Augmentation of Murine Natural Killer Cell Activity by Swainsonine, a New Antimetastatic Immunomodulator

Martin J. Humphries, Kazue Matsumoto, Sandra L. White, Russell J. Molyneux, and Kenneth Olden

Departments of Oncology [M. J. H., K. M., S. L. W., K. O.] and Microbiology [S. L. W.], Howard University Cancer Center, Washington, DC 20060, and Western Regional Research Center, Agricultural Research Service, USDA, Albany, California 94710 [R. J. M.]

ABSTRACT

Swainsonine, an indolizidine alkaloid, has been found to inhibit the experimental metastasis of B16-F10 melanoma cells when administered systemically to syngeneic C57BL/6 mice. The inhibition was both potent and dose dependent with >80% reduction in pulmonary colonization being observed after only 24-h exposure to 3 μg/ml of swainsonine in drinking water. In contrast, the inhibitory activity of swainsonine was completely abrogated when assays were performed in mice depleted of their natural killer (NK) cell activity either experimentally (anti-asialo-GM1, antibody- or cyclophosphamide-treated C57BL/6 mice) or as a result of genetic mutation (homozygous C57BL/6 beige mice). Swainsonine elicited a 32.0% increase in spleen cell number 2 days after administration and induced a concomitant 2- to 3-fold increase in splenic NK cell activity. These results indicate (a) an absolute requirement for a functional NK cell population in order for swainsonine to exert its inhibitory effects on experimental metastasis, and (b) that the antimetastatic activity of swainsonine is mediated primarily through the ability of the drug to augment NK cell reactivity. On the basis of these findings, swainsonine can be classified as a new immunomodulator that has the ability, at least in a prophylactic setting, to block tumor metastasis.

INTRODUCTION

The development of therapeutic modalities which lead to either prevention or elimination of tumor metastases is currently one of the principal challenges facing oncologists. One promising approach is that of immunotherapy. The administration of various naturally occurring or synthetic biological response modifiers has been reported to enhance host immune reactivity and, in combination with systemic transfer of immune cell populations, has been shown to elicit antitumor activity (1-6). In patients, however, the maximum tolerated dosage of biological response modifiers such as interferon and interleukin 2 is lower than that which exhibits maximum biological activity (7-9). Consequently, the full therapeutic potential of these agents has yet to be realized. Development of strategies to overcome the toxicity of biological response modifiers or, alternatively, identification and characterization of new agents that do not exhibit such side effects would greatly enhance the prospects for immunotherapy of human patients.

In previous studies from this and other laboratories, it has been reported that pretreatment of highly metastatic B16-F10 murine melanoma cells with inhibitors of protein glycosylation or processing (the nucleoside analogue tunicamycin (10, 11) and the plant alkaloids swainsonine (12, 13) and castanospermine (14, 15)) causes a substantial impairment of their pulmonary colonization potential following i.v. injection into mice (16-20). Furthermore, the inhibition arising from pretreatment of cells with one of these agents (swainsonine) has been found to be enhanced by simultaneous systemic administration of free drug to mice (21). In this report we have further investigated the antimetastatic activity of swainsonine and now report that, even when administered alone without pretreatment of tumor cells, the drug possesses potent inhibitory activity in experimental metastasis assays. Furthermore, by testing the effect of swainsonine on metastasis of B16-F10 cells in NK-deficient mice, we present evidence that a principal mode of action of the drug is to enhance NK cell function in vivo.

MATERIALS AND METHODS

Materials. The swainsonine used in these studies was isolated from diablo locoweed (Astragalus oxyphysus) using the procedure described previously (22) or was purchased from Calbiochem (San Diego, CA). Similar results to those presented here on immunomodulation in mice were obtained using chemically synthesized swainsonine, thereby discounting a role for endotoxin in the biological effects of the drug. Furthermore, the swainsonine isolated from plant sources was found to contain undetectable levels of endotoxin based on the Limulus Amebocyte Lysate assay.

Pulmonary Colonization. B16-F10 murine melanoma cells (from Dr. L. J. Fidler, M. D. Anderson Hospital, University of Texas, Houston, TX) were cultured and tested for their pulmonary colonization capacity in C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) exactly as described previously (19, 23). Cells were found to be free of both Mycoplasma and mouse antibody production activity by routine testing. The NK-deficient mice used in these studies were: (a) C57BL/6 mice given injections i.v. of 0.2 ml of anti-asialo-GM1 antibody (Wako Fine Chemicals, Dallas, TX; 1:50 dilution of IgG with phosphate-buffered saline) 2 days before tumor cell injection; (b) C57BL/6 mice given injections i.p. of 0.2 ml of 25 mg/ml of cyclophosphamide (Sigma, St. Louis, MO) in Hanks' balanced salt solution 4 days before tumor cell injection; and (c) homozygous C57BL/6 beige mice (Jackson Laboratory, Bar Harbor, ME).

