Inhibitors of Carbohydrate Processing: A New Class of Anticancer Agents\textsuperscript{1,2}

Paul E. Goss,\textsuperscript{3} Michael A. Baker, Jeremy P. Carver, and James W. Dennis

The Toronto Hospital, Department of Medical Oncology, Faculty of Medicine [P. E. G., M. A. B.] and Department of Medical Genetics [J. P. C., J. W. D.], University of Toronto, Toronto, Ontario, and Samuel Lunenfeld Research Institute, Mt. Sinai Hospital [J. W. D.], Toronto, Ontario M4X 1K9, Canada

Abstract

There is a need for anticancer agents with novel mechanisms of action. Recently identified molecular targets for new anticancer agents include inducers of cell differentiation, cell cycle arrest, and apoptosis, as well as signaling pathways for growth factors and cytokines.

Another unexplored opportunity is presented by the ubiquitous intracellular glycoprotein glycosylation pathway. This complex process, concerned with the addition of sugars onto newly synthesized proteins, occurs in the lumen of the rough endoplasmic reticulum and in the Golgi. There are estimates of over 200 glycosyltransferase enzymes in this pathway, which results in considerable structural diversity of carbohydrates found on secreted and transmembrane glycoproteins. The specificity of glycosyltransferases for acceptors and sugar-nucleotide donors dictates linkage positions between sugars, anomeric configuration of linkages, and monosaccharide composition. Specific carbohydrate structures participate in cell-cell and cell-substratum interactions affecting processes such as lymphocyte trafficking, immune cell stimulation, embryogenesis, and cancer metastasis.

Of the carbohydrate-processing inhibitors presently available, the alkaloid swainsonine, a Golgi \(\alpha\)-mannosidase II inhibitor, is the first to have been selected for clinical testing based on its anticancer activity, p.o. availability, and low toxicity in mice. Herein, we review the rationale for targeting Golgi carbohydrate processing pathways in the treatment of cancer, and summarize the preclinical and clinical results with swainsonine. Prospects for the development of second generation inhibitors with improved specificity for Golgi-processing enzymes are discussed. Potential clinical applications of this new class of anticancer agents are emphasized.

Carbohydrate Processing and Malignancy

Malignant transformation is associated with a variety of structural alterations in the carbohydrate portion of glycopeptides (1–4). Glycoprotein glycosylation (Fig. 1) begins in the lumen of the rough endoplasmic reticulum (5–8) where a subset of Asn (i.e., N-linked) and Ser/Thr (i.e., O-linked) residues on newly synthesized proteins are subject to the addition of sugars (reviewed in Ref. 6). For \(N\)-linked carbohydrates, Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2} is preassembled on dolichol PP, and then transferred as a unit to Asn-X-Set/Thr sequences of glycoproteins while they are being synthesized. This initial glycosylating event is required for many glycoproteins to fold into their native or active conformation. The Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2} structures are then remodeled or processed as the newly synthesized glycoproteins are transported through the Golgi on route to the cell surface. As depicted in Fig. 1, this begins with trimming by \(\alpha\)-glucosidases and \(\alpha\)-mannosidases I, leaving Man\textsubscript{5}GlcNAc\textsubscript{2}-N which is then substituted by GlcNAc-T1 and trimmed by \(\alpha\)-mannosidase II producing GlcNAcMan\textsubscript{5}GlcNAc\textsubscript{2}-N. The latter structure is a substrate for the GlcNAc-Ts (i.e., GlcNAc-T-II, -IV, -V); each enzyme substitutes a distinct position on the trimannose core to initiate “branches” (6). Cancer cells commonly show increased \(\beta\)-1-6-N-acetylglucosamine (GlcNAc) branching at the trimannosyl core of \(N\)-linked carbohydrates (9–12). For example, increased branching has been noted in primary tumors of human carcinoma of the breast, colon, and skin, and appears also to correlate with disease progression (13, 14). In normal tissues, the \(\beta\)-1-GlcNAc branched carbohydrate structures are restricted to cells capable of invasion including trophoblasts, endothelial cells, interstitial fibroblasts, and activated lymphocytes (14, 15). The key enzyme which initiates the \(\beta\)-1-6 branching is \(\beta\)-1-GlcNAc TV\textsuperscript{4} (see Fig. 1 and Ref. 9).

