ABSTRACT

Increased sialylation and branching of asparagine-linked oligosaccharides have recently been associated with both neoplastic transformation and the metastatic phenotype. Swainsonine, an inhibitor of Golgi α-mannosidase II blocks the synthesis of sialylated tri- and tetraantennary asparagine-linked oligosaccharides and results in the expression of hybrid-type oligosaccharides at the cell surface. Both the lymphoid tumor line MDAY-D2 and B16F10 melanoma cells were less metastatic when grown in swainsonine (0.3 μg/ml) for 48 h prior to injection of the cells into the lateral tail veins of mice. The addition of swainsonine (2.5 μg/ml) to the drinking water of the mice further reduced the incidence of lung colonization by B16F10 melanoma cells.

MDAY-D2 tumors removed from mice on swainsonine-supplemented drinking water showed a loss of leukoagglutinin-binding complex-type oligosaccharides similar to that of tumor cells cultured in medium containing swainsonine. The growth rate of s.c. MDAY-D2 tumors was not reduced by the addition of swainsonine to the drinking water of the host; however, when mice were given two i.p. injections of the interferon-inducing agent polyinosinic-polyribocytidylic acid in addition to swainsonine, the primary tumor grew at a reduced rate compared to either treatment alone. Swainsonine alone did not inhibit tumor cell growth in vitro; however, the drug enhanced the antiproliferative effect of interferon. The survival time of mice bearing established MDAY-D2 metastases was extended by treating the animals with swainsonine and polyinosinic-polyribocytidylic acid; however, the number of long-term survival was unchanged. Swainsonine-treated tumor cells appeared to be compromised in two ways: (a) reduced organ colonization potential; and (b) drug-treated MDAY-D2 cells were more sensitive to the antiproliferative effects of interferon in vitro and in vivo.

INTRODUCTION

The Asn-linked oligosaccharides in human (1) and rodent cells (2-4) show qualitative differences following neoplastic transformation with chemicals (2), oncogenic viruses (3), or transfection with oncogenes (4). The complex-type oligosaccharides appear to be larger due to increased sialylation (5) and increased branching of the trimannosyl core (6). A number of lines of evidence suggest that certain of these structural changes may be required for expression of the metastatic phenotype. Sialylation of available cell surface galactose and N-acetylglactosamine has been correlated with metastatic potential for a number of murine tumor cell lines (7). Similarly, loss of sialylated Asn-linked oligosaccharides in lectin-resistant mutants of the B16 melanoma and the MDAY-D2 tumor cell line has also been associated with decreased metastatic potential (8, 9).

The Asn-linked oligosaccharide structures isolated from MDAY-D2 cells and the nonmetastatic class 1 mutant MDW4 have recently been compared (9). MDAY-D2 cells synthesize mainly tetraantennary complex-type oligosaccharides containing the β1-6-linked lactosamine antennae, the preferred ligand for L-PHA binding (10).

\[
\begin{align*}
\text{Galβ1-4GlcNAcβ1-6} & \text{ NeuN Aca2-3} \\
\text{Galα1-3} & \text{ Manβ1-4R} \\
\text{Galβ1-4GlcNAcβ1-2 Manα1-6} & \text{ Manα1-3} \\
\text{Galβ1-4GlcNAcβ1-2 Manα1-3} & \text{ NeuN Aca2-3 Galβ1-4GlcNAcβ1-2 Manα1-3}
\end{align*}
\]

These structures also contain repeating sequences of Galβ1-4GlcNAc (i.e., polylactosamine). The class 1 lectin-resistant mutants lack sialic acid and galactose on complex-type oligosaccharides, and the cells were shown to be nonmetastatic from a s.c. location and poorly metastatic when the cells were injected i.v. (9). The relatedness of the class 1 mutation and the nonmetastatic phenotype has been confirmed by selecting revertants in vitro that regain both the wild-type oligosaccharide structures and the metastatic phenotype (11).