Pulmonary Retention. The arrest and subsequent clearance of 125I-iododeoxyuridine-labeled B16-F10 cells from the lungs of C57BL/6 mice were examined exactly as described previously (19, 23).

Cytotoxicity Assay. Spleen cells were prepared from either control, NK-deficient, or swainsonine-treated mice and tested in a standard 4-h cytotoxicity assay using 51Cr-labeled B16-F10 cells or YAC-1 cells as the target (24).

RESULTS AND DISCUSSION

Antimetastatic Effect of Systemic Swainsonine Administration. As an initial step towards examining the effects of systemic administration of swainsonine on experimental metastasis of B16-F10 cells, the drug was solubilized, diluted into drinking water, and provided to mice ad libitum for varying periods of time immediately prior to tumor cell injection. As shown in Fig. 1, swainsonine was highly inhibitory; maximal activity of the drug was observed when mice were on swainsonine-supplemented drinking water for only 24 to 48 h. In other experiments, a similar degree of inhibition was found when swainsonine was present not only for 24 h prior to injection of tumor cells but also for the entire postinjection incubation period (data not shown). Half-maximal inhibition of colonization was
ENHANCEMENT OF NK CELL ACTIVITY BY SWAINSONINE

Fig. 1. Effect of swainsonine on experimental metastasis of B16-F10 cells. Swainsonine (SW) was diluted to 3 µg/ml in water and supplied ad libitum for the indicated times to groups of 8 C57BL/6 mice. Aliquots of 9 × 10⁴ B16-F10 cells were then injected into the lateral tail vein, and animals were returned to unsupplemented drinking water. Two wk later, surface melanotic colonies were counted at necropsy. Bars, SE.

Fig. 2. Dose dependence of swainsonine-mediated inhibition of B16-F10 pulmonary colonization. Swainsonine, diluted into drinking water at the indicated concentrations, was supplied to mice for 24 h prior to injection with 9 × 10⁴ B16-F10 cells. Bars, SE. The inhibition obtained by treatment with 3 µg/ml of swainsonine was significant at P < 0.001 using the Mann-Whitney U test.

Fig. 3. Effect of swainsonine on pulmonary retention of B16-F10 cells. In A, mice were provided with unsupplemented drinking water (C, ●) or 3 µg/ml of swainsonine in drinking water (SW, ○) for 24 h prior to injection of 8 × 10⁴ [¹²⁵I]-iododeoxyuridine-labeled tumor cells. At the indicated times, mice were sacrificed by cervical dislocation, and their lungs were excised, exsanguinated, and counted for radioactivity. In B, drinking water containing 3 µg/ml of swainsonine was administered to mice for various times before injection of 5 × 10⁴ radiolabeled tumor cells. Seventeen h later, pulmonary radioactivity was determined as above. Bars, SE.

The effect of swainsonine on the time course of pulmonary retention of B16-F10 cells is shown in Fig. 3a. Cells were radiolabeled in culture with [¹²⁵I]-iododeoxyuridine and then injected i.v. as for experimental metastasis assays. At various swainsonine concentration of 100 ng/ml, and approximately 80% inhibition was obtained at 3 µg/ml (Fig. 2). In other experiments using lower tumor cell inocula, as much as 96% inhibition of colonization was obtained. A similar dose-response curve was found for longer periods of drug administration to mice, demonstrating that prolonged exposure at low concentration had no further beneficial effect (data not shown). For the experiments reported in this study, at least seven different batches of swainsonine from four independent sources have been tested with virtually identical results.

The effect of swainsonine on the time course of pulmonary retention of B16-F10 cells is shown in Fig. 3a. Cells were radiolabeled in culture with [¹²⁵I]-iododeoxyuridine and then injected i.v. as for experimental metastasis assays. At various
times, mice were sacrificed, and the number of cells remaining in the target organ was determined by γ-counting. For control mice on unsupplemented drinking water, there was almost quantitative recovery of injected cells in the lungs 2 min after injection. After a characteristic negative exponential loss of cells over the immediate postinjection period, 2.0% of injected cells remained 24 h later (Fig. 3a). For mice receiving swainsonine in their drinking water, the initial level of retention was similar to that of control mice (≥95%). Furthermore, from 0 to 6 h postinjection, the profile of cell loss was also similar (e.g., at 6 h, 9.3% of control cells remained in the lungs compared to 6.3% in mice treated with swainsonine; Fig. 3a).

