilodalton.
threonine as well as tyrosine phosphorylation on CD1, CD2, CD3, instead CDK2. Although there is no predicted phosphorylation site for tyrosine in CD1 as per its sequence analysis (netPhos), we did observe a band at the appropriate site. This band may represent a CD1 co-immunoprecipitated protein other than CD1 that may have tyrosine phosphorylated sites. While CD2 has an equal number of predicted phosphorylation sites to those of CD1, there appeared to be a single band of CD2 in all the cell lines treated (H661, H2373, H2461). It could be possible that a) there is only weak phosphorylation and the bands remained non-highlighted in the blot; b) CD2 may be phosphorylated as soon as it is synthesized; or c) glycosylation may have prevented phosphorylation at these sites, a mechanism named yin-yang regulation. However, its role as phosphorylated or unphosphorylated remains to be elucidated. Although CDK4, as well as cyclin E, has a fairly large number of predicted phosphorylation sites, they were not included in the immunoprecipitation/Western blot study, since there was no apparent change in cyclin E in any of the cell lines treated with ASO. Anti-CDK4 showed a number of bands in the blots where the data from immunoprecipitation would have been inconclusive.

Table I. Predicted phosphorylation sites on the cyclin and cyclin-dependent kinase (CDK). The predicted phosphorylation sites of cyclins and CDK were determined by loading their deduced amino acid sequence onto the NetPhos server: (http://www.cbs.dtu.dk/services/NetPhos/).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Serine</th>
<th>Threonine</th>
<th>Tyrosine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>14</td>
<td>5</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>12</td>
<td>4</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>CDK2</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>CDK4</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>

There are several families of kinases involved in phosphorylation of these various sites (http://www.cbs.dtu.dk/services/NetPhos/K). Among these, protein kinase C (PKC) has the highest score for phosphorylation prediction for CD1 (0.83, S55), CD3 (0.87, S263), cyclin E (0.87, S405), CDK2 (0.87; S206), and CDK4 (0.89, S36); while protein kinase A
(PKA) involvement (0.71) at S276 is predicted in CDK2. Although the phosphorylation of threonine and its role in CD1 (T286) and CD3 (T283) is documented (37-39), the role of phosphorylation at the serine that accounts for the highest scores for predicted phosphorylation via PKC lacks evidence. While there are plenty of predicted sites for threonine, there are no reports on the predicted serine- or tyrosine-phosphorylation sites. Phosphorylation inhibition or dephosphorylation of these cyclin proteins would appear to have potential as anticancer therapy.

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**Conflict of Interest**

The Authors have no conflict of interest.

**References**