The loss of sialylated antennae in complex-type oligosaccharide and a simulated lectin-resistant phenotype can be induced in wild-type cells grown in the presence of swainsonine, an inhibitor of the Golgi oligosaccharide-processing enzyme α-mannosidase II (12). In the presence of swainsonine, hybrid-type oligosaccharides structures are synthesized (13):

\[
\begin{align*}
\text{Manα1-6} & \text{ NeuN Aca2-3 Galβ1-4GlcNAcβ1-2 Manα1-3} \\
\text{Manα1-6} & \text{ NeuN Aca2-3 Manα1-3 Manβ1-4R}
\end{align*}
\]

where the presence of the terminal α1-6- and α1-3-linked mannoses prevented the addition of β1-2- and β1-6-linked sialyllactosamine antennae (i.e., NeuN Aca-Gal-GlcNAc). Swainsonine is found in spotted locoweed and when ingested by domestic farm animals, the drug inhibits lysosomal mannosidase as well as Golgi α-mannosidase II (14). The brain tissues accumulate lysosomal vesicles containing oligomannose structures similar to that observed in hereditary lysosomal storage diseases. There are a number of tissue-specific mannosidases, and interestingly, rodents appear to have a brain enzyme that is not inhibited by swainsonine since rat brain does not accumulate oligomannose structures and the animals do not show neurological symptoms when fed the drug (15). Tulisiani and Touster (16) found that rats on swainsonine-supplemented drinking water had increased levels of plasma acid hydrolases and clearance of injected glucuronidase from the serum occurred at a reduced rate. However, rodents can be maintained on swainsonine-supplemented drinking water for weeks with no apparent ill effects which has allowed us to test the drug in situ for inhibition of solid tumor growth and metastasis.

Received 3/27/86; revised 6/27/86; accepted 7/14/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the National Cancer Institute of Canada.

2 To whom requests for reprints should be addressed, at Division of Cancer Research, Mount Sinai Hospital Research Institute, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5.

3 The abbreviations used are: Asn, asparagine; Con A, concanavalin A; GlcNAc, N-acetylglucosamine; Gal, galactose; Man, mannose; NeuN Aca, N-acetylneuraminic acid; L-PHA, leuкоagglutinin; WGA, wheat germ agglutinin; Poly(1)-poly(C), polyinosinic-polycytidylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS, sodium dodecyl sulfate; R, GlcNAcβ1-4GlcNAc.
In a recent report by Humphries et al. (17) swainsonine treatment of B16F10 melanoma cells was shown to inhibit experimental metastasis. In this study, we show that inhibition of lung colonization by B16F10 cells can be enhanced by supplementing the hosts’ drinking water with swainsonine. In addition, swainsonine in combination with the interferon-inducing agent poly(I)-poly(C) inhibited solid tumor growth in vivo. Swainsonine also enhanced the antiproliferative effects of interferon in vitro.

MATERIALS AND METHODS

Cell Lines. The lymphoreticular tumor line MDAY-D2 is a highly metastatic DBA/2 tumor line (18, 20) and the B16F10 is a metastatic variant of the B16 melanoma (19). Tumor cells were grown in α-minimal essential medium (Eagle’s) plus 7% fetal calf serum either with or without swainsonine, 0.3 μg/ml (Calbiochem) for 48 h before harvesting for experiments. Staining with Hoechst 33258 showed all cell lines to be free of Mycoplasma.

Lectin Sensitivity. The ability of tumor cells to proliferate in the presence of increasing concentrations of L-PHA, Con A, poly(I)-poly(C) or mouse α/β-interferon (Sigma) was determined by measuring [3H]thymidine incorporation into DNA. Cells were cultured at 3 × 10^4/ well in 96-well microtreat plates containing serial dilutions of either L-PHA or Con A for 16 h. The cells were pulsed with 1 μCi of [3H] thymidine and harvested 4 h later onto glass fiber disks using a Titrakit harvester, and the disks were counted in a liquid scintillation counter.

Interferon Sensitivity. Tumor cells were seeded into 24-well lymphoplates at 10^4/ml and serial dilutions of mouse α/β-interferon (Sigma) were added. The cells were cultured in the presence and absence of swainsonine (1 μg/ml) and on day 5 the cell number was determined using a Coulter Counter.

