Propagation of Waldenström’s Macroglobulinemia Cells In Vitro and in Severe Combined Immune Deficient Mice: Utility as a Preclinical Drug Screening Model

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Waldenström’s macroglobulinemia (WM) represents an indolent incurable human B-cell tumor. We have successfully established a permanent cell line, WSU-WM, without growth factors or viral transformation, from the pleural effusion of a 60-year-old man with IgM LM. Phenotypic characterization of WSU-WM shows IgMx and expression of other B-cell markers. Karyotypic analysis shows a male chromosome complement with several clonal aberrations, including t(8;14)(q24;q32). Molecular characterization shows deletion of K and rearrangement of V light chain genes indicating a class switching. Both the secretory and membrane (mu) components of IgM are expressed. In addition, the breakpoint on q24 is downstream of exon 3 of the c-myc oncogene. WSU-WM grows in liquid culture and soft agar. When cells were injected subcutaneously in immune deficient mice, six of seven SCID mice developed subcutaneous tumors as opposed to three of seven in the athymic nude mice. When a WM-WM SCID tumor was passaged in vivo in the SCID mice, the take rate was 100%. This xenograft model and a soft agar disk-diffusion assay were used to test the efficacy of standard chemotherapy agents against this tumor in vivo and in vitro, respectively. The cell line and the assays described herein can be used as a model to facilitate the discovery of new therapeutic agents or modalities for this disease.

CASE REPORT

In 1980, an African-American man presented with lymphadenopathy and splenomegaly and had a diagnosis of WM made at age 51. Laboratory studies were significant for the presence of normocytic anemia and a monoclonal IgM monoclonal serum protein, now known as Waldenström's macroglobulinemia. His disease course was marked by multiple relapses controlled by different chemotherapy agents and plasmapheresis for hyperviscosity syndrome. In May 1990, the patient developed malignant right-sided pleural effusion containing IgMx clonal lymphocytes from which the WSU-WM line was established. The patient was treated with multiagent chemotherapy, including cyclophosphamide and doxorubicin, which showed some disease control. However, 3 months later, he presented with third nerve palsy due to meningeal lymphomatosis related to the underlying disease. The patient died after his last discharge from Harper Hospital on August 25, 1990. His last immunoelectrophoresis of the serum in March 1990 continued to show an IgM monoclonal spike with an IgM level of 4,650 mg/dL.

MATERIALS AND METHODS

Establishment and maintenance of the cell line. Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation of the malignant pleural effusion obtained in May 1990. Cells were immediately washed twice with Hanks’ Balanced Salt Solution (HBSS) and resuspended at a concentration of 5 x 10^6 cells/mL in RPMI-1640 medium containing 20% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin G, and 200 μg/mL streptomycin. Five- to 7-mL aliquots were then cultured in each 25-cm² flask and incubated in a...
humidified 5% CO₂ atmosphere at 37°C. The medium was partially replaced with a similar fresh preparation every 3 to 6 days as indicated by change in the color of media to yellow (acidic pH) until a steady cell growth was noted. After establishment, cells were maintained in RPMI-1640 medium with 10% FBS. Throughout the entire incubation period, no mitogens, growth factors, or EBV was added to the cell culture system.

*Plating efficiency.* WSU-WM cells in single cell suspension were plated in 0.3% agar, supplemented with 20% FBS at low cell density (20 cells/cm²) in 35-mm plastic Petri dishes as previously described. The dishes were then incubated in a 5% CO₂ incubator at 37°C. The number of colonies formed were counted at day 14. Plating efficiency equaled the number of colonies formed divided by the number of cells plated multiplied by 100.