The antenna or branch initiated by GlcNAc-TV is preferred by subsequent enzymes in the pathway for extension with polygalactosamine (i.e., repeating Gal\(\beta\)-1-4GlcNAc\(\beta\)-1–3), Lewis antigens, and blood-group sequences (Refs. 16–18 and Fig. 1). These sequences are developmentally regulated structures with limited distribution in normal tissues, but they are expressed in human carcinoma and therefore appear to be markers of malignancy (19, 20). Polylactosamine and Lewis antigen expression on tumor cells may contribute to cell-cell adhesion via selectins and \(\beta\)Gal-binding lectins, which are found on the vascular endothelium, and thereby enhance invasion and metastasis by

---

\textsuperscript{1} This work was supported by research grants from the National Cancer Institute of Canada and Medical Research Council of Canada. J. W. D. is a senior research scientist of the National Cancer Institute of Canada.

\textsuperscript{2} We dedicate this review to the memory of Dr. Martin L. Breitman, a colleague and good friend who died of cancer February 13, 1994 at the age of 41 years. Among the many accomplishments in Martin’s scientific career, we are grateful for his contribution to the development of this area, and we will greatly miss his inspiration and friendship.

\textsuperscript{3} To whom requests for reprints should be addressed, at The Toronto Hospital, General Division, 200 Elizabeth Street, mlw 2-022, Toronto, Ontario M4X 1K9, Canada.

\textsuperscript{4} The abbreviations used are: TV, transferase V; CPI, carbohydrate-processing inhibitor; TIMP, tissue inhibitor of metalloproteinases; NK, natural killer; IL, interleukin; LAK, lymphokine-activated killer; TNF, tumor necrosis factor; PBL, peripheral blood lymphocytes; L-PHA, leucophytohemagglutinin.
GIcNAc
(1-NeuNAc and NeuNGc are two naturally occurring and developmentally regulated forms of sialic acids, N-acetylneuraminic acid and N-glycolylneuraminic acid, respectively. The NeuNGc mutation is due to expression of the enzyme CMP-SA hydroxylase, resulting in glycoconjugates with NeuNGc rather than NeuNAc. The a2-6SA-T mutation is due to overexpression of a2-6SA transferase, resulting in SA-linked a2-6 rather than a2-3; the GlcNAc-TV mutation is due to loss of this activity, resulting in decreased I-6GlcNAc-branching of N-linked oligosaccharides; and the UDP-Gal mutation is a defect in transport of this sugar nucleotide into the Golgi, resulting in loss of Gal and SA from glycoproteins.

Metastasis and solid tumor size were measured 17 days after tumor cells were injected. Summary based on data from multiple experiments.

Table 1 Studies on the MDAY-D2 lymphoma

<table>
<thead>
<tr>
<th>Phenotypea</th>
<th>Tumor (cm³)</th>
<th>Spontaneous (s.c.)</th>
<th>Metastasisb</th>
<th>Experiments (i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>4.55 ± 0.40</td>
<td>100, 173, 227, 464, 446</td>
<td>&gt;500, 500, 500, 500, 500</td>
<td></td>
</tr>
<tr>
<td>NeuNGc</td>
<td>2.13 ± 0.49</td>
<td>3, 243, 308, 409, 500</td>
<td>312, 400, 410, &gt;500, 500</td>
<td></td>
</tr>
<tr>
<td>a2-6SA-T</td>
<td>1.81 ± 0.39</td>
<td>61, 98, 137, 144</td>
<td>0, 3, 8, 8, 11</td>
<td></td>
</tr>
<tr>
<td>GlcNAc-TC</td>
<td>1.57 ± 1.32</td>
<td>0, 0, 0, 0, 0, 1, 1, 1</td>
<td>0, 0, 0, 0, 0</td>
<td></td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>0.10 ± 0.05</td>
<td>0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0</td>
<td></td>
</tr>
<tr>
<td>Swainsonine-treated</td>
<td>1.53 ± 0.50</td>
<td>0, 0, 0, 0, 0</td>
<td>50-90% decrease</td>
<td></td>
</tr>
</tbody>
</table>

a NeuNAc and NeuNGc are two naturally occurring and developmentally regulated forms of sialic acids, N-acetylneuraminic acid and N-glycolylneuraminic acid, respectively. The NeuNGc mutation is due to expression of the enzyme CMP-SA hydroxylase, resulting in glycoconjugates with NeuNGc rather than NeuNAc; the a2-6SA-T mutation is due to overexpression of a2-6SA transferase, resulting in SA-linked a2-6 rather than a2-3; the GlcNAc-TV mutation is due to loss of this activity, resulting in decreased I-6GlcNAc-branching of N-linked oligosaccharides; and the UDP-Gal mutation is a defect in transport of this sugar nucleotide into the Golgi, resulting in loss of Gal and SA from glycoproteins. 

b Metastasis and solid tumor size were measured 17 days after tumor cells were injected.

c Summary based on data from multiple experiments.

blood-borne tumor cells (21–23). However, it is important to note that cancer-associated changes in carbohydrate processing appear to affect not only adhesive phenomena, but also multiple other aspects of tumor cell phenotype as discussed below (3).