Electrophoresis and Glycoprotein Staining. Tumor cells were disrupted by nitrogen cavitation and the plasma membrane fraction was separated on a discontinuous Ficoll gradient. Approximately 3 × 10^8 washed cells were suspended in 4 ml of 20 mM HEPES, pH 7.4-0.5 mM MgCl₂-0.13 M NaCl and stirred while 5 ml or 0.5 ml sucrose was added slowly. After adding 100 μl of Trasylol (Sigma) and 50 μl of 200 mM phenylmethylsulfonyl fluoride the cells were placed in a nitrogen cavitation bomb (Artisan Metal Products) and equilibrated with 600 psi of N₂ for 20 min at 4°C with constant stirring. Cell disruption occurred after dropwise release of the suspension from the bomb. The lysate was made with 1 mM disodium EDTA centrifuged at 1,000 × g for 10 min and the resulting supernatant was centrifuged at 20,000 × g for 20 min. Membrane vesicles were pelleted at 120,000 × g for 1 h, resuspended in 0.5 ml of 1 mM HEPES, pH 8.2-1 mM MgCl₂ and layered on top of 4 ml of Ficoll (1.09 g/ml). The samples were centrifuged for 5 h at 300,000 × g in a SW50.1 rotor. The plasma membrane fraction was removed from the buffer-Ficoll interphase, diluted with HEPES buffer, and pelleted at 120,000 × g for 1 h. The pellet was suspended in 0.5 ml HEPES buffer and stored at −20°C. Protein concentrations were determined using the Bio-Rad protein assay reagent and bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis was performed on 12.5% resolving gels under reducing conditions using 40 μg of protein/lane. WGA was iodinated with 500 μCi of Na¹²⁵I and three iodogen beads (Pierce) in 0.5 ml phosphate-buffered saline, pH 7.0. L-PHA was iodinated with 500 μCi of Na¹²⁵I Bolton-Hunter reagent. The iodinated lectins were passed over a 1- x 30-cm P10 column (Bio-Rad) equilibrated in sodium phosphate, pH 6.8-0.2 M NaCl-1 mM CaCl₂-1 mM MgCl₂-0.2 mM Na₃VO₄. Gels that had been fixed and stained with Coomassie brilliant blue were equilibrated in the phosphate buffer and incubated with the iodinated lectins for 16 h. The gels were then washed exhaustively over a 3-day period, dried, and exposed to X-ray film at −70°C for 1.5 days.

Aliquots of plasma membrane suspended in 30 μl of 50 mM sodium citrate, pH 5.1-1 Triton X-100-1 mM phenylmethylsulfonyl fluoride-0.3 μl Trasylol-0.02 mM NaN₃ were digested overnight with 0.1 unit neuraminidase (type X; Sigma) or endoglycosidase H (Boehringer Mannheim). Two times concentrated SDS sample buffer was added and the samples were heated to 95°C for 5 min and then separated by SDS gel electrophoresis.

Metastasis Assay. Experimental metastasis was performed by injecting 10⁴ MDAY-D2 cells or 10⁴ B16F10 melanoma tumor cells into the lateral tail veins of 6- to 8-week-old syngeneic DBA/2 or C57BL mice, respectively. Mice in the experimental groups were provided with drinking water containing swainsonine (2.5 μg/ml) 2 days before administering tumor cells. The mean survival time of mice given injections of MDAY-D2 cells was determined up to 90 days and mice surviving more than 90 days were considered long-term survivors. Mice that survived more than 90 days were consistently tumor free when autopsied and have been kept for 6 months with no evidence of tumor growth. Mice given injections of B16F10 cells were sacrificed 22–24 days after tumor cell inoculation and the number of metastatic lesions in the lungs was counted with the aid of a dissection microscope.

The effects of swainsonine-supplemented drinking water on the oligosaccharide structures in s.c. growing MDAY-D2 tumors were assessed. Tumor-bearing mice on normal and swainsonine-supplemented drinking water were sacrificed 15 days after tumor cell inoculation and the tumors were weighed, minced with scissors in phosphate-buffered saline and digested with collagenase (type II-5; Sigma) to produce a single-cell suspension (21). Viable tumor cells were harvested from the surface of a Ficoll-Urovision (1.09 g/cm³) cushion after spinning at 1500 × g for 15 min and microsomal membrane vesicles were prepared as described above. The membrane preparations were solubilized on SDS-polyacrylamide gels and compared to the L-PHA binding glycoproteins in tumors from control and drug-treated mice.