*Immunophenotyping of the cell line.* Surface phenotypes of the primary pleural fluid cells and the established cell line were determined using indirect immunofluorescence staining and flow cytometry techniques as routinely performed in our laboratory. The same techniques were also applied in the phenotyping of the subcutaneous xenografts after mechanical dispersion of the tumors to single-cell suspension. For cytoplasmic Ig (CiG) staining, cells were fixed with 0.25% saponin in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 5 minutes before the staining, as previously described. The sources of the monoclonal antibodies used in this study were as follows: anti-CD10 (CALLA), CD11 (MO1), CD19 (B4), CD20 (B1), CD21 (B2), CDw13 (MY7), CDw14 (MO2), and CD33 (MY9) from Coulter Immunology (Hialeah, FL); CD37 (IoB1) from AMAC, Inc (Westbrook, ME); and the remainder were from Becton Dickinson Immunodiagnostics (San Jose, CA). Flow cytometric analysis was performed using the Coulter Epics 753 or the Becton Dickinson FACS 440.

*EBV nuclear antigen (EBNA).* Assay for EBNA was kindly performed by the EBV laboratory at the Joseph Stoke Research Institute (Philadelphia, PA).

*Cytogenetic studies.* The established cells in log phase of growth were exposed to colcemid (0.05 µg/mL) for 1 hour. Cells were then harvested and treated with 0.075 mol/L potassium chloride (KCl) for 15 minutes before they were fixed with 3:1 methanol:acetic acid. Slides were prepared by dropping cell suspension onto ethanol-cleaned glass microscope slides. Q- and G-banding methods were applied in the staining of chromosomes. From each culture, 20 to 30 cells were analyzed microscopically and karyotypes prepared.

*DNA and RNA extraction and analysis.* High molecular weight DNA was prepared by standard techniques from the WSU-WM cell line and normal placenta. DNA was digested with EcoRI, HindIII, and XhoI, fractionated on a 0.7% agarose gel, and transferred to Nytran nylon filter. Hybridization and autoradiography were performed as previously described. Probes of human c-myc exon 3 (Cla I-EcoRI fragment) and α and λ light chain constant region genes obtained from American Type Culture Collection (ATCC; Rockville, MD) were gel purified and labeled by random priming with α-32P.
deoxyctydine triphosphate (dCTP). RNA was isolated as previously described.

Polymerase chain reaction (PCR). Reverse transcriptase PCR was performed on 1 µg of total RNA per manufacturer’s directions using avian leukosis virus (ALV) reverse transcriptase and Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT), with 30 ng of each downstream antisense primer used 20 µL per cDNA reaction. PCR conditions were 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes. Primers were based on published sequence for the μ constant region gene: μ membrane antisense, 5’ CTTGAACAAGGTGACGGTGG 3’; μ secretory antisense, 5’ CTGTGTCGGACATGACCAGG 3’; μ sense, 5’ GCATCTGCGAGGATGACTGG 3’. Analysis of 8 µL of

Table 1. Phenotypic Characterization of the Waldenstrom Tumor

<table>
<thead>
<tr>
<th>Source</th>
<th>Pleural Fluid*</th>
<th>Established WSU-WM Line in Culture</th>
<th>WSU-WM SCID-Xenograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dates</td>
<td>5/29/90</td>
<td>9/6/90†</td>
<td>7/1/91</td>
</tr>
<tr>
<td>CD19 (B4)</td>
<td>2.9</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>CD20 (B1)</td>
<td>0</td>
<td>94.5</td>
<td>34.8</td>
</tr>
<tr>
<td>CD22 (LEU14)</td>
<td>ND</td>
<td>17.8</td>
<td>47.3</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>17.5</td>
<td>97.4</td>
<td>87.5</td>
</tr>
<tr>
<td>LEU10</td>
<td>ND</td>
<td>38.5</td>
<td>40.6</td>
</tr>
<tr>
<td>CD10</td>
<td>ND</td>
<td>55.4</td>
<td>49.6</td>
</tr>
<tr>
<td>SlgM</td>
<td>30</td>
<td>16.4</td>
<td>ND</td>
</tr>
<tr>
<td>Slgκ</td>
<td>26.2</td>
<td>5.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Slgλ</td>
<td>1.1</td>
<td>12.4</td>
<td>73.6</td>
</tr>
</tbody>
</table>