By 24 h postinjection, however, the percentage of cells retained in the lungs of swainsonine-treated mice was only 0.46% compared to 2.0% for mice on unsupplemented drinking water (Fig. 3a). Thus, swainsonine appears to elicit accelerated removal of tumor cells that is manifested primarily ≥24 h postinjection.

As expected from the data presented in Fig. 1, the swainsonine-induced decrease in pulmonary retention of tumor cells was also dependent on the time of exposure of mice to drug-supplemented drinking water (Fig. 3b). In this experiment, after 24-h administration of swainsonine, 2.6% of radiolabeled B16-F10 cells were retained in the lungs 17 h after injection compared to 14.1% for the same cells injected into mice receiving unsupplemented drinking water; longer periods of administration had no greater effect (data not shown). An approximate half-maximal effect was obtained by administration of swainsonine for 2 h, a result consistent with the data presented in Fig. 1. Thus, although swainsonine prevents pulmonary colonization after only a brief exposure to mice, the inhibition is not mediated primarily through early effects on injected tumor cells (0 to 10 h postinjection). Instead, swainsonine appears to exert most of its biological activity at least 24 h after administration to mice.

Considering the time course over which swainsonine acts, one potential mechanism of action of the drug could be stimulation of immune system function. Interestingly, there is now substantial experimental evidence implicating carbohydrate residues in immune cell recognition processes (26, 27). In particular, the relationship of cell surface oligosaccharides to NK cell function has been investigated in some detail (28–31). In a related approach to our own, depression of murine lymphocyte proliferation caused by exposure to an endogenous immunosuppressive factor has been reported to be restored by swainsonine treatment (32). Furthermore, the time course over which swainsonine inhibits experimental metastasis [≥24 h after addition, with a peak of activity 2 to 3 days after administration (see Fig. 5 below)] parallels previous reports of the kinetic profile of NK cell activation (33–36). It should also be noted, however, that an equally plausible explanation for the antimitastatic activity of swainsonine is that the drug causes structural alterations in the tumor cell surface oligosaccharides in vivo which in turn lead to an increase in their susceptibility to NK cell lysis.

We have used two approaches to directly test the hypothesis that swainsonine-mediated inhibition of B16-F10 experimental metastasis might be mediated at least partly through its ability to enhance NK cell reactivity. (a) The pulmonary colonization potential of B16-F10 cells was measured in the presence or absence of swainsonine in mice with compromised NK cell function. (b) The effect of systemic swainsonine administration on NK cell activity was examined in a standard 4-h cytotoxicity assay.

Antimetastatic Effect of Swainsonine in NK-deficient Mice.

For the first approach, C57BL/6 mice treated with either anti-asialo-GM1 antibody or cyclophosphamide and C57BL/6 mice homozygous for the beige mutation were used as models for NK deficiency. Asialo-GM1 is a marker for murine NK cells (37, 38), and anti-asialo-GM1 antibody treatment currently represents one of the most specific methods available for removal of NK cells from a mixed population, since immature T-cells are the only other cell type known to express asialo-GM1, and this is at a much lower level than NK cells (37–42). Cyclophosphamide treatment has been reported to reduce NK cell activity (43, 44), but it is also known to suppress other immune cell populations (45–47). Beige mice possess a mutation in an autosomal recessive gene that results in a relatively selective decrease in their NK cell activity (48–51). This defect appears to be linked to an inability of the NK cells in beige mice to secrete cytotoxic factors required for target cell lysis (48). To demonstrate that each of these types of mice were indeed defective in NK function, we measured their endogenous levels of NK cell activity in a 4-h cytotoxicity assay against 51Cr-labeled YAC-1 cells. Consistent with previously published data (37–39, 48–55), both anti-asialo-GM1 antibody and cyclophosphamide treatment almost completely abolished NK activity, and the cytolytic activity of spleen cells from beige mice was approximately 30% that of spleen cells from control C57BL/6 mice (data not shown).