Interestingly, transformation of cells in tissue culture by activated oncogenes in the ras-signaling pathway (i.e., T24 H-ras, v-src, v-fps, middle T of polyoma virus; Refs. 9, 10, 24, and 25) as well as c-myc (26) has been shown to increase GlcNAc-TV activity. Thus, up-regulation of this and possibly other enzymes in the pathway may represent mandatory steps in the phenotypic expression of some malignancies.

Somatic mutant cell lines with defects in the N-linked carbohydrate processing pathway have been selected from highly malignant tumor cell lines (27, 28). By examining the biochemical defects and malignant properties of independent genetic mutations, a direct association has been observed between expression of B1-6GlcNAc-branched complex-type carbohydrates and malignant potential in these mutant cell lines (28, 29). Mutations selected in the highly metastatic MDAY-D2 lymphoma cell line are listed in Table 1 in order of increasing effect on the malignant phenotype. Mutants with minor structural changes in sialic acid showed slower solid tumor growth but remained metastatic. In contrast, the deficiency in Golgi UDP-Gal transport activity resulted in loss of Gal and sialic acid from both O- and N-linked oligosaccharides, and clearly had the most marked effect on solid tumor growth and metastasis (28).
Mutants deficient in GlcNAc-TV activity affected only the N-linked pathway, and this also profoundly suppressed tumor cell metastasis (24, 28). These mutations are causally related to the loss of malignant potential based on several observations. Examination of independent isolates with mutations in a common gene showed the same phenotype. The mutations have been isolated and characterized several times in independent tumor cell lines, both mouse and human (24, 28, 30, 31). Finally, revertants of the UDP-Gal transport mutation, i.e., mutant cells that had regained the UDP-Gal transport function, also regained the malignant phenotype (32, 33).

More recently, experiments using expression vectors for glycosyltransferases support a direct relationship between 31-GlcNAc-branched oligosaccharides and the malignant phenotype. Immortalized (i.e., premalignant) lung epithelial cells transfected with a GlcNAc-TV expression vector showed relaxed growth controls, increased cell motility, reduced substrate adhesion, and increased incidence of tumorigenesis in nude mice (34). B16 melanoma cells transfected with GlcNAc-TIII, an enzyme that competes with GlcNAc-TV for acceptor substrate, and thereby suppresses the expression of 31-GlcNAc-branched oligosaccharide, has recently been shown to inhibit lung metastasis (35). These observations suggest that up-regulation of GlcNAc-TV can contribute to features of the premalignant cellular phenotype as well as tumor cell metastasis, and may be an important downstream effector of transformation by oncogenes in the ras-signaling pathway.

A number of CPIs isolated from natural sources have been identified, including tunicamycin and several a-glucosidase and a-mannosidase inhibitors (Figs. 1 and 2; Refs. 36 and 37). Indeed, murine tumor cells cultured in the presence of the dolichol-oligosaccharyltransferase inhibitor, tunicamycin, the a-glucosidase inhibitors, castanospermine, 1-deoxynojirimycin, or 1,6-epi-cyclophellitol; the Golgi a-mannosidase I and II inhibitors, 1-deoxymannojirimycin, mannostatin, or swainsonine showed a marked decrease in metastatic potential when injected i.v. into mice (38-46). The position in the pathway where these inhibitors block N-linked carbohydrate processing is shown in Fig. 1. In Fig. 2, the structures of these a-mannosidase and a-glucosidase inhibitors are shown in an orientation that compares them to the a-linked substrate normally cleaved by the enzymes. Table 1 provides an example of the antitumor effect of these inhibitors. Swainsonine-treated tumor cells showed much reduced organ colonization, and tumor-bearing mice treated with the drug showed reduced solid tumor growth and metastases similar to that observed for the GlcNAc-TV mutants (38, 39, 47-49).