Numbers represent percent positive cells for the given markers.
Abbreviation: ND, not done.
*Note that approximately 60% of lymphocytes in the primary pleural fluid were normal T cells (see text).
†Other markers tested initially but not shown are: CD5 (Leu1), 5.1%; CD4 (Leu3), 4.2%; CD11c (LeuM5), 4.5%; CDW14 (MO2), 3.3%; BL7, 3.3%; CD21 (B2), 3.1%; CDW13 (MY7), 4.1%; CD33 (MY9), 0.8%; CD25 (IL2R), 3.9%; SlgG, 3.9%; CD37 (IOB1), 27.7%; SlgM, 52.5%; Slgλ, 71.0%.
The PCR reaction was by electrophoresis on 4% 3:1 Nusieve agarose gels in Tris-Acetate-EDTA buffer, ethidium bromide staining, and photography under UV transillumination.

**Soft agar disk diffusion colony-formation assay.** The techniques used in this assay were similar to those described by the drug discovery group in our institution. Three grams of Noble-agar (Difco, Detroit, MI) were dissolved in 100 mL of distilled water and boiled for 30 minutes. The preparation was then cooled to 43°C and left in a water bath at that temperature. WSU-WM cells (10^9) were then suspended, as single cells, in 9 mL of regular culture medium (RPMI-1640 with 10% FBS) at 37°C. Finally, 0.3% agar medium is prepared by mixing the cell suspension with 1 mL of the 3% soft agar. Three milliliters of the preparation is then poured into each of 60 x 15 mm tissue culture Petri dishes (Becton Dickinson Labware, Lincoln Park, NJ) and left at room temperature for 20 minutes. During this time, the drug-containing disks are prepared. The disks are standard hole punch (6.5 mm) of Whatman #1 filter paper (Maidstone, England) washed in water and ethanol and autoclaved before loading with a given chemotherapy agent. Drugs are freshly prepared at a concentration of 5 mg/mL and a volume of 0.05 mL (250 µg) is placed on the disk and left to dry before placing the disks on the soft agar dishes. These dishes are then transferred to a 5% CO₂ 37°C incubator.

For determining cytotoxicity of the drugs against human colony-forming unit granulocyte-macrophage (CFU-GM), specimens of aspirated marrows were obtained from healthy donors for allogeneic bone marrow transplantation according to Institutional Review Board (IRB)-approved protocol. Mononuclear cells were then obtained by Ficoll-Hypaque density centrifugation. The monolayer is washed and then the cells are counted and mixed with soft agar. The rest of the procedure is the same as that described for the WSU-WM cells.

To determine the “activity” of the drug, the Petri dishes were examined 10 to 14 days after plating on an inverted microscope (X40 to 60). If the agent is toxic to the test cells, a zone of inhibition of colony formation will occur. Two hundred zone units (ZU) equals 6.5 mm. The differential zone is the ZU value in WSU-WM minus the ZU value of CFU-GM. An agent is active if the differential zone is ≥250 ZU.

**Xenografts.** Four-week-old female Fox Chase C.B.17 SCID and Swiss athymic nude mice were obtained from Taconic Laboratory (Germantown, NY) and maintained under specific pathogen-free conditions in the animal facility of Wayne State University School of Medicine (Detroit, MI). After an adaptation period of a few days, the mice were injected with one dose of cyclophosphamide (150 mg/kg) subcutaneously (SQ). Seventy-two hours later, each animal received 10^7 WSU-WM cells (in serum-free RPMI-1640) SQ in each flank area. When tumors developed, to the point of approximately 1,500 mg, animals were killed and tumors were dissected and mechanically dissociated into single-cell suspension in RPMI-1640 me-

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**Fig 3.** Partial Q-banded karyotype of WSU-WM on September 1990 (insert) showing t(8;14). The complete G-banded karyotype on July 1992 shows further rearrangements, including 10p-, t(Y;8), and t(12;17).
As follows: \( (T - C) - (\text{duration of treatment in days})/(3.32) \) (tumor doubling time [Td]).