Methylation Analysis. MDAY-D2 tumor cells were expanded for 48 h in either the presence or absence of swainsonine (0.3 μg/ml); then 2.5 × 10⁶ cells were placed in 5 ml of low glucose RPMI 1640 plus 200 μCi of [2-¹⁴C]mannose and cultured for 16 h. The cells were washed, combined with 20 × 10⁶ unlabeled cells followed by extraction of glycolipids with chloroform/methanol, 2:1 (v/v), and the protein residue was Pronase digested as previously described (9). The peptide was passed over a 2-ml DEAE-cellulose column in 10 mM Tris, pH 7.0, and sialylated oligosaccharides were eluted with 250 mM NaCl in the same buffer. To eliminate neutral oligosaccharides containing charged peptides, samples were digested with 100 milliunits neuraminidase (Clostridium perfringens) and passed over DEAE-cellulose a second time. Glycopeptides excluded from the column were methylated, acid hydrolyzed, reduced with NaBH₄ (22), and methylated derivatives of [2-¹⁴C]mannose were separated on an Alltech C18 high-performance liquid chromatography column as previously described (23).

RESULTS

Swainsonine-inhibited Expression of Complex-type Oligosaccharides. Swainsonine-induced inhibition of mannosidase II in fibroblast cells has been shown to result in the synthesis of hybrid type structures rather than the normal complex-type oligosaccharides (12). These structures have terminal mannose residues attached to the α-1-6 side of the trimannosyl core rather than β1-2- and β1-6-linked lactosamine antennae required for L-PHA binding (see structure in "Introduction”); therefore, loss of L-PHA binding and increased Con A binding to membrane glycoconjugates were used to monitor inhibition of oligosaccharide processing by swainsonine.

Fig. 1 shows that concentrations of swainsonine as low as 0.03 μg/ml inhibited synthesis of L-PHA-binding glycoproteins in MDAY-D2 cells and concentrations between 0.03 and 1 μg/ml showed similar levels of inhibition. MDAY-D2 cells grown in swainsonine (0.03 μg/ml) were partially resistant to the toxic effect of L-PHA, and at 0.3 μg/ml the cells were completely resistant to L-PHA (Fig. 2); therefore, tumor cells were grown in swainsonine (0.3 μg/ml) to assess the effects of the drug on oligosaccharide structures in vitro and the organ colonization potential of the cells in vivo. The plasma membrane fraction was separated on an Alltech C18 high-performance liquid chromatography column as previously described (23).
isolated from MDAY-D2 cells grown in swainsonine (0.3 µg/ml) showed a large increase in Con A-binding glycoprotein and a parallel loss of L-PHA-binding material indicating that hybrid oligosaccharides were expressed at the cell surface (Fig. 1).

To confirm that inhibition of α-mannosidase II by swainsonine also inhibits the addition of lactosamine antennae to the α1-3 side of the trimannosyl core, MDAY-D2 cells were grown in the presence of [2-3H]mannose, and sialylated oligosaccharides were isolated for methylation analysis. The results in Table 1 show that the distribution of di-o-methylmannitol derivatives in sialylated glycopeptides from MDAY-D2 was similar to that previously reported using gas-liquid chromatography/mass spectrometry and indicated that the tetraantennary complex was the predominant complex-type oligosaccharide present. MDAY-D2 cells grown in the presence of swainsonine showed a decrease in 3,4-di-o-methylmannitol and the presence of 3,4,6- and 2,3,4,6-methylmannitols consistent with the presence of hybrid structures with only a single lactosamine antenna. This finding suggests that the addition of the β1-4 antennae to the α1-3 side of the trimannosyl core was also inhibited by swainsonine. Swainsonine did not completely block synthesis of complex as indicated by the residual 3,4-di-o-methylmannitol (Table 1).