Swainsonine, A Prototypic CPI for Cancer Therapy

Swainsonine, or 8ββ3-indolizidine-1α,2α,ββ-triol, is an indolizidine alkaloid first isolated from the Australian plant Swainsona canescens (50), and later from North American plants of the genera Astragalus and Oxypotis (51). Ingestion of these plants by grazing farm animals is known to result in a neurological condition called "loco syndrome" (52, 53). Purified swainsonine alone is now known not to be neurotoxic, but rather loco syndrome may be caused by ingestion of a combination of alkaloids found in Swainsona canescens or Astragalus (54). Swainsonine has been administered to adult mice and rats for periods of up to 2 months with no mortality (47, 55, 56). One effect is that it causes carbohydrate accumulation (i.e., lysosomal storage) in tissues, the least of which occurs in the brain. In a recent report of the distribution of swainsonine when administered to mice, brain levels were noted to be 5-20-fold lower than those measured in other organs such as the bladder, kidney, or thymus gland (57).

Swainsonine is a potent, competitive inhibitor of Golgi a-mannosidase II (Ki = 40 nM; Refs. 58-60) but not a-mannosidase I, and trimming therefore proceeds as far as Man3GlcNAc2 (Fig. 1). Inhibition of a-mannosidase II prevents substitution of the structures by GlcNAc-TII and GlcNAc-TV, thereby diverting the pathway to produce "hybrid-type" carbohydrates (36). Swainsonine also inhibits lysosomal a1-3- and a1-6-mannosidases (IC50 = 70 nM), the degradative pathway for glycoproteins (61-64). This latter activity may produce unwanted clinical side effects and might be eliminated in second generation inhibitors as discussed further below.

As a potential therapeutic agent, swainsonine has several apparent advantages, most notably low preclinical toxicity, compared to inhibitors that block processing earlier in the pathway. For example, tunicamycin, which blocks the transfer of Glc3Man9GlcNAc2 from dolichol to Asn-X-Ser/Thr, is highly toxic in cell culture and when given to mice (65). Toxicity is probably as a result of depletion of the membrane glycoproteins due to the inability of some underglycosylated glycoproteins to fold properly in the endoplasmic reticulum (66). Castanospermine, 1-deoxynojirimycin, and 1-deoxymannojirimycin block processing prior to trimming of the oligomannose chains (Fig. 1), and can also adversely affect intracellular movement of glycoproteins (67-69) or the function of some glycoproteins (68, 70, 71). In contrast, swainsonine does not appear to block membrane localization or secretion of glycoproteins (69, 72-74), and shows low toxicity in tissue culture (38, 48, 75) and in mice (47, 55, 56). Swainsonine is also 20-100-fold more potent than castanospermine, 1-deoxynojirimycin, or 1-deoxymannojirimycin as an inhibitor of carbohydrate processing in cell culture (IC50 = 0.1 μM; Refs. 48 and 76).

Antitumor Effects in Mice

Swainsonine has been administered to tumor-bearing mice in a wide range of dosages by either p.o. administration (39, 48, 77), i.p. injection (78), or infusion with miniosmotic pumps (47). It has been shown to inhibit organ colonization and solid tumor growth rate, and to extend survival of mice following surgical removal of primary murine tumor implants (39, 47, 48, 77, 79). Human colorectal carcinoma tumors growing in athymic nude mice were growth rate inhibited by approximately 50% when mice were provided with drinking water containing 2.5 μg/ml swainsonine (estimated to be 0.3-0.5 mg/kg/day; Ref. 48). Oral administration of swainsonine also inhibited the growth of human melanoma MeWo tumors (47) and human breast carcinoma xenografts in nude mice (80). In another series of experiments, dose-dependent inhibition of carbohydrate processing and tumor inhibition was observed in athymic nude mice bearing either human MeWo melanoma xenografts (47) or metastatic murine mammary carcinoma tumors (81).
Anticancer Mechanisms: Effects on Tumor Cells

Tumor Cell Adhesion to Endothelium. Blood-borne tumor cells escape into secondary tissue by attaching to endothelium, either as single cells or by clumping with circulating host cells, and then extravasating and invading through the extracellular matrix (82). We and others (21, 83, 84) have observed that αGal-binding lectins present on both tumor cells and endothelial cells contribute to the retention of blood-borne tumor cells in the microvasculature (83). As noted above, swainsonine treatment of tumor cells reduces the number of Galβ1-4GlcNAc antennae in N-linked carbohydrates and also reduces attachment of the MDAY-D2 tumor cells to endothelial cell monolayers in vitro (21). In vivo, swainsonine-treated wild-type tumor cells show reduced organ retention on initial pass of the cells through the lung5 (85). As additional evidence of the importance of Galβ1-4GlcNAc antennae, mutant lymphoma cells which lack the Golgi UDP-Gal transporter activity are deficient in organ colonization in vivo, and attach weakly to monolayers of vascular endothelial cells in vitro (21). When these mutant cells were treated with purified β1-4-galactosyltransferase and UDP-Gal to restore βGal to the surface, both organ colonization and adhesion to endothelial cells were partially restored.