**Calculation of the Td in SCID mice.** Tumor weights were plotted against time on a semilog plot. The growth pattern gave an S-shaped plot. Td is the time (in days) required for the tumor to double in weight during the exponential phase of growth.

**RESULTS**

**Establishment and description of the WSU-WM cell line.** The cells did not proliferate during the first 4 weeks in culture. In fact, there was a decline in cell number seeded. After this initial decline, a gradual but definite cell proliferation was noted. By 8 weeks, a continuous cell line had been established and designated WSU-WM. Cells grow in suspension forming large clumps. Once stabilized, the cell line grows rapidly, with a doubling time of approximately 16 hours and a saturating density of 3.5 \( \times 10^6 \) mL. Light microscopic examination of cytospin smears shows lymphoblastoid cells of fairly large size having moderate amounts of cytoplasm, a large nucleus with open chromatin network, and a prominent nucleolus. Some cells have occasional "blebs" in the cytoplasmic membrane (Fig 1). The plating efficiency of the cell line in soft agar is 21%, forming fairly compact colonies (Fig 2).

**Phenotypic characterization.** Comparison of the phenotypes of primary pleural fluid cells, the established WSU-WM line in culture, and the WSU-WM line in the SCID mice as a xenograft is shown in Table 1. Approximately 60% of the pleural fluid lymphocytes were normal T cells, as indicated by the following markers: CD5 (Leu1), 54%; CD2 (Leu5B), 59.7%; CD4 (T4), 34.7%; and CD8 (T8), 19.7%. The most remarkable difference between the pleural fluid and the established WSU-WM phenotype is in the Ig light chain expression. Although the primary cells were \( \kappa^+\lambda^- \), WSU-WM is \( \kappa^+\lambda^- \). Review of the latest serum immunoelectrophoresis available on the patient (March 1990) showed monoclonal IgM bands, supporting the surface phenotype finding of the pleural fluid. Another difference between the phenotype of the primary cells and WSU-WM at establishment is the expression of CD20 in the cell line but not in the primary cells. Although there were differences in the proportion of cells expressing different markers on subsequent characterizations, the cell line generally maintained its phenotype subsequent to its establishment except for the additional expression of CD19.

**Cytogenetic characterization.** Chromosome analysis at early passages showed a male karyotype with chromosome number ranging from 46 to 54. All cells exhibited t(8;14)(q24;q32), t(12;17)(q24;q21), and 2P-. Extra copies of chromosomes 1, 3, 4, 7, 11, 17, 18, and X were observed in the hyperdiploid cells. With further passages, the near-diploid population became predominant. Additional acquired aberrations including deletion of the short arm of chromosome 10 and translocation of the long arm of the Y chromosome to the aberrant chromosome 8 were observed (Fig 3). Since then the karyotype has maintained these features.

**Molecular-genetic characterization.** Since its establishment, genomic DNA analysis of the WSU-WM line by Southern blotting showed deletion of the \( \kappa \) and rearrangement...
Human c-myc gene

Fig 5. Southern analysis of WSU-WM DNA. Genomic DNA was isolated and 10 μg digested with 50 U of the restriction endonucleases Xba I (X), HindIII (H), or EcoRI (E). After electrophoretic separation and transfer to Nytran, the filter was hybridized with the c-myc exon 3 Cta I-EcoRI probe and autoradiographed.

The presence of monoclonal IgM was detected in the culture supernatant of WSU-WM by Ouchterlony technique, supporting the molecular finding. The WSU-WM cells also showed rearrangement of one allele of the c-myc proto-oncogene (Fig 5). The breakpoint on chromosome 8 is downstream of exon 3 of c-myc and located between the Xba I and HindIII restriction enzyme sites. Additional digests with Kpn I or BamHI confirmed that this was a rearrangement, not merely a HindIII restriction site polymorphism. Northern analysis showed abundant expression of c-myc transcripts of the expected size (data not shown).