Swainsonine-inhibited Experimental Metastasis. Poly(I)-poly(C) is an effective inducer of interferon which in turn acts as an immunoadjuvant (24) as well as an inhibitor of cell proliferation (25). For the class 1 lectin-resistant mutants, the loss of sialic acid and exposure of incomplete complex-type oligosaccharides appeared to enhance the susceptibility of the cells to natural killer cell lysis in vitro (26); therefore, swainsonine and poly(I)-poly(C) as well as a combination of these agents were tested for inhibition of tumor cell growth and metastases.

MDAY-D2 tumor cells were grown in medium with or without swainsonine (0.3 µg/ml) for 48 h and then were injected into mice that had been given an i.p. injection of either poly(I-C) or water 24 h earlier (Table 2). Since MDAY-D2 cells produce many diffuse nodules in the organs which are difficult to count accurately, experimental metastasis of control and swainsonine-treated cells were compared by injecting a low dose of tumor cells and monitoring the number of deaths due to tumor growth (11). Both poly(I)-poly(C)-treated and control mice died of metastatic tumor growth whereas approximately 30% of the mice (i.e., 10 of 30; P < 0.005) given injections of swainsonine-treated cells were tumor free at 100 days (Table 2); however, poly(I)-poly(C) treatments did not increase survival in groups given injections of swainsonine-treated tumor cells nor were swainsonine-treated MDAY-D2 cells significantly more sensitive to natural killer cell lysis in vitro (data not shown).

Swainsonine-treated F16F10 melanoma cells were also less metastatic in the experimental metastasis assay (Table 3). C57BL mice given in i.v. injection of swainsonine-treated B16F10 cells showed fewer lung nodules (i.e., 30-40% of controls) and mice on swainsonine-supplemented drinking water.

Table 1  Methylation analysis of sialylated glycopeptides from MDAY-D2

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>Without swainsonine</th>
<th>With swainsonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>3,4-di-o-methyl</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td></td>
<td>2,4-di-o-methyl</td>
<td>1.0 (2.0)</td>
</tr>
<tr>
<td></td>
<td>3,6-di-o-methyl</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td></td>
<td>3,4,6-tri-o-methyl</td>
<td>0.3 (0.9)</td>
</tr>
<tr>
<td></td>
<td>2,3,4,6-tetra-o-methyl</td>
<td>0.0 (1.0)</td>
</tr>
</tbody>
</table>

Table 2  Experimental metastasis of MDAY-D2 tumor cells

MDAY-D2 tumor cells were cultured for 48 h in the presence or absence of swainsonine (0.3 µg/ml) before injecting 10^6 cells into the lateral tail veins of mice on day 0. Mice were given drinking water with swainsonine (2.5 µg/ml) 2 days before tumor cells were injected and maintained on the drug for 17 days. Mice were given an i.p. injection of poly(I)-poly(C) (100 µg) the day before tumor cells were injected and again on day 3. Those that survived longer than 100 days were termed survivors and scored as long-term survivors. Mice with metastatic lesions died between 25 and 48 days after tumor cell injection. Mice given injections of swainsonine-treated cells (Lines 3-6) showed a significantly higher frequency of long-term survivors compared to those given injections of untreated cells (Lines 1 and 2); P < 0.005.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice</th>
<th>Cells</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Nil</td>
<td>Poly(I)-poly(C)</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Swainsonine</td>
<td>Nil</td>
<td>Poly(I)-poly(C)</td>
<td>3/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Swainsonine</td>
<td>Poly(I)-poly(C)</td>
<td>2/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Swainsonine</td>
<td>Swainsonine</td>
<td>Not done</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Swainsonine</td>
<td>Swainsonine + poly(I)-poly(C)</td>
<td>Not done</td>
<td>3/5</td>
<td></td>
</tr>
</tbody>
</table>
EFFECTS OF SWAINSONINE AND POLY(I)-POLY(C) ON MURINE TUMORS