Tumor Cell Invasion. Swainsonine is a very effective inhibitor of murine and human tumor cell invasion through extracellular matrix in vitro (86–88). It appears to affect the expression of genes encoding proteins involved in extracellular matrix proteolysis. For example, mouse mammary carcinoma cells cultured in the presence of swainsonine show increased transcription rates for the tissue inhibitor of the metalloproteinase (TIMP-1) gene, while the urokinase plasminogen activator, transcin, and actin are not altered (89). A similar up-regulation of TIMP is observed in the GlcNAc-TV and UDP-Gal transporter glycosylation mutants of MDAY-D2, MeWo, and Chinese hamster ovary cells, suggesting the effect was due to swainsonine’s primary action as an inhibitor of carbohydrate processing (89). TIMP proteins bind to collagens and inhibit their activity, and have been shown to block tumor cell invasion in vivo as well as metastasis in vivo (90, 91). Seftor et al. (87) reported that both swainsonine and castanospermine block invasion through extracellular matrix by human tumor cells. In these cell lines, the drugs also suppressed expression of collagenase type IV (87). Therefore, the effects of swainsonine and other CPIs on tumor cell invasion may be largely due to altered TIMP and collagenase gene expression, which effectively reduces the hydrolysis of extracellular matrix.

Oncogenic Glycoproteins

There is considerable interest in determining which glycoproteins in malignant cells have α-6GlcNAc-branched carbohydrates and require this posttranslational modification to develop the invasive and metastatic phenotype. Since most growth factors and their receptors are glycoproteins, it is possible that cancer-associated changes in glycosylation of certain ones of these molecules may affect signaling pathways in a manner that promotes the malignant phenotype. In this regard, the transmembrane receptor tyrosine kinases such as fms and sea are glycosylated in their extracellular domains, and in addition to constitutively activating mutations, their transforming activity is dependent on glycosylation.

5 S. Yagel and J. W. Dennis, unpublished data.

Fig. 2 Glycosidase inhibitors. Mannose and glucose shown in an α-glycosidic linkage to R are positioned above their analogues swainsonine and 1-deoxymannojirimycin, and castanospermine and 1-deoxynojirimycin, respectively. The structures are vertically aligned and oriented to show their similarity about the forward half of the molecules.
(68, 69). Indeed, castanospermine administered p.o. to nude mice has been shown to reduce the growth of v-fms-induced tumors (92). Swainsonine has been shown to inhibit the growth of T24 H-ras-transfected NIH 3T3 cells in an anchorage-independent manner in soft agar (93). In general, N-linked carbohydrate processing inhibitors appear to induce subtle alterations in the transformed phenotype. Although H-ras and many other oncoproteins are not glycoproteins, they directly or indirectly regulate the expression of many glycoproteins, which in turn are required to create the malignant phenotype (94).

Anticancer Mechanisms: Effects on the Host

Immune Stimulation. Swainsonine, which alters glycosylation of tumors in situ, is not tumor specific, and therefore would be expected to also have effects on host tissues. Thus, in addition to its direct action on tumor cells, its effects in mice and probably in humans include an immune stimulatory activity, which may play a significant role in its antitumor action. The action of swainsonine as an immune modulator has been reviewed by Humphries and Olden (95) and Olden et al. (96). The following section summarizes these effects.

NK Cell Activation. The following observations suggest that antitumor effects of swainsonine in mice are mediated at the level of both the tumor cells and the host immune system. B16F10 melanoma cells grown in the presence of swainsonine show reduced organ colonization when injected i.v. into untreated mice. Untreated tumor cells injected into mice that have been pretreated with swainsonine also produce fewer metastases (39, 77). However, pretreating mice with swainsonine had little effect when NK cells were eliminated in experiments using either bg/bg mice or mice treated with anti-asialo-GM1 antibodies (77). Therefore, in addition to effects on the tumor cells, swainsonine also augments NK cell activity, which participates in the elimination of blood-borne B16F10 tumor cells (79).

Glycosylation mutants of Chinese hamster ovary cells and MDAY-D2 tumor cells have been shown to be more sensitive than wild-type cells to NK cell lysis in vitro (97). In addition, purified oligosaccharides, similar in structure to those found in swainsonine-treated cells, stimulate NK cells in vitro (98). This suggests that NK cell recognition of hybrid-type carbohydrates on target cells may contribute to their activation and/or killing function. In this regard, putative rat and mouse NK receptors have recently been cloned and have been shown to share sequence homology with the family of carbohydrate-binding proteins, the C-type lectins (99).