WSU-WM xenografts. To compare the engraftment rate of WSU-WM in the athymic nude and SCID mice, seven animals of each strain were injected SQ with equal numbers of WSU-WM cells (10⁷) in each flank. Six of the seven SCID mice developed tumors, as opposed to only three of seven in the athymic nude mice. Moreover, there was a difference in the time-to-tumor development. All six SCID mice had developed palpable tumors by the end of the 3 weeks. On the other hand, two athymic nude mice developed the tumors after 4 weeks and the third developed a tumor after 6 weeks. Based on these results, subsequent drug efficacy studies were performed on SCID mice. Dissection of the tumor-bearing animals showed enlargement of some lymph nodes in the abdominal cavity but no hepatosplenomegaly. Histopathologic examination of the tumors showed a diffuse proliferation of medium-sized plasmacytoid lymphocytes with fine chromatin pattern, prominent nucleoli, and a small amount of eosinophilic cytoplasm. Mitoses were numerous, averaging eight per high power field (Fig 1). Enlarged abdominal lymph nodes were involved by the same growth, whereas the liver and spleen were normal. The human origin of the SQ tumors was confirmed by cytogenetic and phenotypic marker studies that showed no significant changes compared with studies performed on the cells in
Table 2. Activity of Standard Chemotherapy Agents Against WSU-WM In Vitro

<table>
<thead>
<tr>
<th>Drug</th>
<th>WSU-WM</th>
<th>huCFU-GM</th>
<th>Differential</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HC</td>
<td>400</td>
<td>600</td>
<td>-200</td>
</tr>
<tr>
<td>L-PAM</td>
<td>1,000</td>
<td>700</td>
<td>300</td>
</tr>
<tr>
<td>Vinristine</td>
<td>1,000</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>600</td>
<td>900</td>
<td>0</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>200</td>
<td>300</td>
<td>-100</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>600</td>
<td>600</td>
<td>0</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>1,100</td>
<td>500</td>
<td>600</td>
</tr>
<tr>
<td>Etoposide (VP-16)</td>
<td>1,000</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are ZU in the soft agar disk diffusion assay.
Abbreviations: 4HC, hydroperoxycyclophosphamide; huCFU-GM, human CFU-GM.

Discussion

In this report, we show that the WSU-WM cell line has several unique features that make it valuable as a research tool. First, this is the first reported Waldenström’s line established without EBV infection or growth factor stimulation. It is well known that c-myc expression confers a proliferative advantage on cells.1,18 Therefore, it is conceivable that c-myc rearrangement and dysregulated expression has contributed to the successful establishment of the cell line and its rapid growth rate in culture and in mice. Because cytogenetic analysis was not performed on the primary pleural fluid cells or other tumor specimens from the patient, it is not known whether t(8;14) and c-myc rearrangement developed in culture. However, this is not likely because these abnormalities were detected at the time of WSU-WM establishment and were maintained to date. Moreover, the clinical behavior of the disease in the patient had changed to a more rapid course between 1980 and the beginning of 1990.

Although translocation between the myc-carrying chromosome and one of the three Ig loci is a regular feature of certain B-cell-derived tumors such as the human Burkitt’s lymphoma, Balb/c mice plasmacytomas, and Louvain rat immunocytomas,18 it is not usually associated with human low-grade B-cell tumors such as WM.19 The 8;14 translocation was found in none of 27 WM patients analyzed in two independent studies at initial presentation and/or disease progression.19,20 In addition to the rarity of the t(8;14) in WM, the breakpoint of chromosome 8 in WSU-WM is unusual. Whereas the breakpoint in t(8;14) of Burkitt’s lymphoma is usually upstream of myc exon 1 or in intron 1,21 it was down-stream of exon 3 in WSU-WM.