Table 3  Lung colonization by B16F10 melanoma cells

B16F10 tumor cells were cultured for 48 h in the presence or absence of swainsonine (0.3 μg/ml) before injection of 10⁶ cells into the lateral tail veins of mice on day 0. Mice were given drinking water with swainsonine (2.5 μg/ml) 2 days before tumor cells were injected and maintained on swainsonine for 17 days. Mice were given an i.p. injection of poly(I)-poly(C) (100 μg) the day before tumor cells were injected and again on day 3. Lung nodules were counted on day 24 and each group consisted of 5 mice. Data were analyzed using the Mann-Whitney test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Nil</td>
<td>Poly(I)-poly(C) 37 ± 40 (No statistical difference)</td>
</tr>
<tr>
<td>Swainsonine Nil</td>
<td>16 ± 12 (P &lt; 0.02)</td>
</tr>
<tr>
<td>Swainsonine Poly(I)-poly(C)</td>
<td>24 ± 18 (P &lt; 0.05)</td>
</tr>
<tr>
<td>Swainsonine Swainsonine</td>
<td>5 ± 6 (P &lt; 0.005)</td>
</tr>
<tr>
<td>Swainsonine + poly(I)-poly(C)</td>
<td>4 ± 4 (P &lt; 0.005)</td>
</tr>
</tbody>
</table>

* Mean ± SD.

had 0–7% the number of lung nodules found in control mice given injections of untreated B16F10 cells. Poly(I)-poly(C) treatment of the mice in conjunction with swainsonine-treated tumor cells appeared to have a synergistic effect in one of the two experiments. It is possible that further optimization of the doses and timing of administration of poly(I)-poly(C) may result in a consistent enhancement of the antineoplastic effects of swainsonine.

B16F10 cells grown in medium containing swainsonine (0.3 μg/ml) showed a marked reduction in two L-PHA-staining glycoproteins (Fig. 3) similar to that observed for MDAY-D2 cells. In addition the WGA-staining sialylated oligosaccharides in swainsonine-treated cells were endoglycosidase H sensitive as expected for hybrid structures. The sialylated lactosamine antennae on the α1-3 side of the trimannosidase H sensitive provides a ligand for WGA binding whereas the terminal mannose residues on the α1-6 side of the trimannosidase core render the structures endoglycosidase H sensitive. The WGA-binding glycoprotein(s) in swainsonine-treated cells was reduced in apparent molecular weight by endoglycosidase H and the residual WGA binding of these structures may be due to the presence of sialylated O-linked oligosaccharides or incomplete inhibition of α-mannosidase II by swainsonine (Fig. 3, lane 4).

Swainsonine- and Poly(I)-poly(C)-inhibited Solid Tumor Growth. Spontaneous metastasis from a s.c. site involves tumor growth and local invasion as well as the steps common to experimental metastasis, namely, organ colonization; therefore the effects of swainsonine and poly(I)-poly(C) on solid tumor growth were also examined. A combination of swainsonine added to the drinking water of the host and two i.p. injections of poly(I)-poly(C) reduced the rate of growth of MDAY-D2 tumors (Fig. 4). Neither poly(I)-poly(C) nor swainsonine alone significantly inhibited tumor growth; however, swainsonine alone inhibited organ colonization, suggesting that the drug had distinct effects on organ colonization and solid tumor growth. Swainsonine may enhance the antiproliferative effects of poly(I)-poly(C)-induced interferon in vivo. To examine this

5134

**Fig. 3.** Endoglycosidase H (Endo H) sensitivity of plasma membrane glycoprotein isolated from B16F10 melanoma cells grown in the presence or absence of swainsonine (SW) (0.3 μg/ml). Samples were digested with endoglycosidase H or neuraminidase (Nanase), separated by SDS-gel electrophoresis, and the gels were stained with 125I-labeled WGA to detect sialylated glycoproteins. K, thousands.

**Fig. 4.** Left, growth of s.c. MDAY-D2 tumors in mice given swainsonine (SW)-supplemented drinking water and/or two i.p. injections of poly(I)-poly(C). Mice were given drinking water with swainsonine (2.5 μg/ml) 2 days before 10⁵ tumor cells were injected and poly(I)-poly(C) (100 μg/ml) was injected i.p. 1 day before and 2 days after tumor cells were injected. Tumors were removed on day 15, weighed, and used for the preparation of microsomal membranes as described in "Materials and Methods." * significant at the level of P < 0.05 using Student's t test. The experiment was repeated three times with six mice/group and similar results were obtained. Right, microsomal membrane proteins separated by SDS-polyacrylamide gel electrophoresis and stained with 125I-labeled L-PHA. K, thousands; bars, SD; C, control.
EFFECTS OF SWAINSONINE AND POLY(I)-POLY(C) ON MURINE TUMORS

possibility, MDAY-D2 cells were cultured in the presence of increasing concentrations of mouse α/β-interferon either with or without swainsonine in the culture medium. The results in Fig. 5 show that swainsonine also enhanced the antiproliferative effect of α/β-interferon in vitro.