IL-2 and LAK Cells

Swainsonine is an immune modulator that can stimulate lymphocyte proliferation (100), activate natural antitumor immunity (77, 101), and enhance T cell stimulation by antigen (102). Although speculative, the basis of immune cell activation and bone marrow proliferation may be related to the observation that some lymphokines and growth factors have carbohydrate binding activities (103). This is true for example of IL-1, IL-2, and TNF, all of which have been shown to bind to oligomannose structures (104).

It has been shown that incubation of human lymphocytes in the presence of swainsonine enhances LAK cell activity measured against human colon carcinoma cells (101). Antibodies to IL-2 abolished swainsonine-induced enhancement of LAK cell killing, but lymphocytes showed no increase in IL-2 production or receptor number. These observations suggest that the α-mannosidase inhibitors increase the sensitivity of LAK cells to IL-2 activation (101). Infiltrating lymphocytes and monocytes in tumors are often inactive, but can be activated in vitro by IL-2 and other lymphokines (105). Therefore, α-mannosidase inhibitors with good bioavailability in tumors might serve to sensitize LAK cells, NK cells, and macrophages to local lymphokines.

TNF/IL-1 and Macrophage Activation

Thiglycollate-elicited peritoneal macrophages are activated by culturing the cells in the presence of swainsonine or by injecting swainsonine into the peritoneal cavity (106, 107). The macrophages show increased tumoricidal activity, IL-1 production, induction of protein kinase C activity, and increased cell surface class II histocompatibility antigen Ia (i.e., HLA-DR in humans, see Phase I trial).

Macrophages can be activated by cytokines, notably IL-1 and TNF, and once activated, the cells then produce these factors. Swainsonine has been shown to enhance the toxicity of TNF-α toward WEHI 164 tumor cells, and to increase tumoricidal activation of human monocytes in vitro (108). These observations suggest that swainsonine and IL-1/TNF-α may synergize to enhance direct lymphokine killing of tumor cells as well as by activating host macrophages. In this regard, certain functions or signaling by IL-1 and TNF-α in vivo may depend on their oligomannose-binding activity (108, 109).

Protection against Immunosuppressive Agents

Cancer patients can exhibit depressed immune responses including delayed-type hypersensitivity, depressed NK cell activity, and decreased macrophage migration and phagocytosis (110, reviewed in Ref. 111). Tumors produce, or induce host tissues to express, immunosuppressive proteins such as transforming growth factor β, which is a broadly immunosuppressive cytokine. In this connection, Kino et al. (43) have shown that swainsonine restores B-cell response to normal levels in mice treated with cyclophosphamide or in mice with immunosuppressive tumor burdens.

Swainsonine administered to mice i.v. enhanced bone marrow cellularity, engraftment efficiency, and colony forming units (112). Furthermore, swainsonine treatment has been reported to decrease lethality of methotrexate, 5-fluorouracil, cyclophosphamide, and doxorubicin in nontumor-bearing mice (113). Increased survival of these mice correlated with stimulation of bone marrow proliferation, bone marrow cellularity, and engraftment efficiency in the mice. These data suggest that swainsonine enhances stem cell proliferation in bone marrow, possibly due to increased responsiveness of precursor cells to endogenous lymphokines.

Phase I Trial of Swainsonine in Cancer Patients

We recently reported the results of our first Phase I trial of swainsonine in patients with advanced malignancies (114, 115). The results are summarized here.
The quantitative and qualitative toxicities of chemically synthesized swainsonine were studied in patients given a continuous i.v. infusion over 5 days. Dose levels were escalated in increments of 100 μg/kg/day from 50 to 550 μg/kg/day. Nineteen patients with both advanced solid tumor and hematological malignancies were given a total of 31 courses. The maximum tolerated dose and, thus, the maximum recommended starting dose (maximum tolerated dose–1 level) by this route of administration were 550 and 450 μg/kg/day, respectively. However, as discussed below, biomarker levels assessed in this study suggest that lower doses of swainsonine may produce the therapeutic effects desired.

Common side effects in patients treated included peripheral edema (n = 11/19), mild liver dysfunction in all patients (aspartate aminotransferase up to 4-fold normal), a rise in serum amylose (n = 8/19), and decreased serum retinol levels. Acute respiratory distress syndrome, probably precipitated by swainsonine in one patient with extensive liver metastases and pre-existing liver dysfunction, resulted in a treatment-related death. Other side effects noted included shortness of breath (n = 3), lethargy (n = 4), nausea (n = 1), and skin rash (n = 1). One patient with head and neck cancer showed >50% shrinkage of tumor mass 3 weeks after treatment. Two patients with lymphangitis carcinomatosis on chest X-ray noted improvement in cough and shortness of breath during the infusion of swainsonine and for 1 week thereafter.