We feel that the difference in the Ig light chain expression between the primary cells and the established line represents a light chain class switching phenomenon. The switching of heavy chain class (from μ to γ) has been reported.21 Therefore, it is conceivable that similar switching can take place in the light chains. This is especially true for switching from κ to λ as it is currently believed that κ-gene rearrangements precede those of λ-gene.22

Another unique feature of this cell line is its ability to grow in liquid culture, in soft agar (with a rather high plating efficiency), and in immune-deficient mice. This feature allows for experimentation on cells under different environmental conditions. For example, an established procedure for evaluating prospective anticancer agents used by our laboratory and other drug discovery groups23 is the disk diffusion in vitro assay. This assay depends on the ability of the cells to grow in soft agar. Agents identified in this assay as active usually undergo preclinical in vivo testing. The mouse is the most commonly used animal model for anticancer drug development. The availability of human tumor xenografts in mice, such as the WSU-WM, brings the preclinical model closer to the human situation and perhaps makes it more predictive. Although the athymic nude mice have been successfully used for human hematopoietic engraftment,24 more recent reports indicate a superiority of the SCID mice.25,26 The SCID mice have been recently successfully used as models for human non-Hodgkin’s lymphoma27 and acute lymphoblastic leukemia28 of T- and pre-B-cell origins, respectively.

Using the WSU-WM cell line as a model, we showed that, of the standard agents tested in vitro and in vivo, both vincristine and doxorubicin were active against this tumor. On the other hand, cyclophosphamide, nitrogen mustard, bleomycin, and methotrexate are inactive based on the criteria set in Materials and Methods. Results of selected “standard” agent activity against WSU-WM in vivo are shown in Table 3.

Table 3. Preclinical In Vivo Trials of Standard Chemotherapy Agents in WSU-WM-Bearing SCID Mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose Level (mg/kg)*</th>
<th>No. of Animals</th>
<th>T/C (%)</th>
<th>Log10 Kill (net)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0</td>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>40</td>
<td>5</td>
<td>87</td>
<td>-1.175</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5</td>
<td>88</td>
<td>-1.175</td>
</tr>
<tr>
<td>L-PAM</td>
<td>4.4</td>
<td>5</td>
<td>19</td>
<td>1.322</td>
</tr>
<tr>
<td></td>
<td>7.1</td>
<td>5</td>
<td>12</td>
<td>3.967</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.5</td>
<td>5</td>
<td>19</td>
<td>0.588</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5</td>
<td>19</td>
<td>2.791</td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>0.7</td>
<td>5</td>
<td>42</td>
<td>-0.44</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>5</td>
<td>42</td>
<td>0.145</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>16</td>
<td>5</td>
<td>31</td>
<td>-0.293</td>
</tr>
</tbody>
</table>

*Doses are determined based on previous experiments with these drugs.
cristine and L-PAM are active (Tables 2 and 3). Based on the soft agar disk diffusion assay in vitro, we predict that doxorubicin and VP-16 will also show activity in the xenograft model. On the other hand, cyclophosphamide, nitrogen mustard, methotrexate, and bleomycin showed no activity. The identification of melphalan, considered the "gold" standard in treatment of immunoproliferative disorders, as active in our assays attests to the validity of our model.

There has been significant debate regarding the best treatment regimen for the immunoproliferative disorders in general. It is not clear if multiagent "intensive" chemotherapy in our assays attests to the validity of our model.

The model described in this report may prove useful in the treatment regimen for the immunoproliferative disorders in general. Hence, available standard chemotherapy regimens are superior to melphalan and prednisone.31 The immunoproliferative disorders (primarily WM and multiple myeloma) remain incurable regardless of the treatment regimen used. Therefore, available standard chemotherapy agents are being used in different combinations in the hope of obtaining better therapeutic results.32 Although such an approach may improve the response rate by a small percentage or decrease toxicity, it has not changed the natural history of these diseases. Based on these observations, a new strategy for tumor-specific, rational drug development is needed. The model described in this report may prove useful for such an endeavor. Testing of new organic synthetic compounds in this model is currently underway in our laboratory.

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