To determine whether swainsonine had similar effects on tumor cell oligosaccharides in vivo as those observed in vitro, MDAY-D2 tumors were removed from mice on normal or swainsonine-supplemented drinking water and compared for the relative amounts of L-PHA-staining glycoproteins (Fig. 4). Microsomal membranes prepared from MDAY-D2 tumor cells removed from mice on swainsonine-supplemented drinking water showed a marked reduction in the M, 90,000–120,000 L-PHA-staining glycoprotein similar to cells cultured in medium containing swainsonine (Fig. 1).

An attempt was made to determine the effects of swainsonine and of poly(I)-poly(C) administration at a time in the course of the disease similar to that when chemotherapeutic drugs might be used in human patients. S.c. growing MDAY-D2 tumors were resected 12 days after tumor cell inoculation when metastases were known to be present (27) and the mice were then treated with swainsonine and/or poly(I)-poly(C). The combination of swainsonine and poly(I)-poly(C) increased the survival time of the mice; however, the number of long-term survivors was not significantly increased by the therapy (Fig. 6). This is consistent with the observation that a combination of swainsonine and poly(I)-poly(C) reduced tumor growth rate (Fig. 4); however, established metastases do not appear to be eliminated.

DISCUSSION

Our recent studies on lectin-resistant tumor cell mutants have suggested that loss of sialylated Asn-linked oligosaccharides is related to decreased metastatic potential (9, 11).4 This association has been strengthened by the observation that revertants of class I nonmetastatic lectin-resistant mutants of MDAY-D2 require the wild-type Asn-linked oligosaccharide structures and the highly metastatic phenotype (11). Therefore induction of a lectin-resistant phenotype in tumor cells using an inhibitor of oligosaccharide processing that decreases the number of sialylated lactosamine antennae might be expected to decrease the metastatic potential of the cells. To test this hypothesis we have used swainsonine, an inhibitor of mannosidase II which blocks synthesis of complex-type structures and results in the expression of hybrid oligosaccharides (12, 13). Swainsonine has the added advantage that it can be administered to mice with no apparent ill effects and therefore the drug can be tested as a potential therapeutic agent.

Tumor cells cultured in swainsonine-containing medium showed a loss of L-PHA-binding glycoprotein in plasma membrane fractions and a concomitant increase in Con A-binding structures consistent with a shift from complex-type to hybrid-type oligosaccharides. The drug-treated cells also showed altered sensitivity to the same lectins suggesting that swainsonine inhibited expression of the normal cell surface oligosaccharides. In addition, methylation analysis of the sialylated fraction from MDAY-D2 cells grown in swainsonine indicated that most of the tetraantennary complex-type oligosaccharides had been converted to monoantennary hybrid-type structures. The absence of the β1–4 antennae in swainsonine-treated cells suggests that its initiation (i.e., addition of the β1–4-linked GlcNAc) may require the prior action of α-mannosidase II.

Both B16F10 and MDAY-D2 tumor cells grown in swainsonine-containing medium were less metastatic in the experimental metastasis assay using untreated syngeneic mice. These results could not be attributed to toxic effects of the drug since neither cell growth (Fig. 5) nor plating efficiency (data not shown) were altered by swainsonine in vitro. The addition of the drug at 2.5 μg/ml to the drinking water of the host dramatically reduced organ colonization by B16F10 melanoma cells. Tumor cell dissemination following an i.v. injection of cells appears to be a rapid process occurring within minutes (28) and may be the step compromised for swainsonine-treated cells in the normal host. The systemic administration of swainsonine may also affect the host microenvironment in a manner that reduces organ colonization; therefore, the dramatic reduction in experimental metastasis observed in mice on swainsonine-supplemented drinking water may be due to drug-induced effects on both the tumor cells and the host.