Clearance and serum half-life for swainsonine were determined to be approximately 2 ml/h · kg and 0.5 days, respectively. Golgi oligosaccharide processing, the putative anticancer target for swainsonine, was inhibited in PBL as evidenced by a decrease in leucoagglutinin (L-PHA) binding after 5 days of treatment. Oligomannosides in patient urine increased 5–10-fold over the 5 days of treatment, indicating that tissue lysosomal α-mannosidases were also blocked by swainsonine. Urine oligomannoside accumulation reached steady state at 3 days, approximately 1 day after serum drug levels reached steady state. The fraction of HLA-DR-positive cells in PBL increased following 5 days of swainsonine treatment, an effect similar to that observed for PBL from normal subjects cultured with swainsonine. In summary, swainsonine treatment resulted in moderate toxicity in cancer patients with normal pretreatment liver function when administered i.v. at dosages that resulted in moderate toxicity in cancer patients with normal pretreatment liver function when administered i.v. at dosages that reached steady state at 3 days, approximately 1 day after serum drug levels reached steady state. The fraction of HLA-DR-positive cells in PBL increased following 5 days of swainsonine treatment, an effect similar to that observed for PBL from normal subjects cultured with swainsonine. In summary, swainsonine treatment resulted in moderate toxicity in cancer patients with normal pretreatment liver function when administered i.v. at dosages that inhibit both Golgi α-mannosidase II and lysosomal α-mannosidases. In patients with liver metastases and/or dysfunction, significant toxicity occurred resulting in one treatment-related death. We are currently studying chronic p.o. administration of swainsonine in cancer patients selected by criteria similar those applied in our first study. Patients are receiving swainsonine p.o. (50, 150, 300, and 600 μg/kg twice weekly). A dose-dependent trend in hepatic dysfunction and fatigue have been determined as the dose-limiting toxicities. L-PHA lectin binding to PBL is significantly suppressed at the 150-μg/kg dose level, and saturation of tissue lysosomal α-mannosidases occurs between 150 and 300 μg/kg twice weekly. The details of this study will be published later.

Considerations in the Design of New CPIs

The desired properties of CPIs to be useful as anticancer agents include: (a) specificity for a Golgi-processing enzyme(s); (b) low IC₅₀, for inhibition of the target enzyme in vitro and in cell culture; (c) activity in vivo by p.o. administration; (d) low toxicity in animals with chronic exposure to the drug; (e) antitumor effects in preclinical experiments; and (f) activity and acceptable toxicity in clinical trials. Swainsonine meets criteria b–e and shows promise in f. The side effects of swainsonine seen in patients may be due to inhibition of lysosomal α-mannosidases. If this is the case, they might be eliminated by developing an analogue that retains α-mannosidase II inhibitor activity but lacks activity on lysosomal enzymes. This could be particularly important in patients chronically treated with swainsonine.

In vitro the IC₅₀ for inhibition of α-mannosidase II and the IC₅₀ for blocking carbohydrate processing in viable cells is similar, suggesting that swainsonine has optimal cell entry properties (75, 76). Thus, an important consideration in designing second generation CPIs is to maintain both the specificity and potency of swainsonine but also the favorable pharmacokinetics and cell entry properties (76).

Based on the preclinical studies described above, an inhibitor of the enzyme GlcNAc-TV should have potent anticancer activity. Accepter analogues for GlcNAc-TV that serve as competitive inhibitors of the enzyme have been developed (116, 117), but entry into the cells of compounds identified to date is poor. An example is GlcNAcβ1-2(6-deoxy)Manα1-6Glcβ1-OCO₂(CH₂)₇CH₃, which lacks the OH normally substituted by the enzyme, and has a Kᵢ of 70 μM, similar to the Kᵢ for the 6-hydroxylated acceptor and thus has poor cell entry (117). In addition, it is an acceptor for B1-4Gal-T which inactivates its GlcNAc-TV inhibitory activity.

It is possible that drug resistance may be encountered for compounds designed as CPIs. For example, minor alternate-processing pathways exist (118–120), one of which transfers an unusual form of Man₃GlcNAc₂ from dolichol to nascent glycoproteins in the endoplasmic reticulum (Fig. 1). This circumvents the need for α-mannosidase II in the biosynthesis of complex-type carbohydrates. Thus, it is possible that swainsonine treatment of cancer patients may result in selection for tumor cells with enhanced use of an alternate processing pathway. However, it may be possible to minimize drug resistance by using inhibitors acting on different enzymes in the pathway such as GlcNAc-TI where the alternate pathway will also be blocked. On the other hand, the alternate processing pathway allows some normal processing in swainsonine-treated cells, and complete blockage of carbohydrate processing may be more toxic in vivo than the selective block produced by swainsonine.

Other Potential Toxicities of CPIs

The serum half-life of many glycoproteins is dependent on glycosylation and is an important factor in the in vivo bioactivity of cytokines such as erythropoietin (121) and peptide hormones (122). For example, serum transferrin-bearing swainsonine-induced hybrid-type structures have a serum half-life of 14 h compared to 24 h for asialotransferrin and 40 h for the normal sialylated biantennary form of transferrin (123). The biological and clinical consequences of this altered glycoprotein clearance with swainsonine treatment remains to be determined.

---

Swainsonine does not appear to be mutagenic in cell culture, and does not cause acute liver cell damage in animals in sharp contrast to the pyrrolizidine alkaloids, a structurally related class of compounds. The pyrrolizidine alkaloids are found in tansy ragwort (Senecio jacobaea), and their consumption by rats and chickens results in hepatotoxicity associated with depletion of liver retinol stores (124). In chickens fed a diet supplemented with tansy ragwort, the liver histopathology can be prevented by a vitamin A supplement, suggesting that its depletion is a significant factor in hepatotoxicity (124). Serum retinol levels were examined in nine patients in our study, and drug-related depletion was noted in four. In a few patients being treated on our current p.o. swainsonine study, depletion of serum retinol has not been observed. In subsequent clinical studies of swainsonine, the effect of vitamin A supplementation on hepatotoxicity should be examined.

A subset of patients with the genetic disorder called hereditary erythroblastic multinuclearity associated with positive acidic serum shows reduced expression of Golgi a-mannosidase II in table erythroblastic multinuclearity associated with positive acidified serum resembles that observed in swainsonine-treated cells (126), and these patients suffer from chronic anemia. It needs to be determined whether similar findings will occur in patients treated chronically with swainsonine.

It may be necessary to eliminate certain plant lectins such as gliadin from the diet of CPI-treated patients to prevent gastrointestinal toxicity. Gliadin, a wheat protein mixture, causes atrophy of the enterocyte villi in the small intestine, hyperplasia of cryptic cells, and lymphatic infiltration in patients with celiac disease. Celiac disease can be induced in rats fed gliadin plus Astragalus lentiginosus as a source of swainsonine, although either supplement alone has no effect on intestinal villi (127). Proteins in gliadin appear to have high mannose-binding lectin activity, and show increased binding to intestinal villi in rats fed A. lentiginosus (127).

Summary

Preclinical studies on swainsonine have now been reinforced by the antitumor and biological effects seen in patients with advanced malignancy. The studies in mice suggest that CPIs in general may be useful in a wide spectrum of malignancies in both the metastatic and (neo)adjuvant clinical setting. Therefore, an opportunity exists for developing and testing both more potent and less toxic antitumor inhibitors than swainsonine. L-PHA binding on PBL provides a simple means to determine the activity of new CPIs. Furthermore, the assay could readily be applied to sequential biopsies of patients' tumors in order to correlate L-PHA binding in the tumors to the biological effects of the inhibitors.

The antitumor activity of swainsonine in mice has been shown to be synergistic with IFN-α and IL-2 (39, 48, 79), and as such, CPIs may be used in combination with other biological agents or existing anticancer therapies. The immune stimulatory activity of swainsonine in mice suggests that the drug may also be used to enhance recovery of bone marrow and immune function in patients following chemotherapy. Bone marrow transplant patients and patients with other immune incompetent states, e.g., AIDS patients, may also benefit from the apparent hematorestitorative action of swainsonine. However, the relative importance of swainsonine's action on tumor cells versus host immunity is not yet clear, and may vary for different cancers. In a more general context, results to date suggest that CPIs that block α-mannosidase II or enzymes adjacent in the pathway may be useful anticancer agents. If this is indeed the case, inhibitors of GlcNAc-TV as well as other glycosyltransferases may also be developed for the treatment of cancer patients.

Acknowledgments

We thank Dr. Lou Siminovitch for helpful suggestions. We also thank Zofia Kryzek and Cathy Bedlington for secretarial assistance.

References


Inhibitors of carbohydrate processing: A new class of anticancer agents.