The growth of solid MDAY-D2 tumors was reduced by approximately 70% in mice given swainsonine and two injections of poly(I)-poly(C). Neither swainsonine nor poly(I)-poly(C) alone inhibited tumor growth indicating that the two agents were acting synergistically. In vitro, swainsonine also enhanced the antiproliferative effects of α/β-interferon on MDAY-D2 cells. Similarly, tunicamycin has been shown to enhance the antiproliferative effects of interferon on 3T3 fibroblasts (29). It is possible that alterations in the carbohydrate

![Fig. 5. Enhancement of the antiproliferative effect of interferon (INF) by swainsonine. MDAY-D2 cells were seeded at 10⁶/ml in the presence or absence of swainsonine (SW) (1 μg/ml) and counted 5 days later using a Coulter Counter. The mean ± SD (bars) range of duplicates has been graphed.]

Fig. 5. Effect of swainsonine (SW) and poly(I)-poly(C) on the survival of mice bearing established MDAY-D2 metastases. MDAY-D2 tumor cells were injected s.c. and the resulting tumors were surgically resected 12 days later. At this time the 40 mice were divided into 4 treatment groups. Poly(I)-poly(C) was administered on days 12 and 15, and swainsonine-supplemented drinking water was provided between days 12 and 30. Survivors were tumor free at 90 days. ∙, the SW and the SW + poly(I)-poly(C) groups differed from the control group at the P < 0.01 level using the Mann-Whitney U test.
structure of the interferon receptor may enhance interferon binding and/or intracellular signaling. The glycoprotein hormone receptors provide a precedent for this type of mechanism as it has been shown that removal of the receptor carbohydrate enhances hormone binding (30).

Finally swainsonine and poly(I)-poly(C) prolonged the life of mice with established MDAY-D2 metastases; however, long-term survival rates were similar to that of the controls. These results are consistent with the observation that swainsonine in conjunction with poly(I)-poly(C) reduced the growth rate of the s.c. tumors but the tumors did not regress. In summary, swainsonine inhibited organ colonization for both MDAY-D2 and B16F10 tumor cells and a combination of swainsonine and poly(I)-poly(C) reduced the growth rate of solid MDAY-D2 tumors.

Tunicamycin and castanospermine have been shown to inhibit the transport of some glycoproteins to the cell surface (31–33). In contrast swainsonine inhibits oligosaccharide processing at a later stage and consequently does not appear to block expression of cell surface glycoproteins (31, 32); therefore, the inhibitory effect of swainsonine on metastasis is more likely to be due to an alteration in the activity of a cell surface glycoprotein rather than the inhibition of its expression at the cell surface. The most prominent L-PHA/WGA binding glycoproteins in both MDAY-D2 and B16F10 were M, 90,000–140,000, similar in size to two glycoproteins that have previously been linked to the malignant phenotype by changes in their WGA and Con A binding (34). The function of these glycoproteins and their carbohydrates as well as their role in the metastatic process remains to be determined.

These studies suggest that swainsonine or other drugs that inhibit Asn-linked oligosaccharide processing might be used to inhibit either tumor growth or metastasis. In most instances chemotherapeutic agents or biological response modifiers are ineffective for treatment of certain types of tumors but effective on others. For example, m-platinum greatly increased the inhibitory effect of swainsonine on metastasis is more likely to be due to an alteration in the activity of a cell surface glycoprotein rather than the inhibition of its expression at the cell surface. The transport of some glycoproteins to the cell surface (31-33).

ACKNOWLEDGMENTS
I wish to thank Kathy Louste for excellent technical assistance and Astrid Eberhart for secretarial help.

REFERENCES
13. Tulsiani, D. R. P., and Touster, O. Swainsonine causes the production of hybrid glycoproteins by human fibroblasts and rat liver Golgi prepara-

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 1986 American Association for Cancer Research.
Effects of Swainsonine and Polyinosinic:Polycytidylic Acid on Murine Tumor Cell Growth and Metastasis

James W. Dennis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/10/5131

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.