Fig. 4 shows data combined from three sets of independent experiments comparing the metastatic activity of B16-F10 cells in NK-deficient and normal mice receiving either unsupplemented or swainsonine-supplemented drinking water. In all experiments, swainsonine was administered at 3 µg/ml for 24 h. For anti-asialo-GM1 antibody-treated mice on control drinking water, the number of melanotic colonies in the lungs 14
days after tumor cell injection was enhanced approximately 4-fold over control mice receiving the same tumor cell inoculum (Fig. 4a). Injection of normal rabbit serum as a control had no effect on colony number (data not shown). In untreated mice receiving an inoculum of $8 \times 10^6$ B16-F10 cells, swainsonine treatment induced the formation of metastases by 87.8%. In anti-asialo-GM$_1$ antibody-treated mice, the drug treatment was completely ineffective (0.87% stimulation of colonization; Fig. 4a). The possibility that the decreased antitumoral activity of swainsonine might be an artifact of the increased colony number in antibody-treated animals was tested by examining the effect of swainsonine on pulmonary colonization obtained with lower numbers of injected tumor cells. These inocula were designed to yield similar numbers of colonies to that produced by $8 \times 10^6$ cells in non-antibody-treated mice. From the data presented in Table 1, a reduction in the size of the tumor cell inoculum from $8 \times 10^6$ to $4 \times 10^6$ or $2 \times 10^6$ had only a small effect on the degree of inhibition of colonization obtained with swainsonine in anti-asialo-GM$_1$ antibody-treated mice. For example, 87.8% inhibition was obtained in non-antibody-treated mice receiving $8 \times 10^6$ cells (40.0 colonies in the control), but only 4.1% inhibition was observed when $4 \times 10^6$ cells were injected into antibody-treated mice (65.1 colonies in the control). Thus, for control mice bearing an approximately equal number of colonies, the inhibitory activity of swainsonine was markedly different between the two sets of animals. This finding demonstrates that the lack of activity of swainsonine in anti-asialo-GM$_1$ antibody-treated mice reported in Fig. 4a was not due to the high colony number in the control but rather to a specific effect of the antibody.

Very similar results to those observed with anti-asialo-GM$_1$ antibody were obtained by treatment of mice with cyclophosphamide (Fig. 4b). Administration of cyclophosphamide i.p. elicited a 5-fold enhancement of B16-F10 colony formation, but at the same time it completely abrogated the antitumoral activity of swainsonine (70.7% inhibition in untreated mice compared to 10.8% stimulation in cyclophosphamide-treated animals; Fig. 4b).

When mice homozygous for the beige mutation were used for metastasis studies, the inhibition obtained by swainsonine treatment was reduced approximately 3-fold (65.8% inhibition in untreated animals compared to 21.1% in beige mice; Fig. 4c). This significant yet incomplete effect of swainsonine is consistent with the intermediate NK activity of beige mice when assayed in the standard cytotoxicity assay (51) and with previous reports that the NK cells in beige mice can be activated with interferon inducers (52). Since swainsonine administration in Fig. 4 was the same for all animals, yet each of the NK-deficient groups of mice was defective in its response to the antitumoral effects of the drug, these findings are consistent with the hypothesis that swainsonine inhibits experimental tumor metastasis by enhancing NK cell function.

**Effect of Swainsonine on NK Cell Activity.** Our second approach to examining the relationship between swainsonine and NK cell activity was to test the effect of drug administration in a standard NK cytotoxicity assay. Initially, two different strategies were used. (a) Groups of mice were fed swainsonine for 24 h, and then spleen cells were removed and tested for their ability to lyse $^{51}$Cr-labeled B16-F10 cells or YAC-1 cells in comparison to spleen cell preparations from mice receiving unsupplemented drinking water. A representative experiment for YAC-1 cells is shown in Table 2. In this assay, swainsonine treatment had no effect on YAC-1 cell lysis, demonstrating that the drug was not functioning as an activator of NK cells. Similar results to those in Table 2 were obtained by exogenous addition of swainsonine to spleen cells from untreated mice prior to and during the cytotoxicity assay. In addition, no enhancement of B16-F10 cell killing was observed in the same assays (data not shown). (b) We examined the possibility that swainsonine might stimulate spleen cell proliferation rather than activate existing NK cells. In this scenario, no difference would be expected in the cytotoxicity assay performed in Table 2 because all results are normalized to standard effector:target cell ratios and therefore do not take into account possible differences in total spleen cell numbers in the original cell preparations. To correct for this, we examined the NK activity of spleen cells prepared from groups of mice either treated or untreated with swainsonine as before, but rather than diluting each preparation to yield the same effector:target ratios, both sets of cells were resuspended to the same volume and then tested in the cytotoxicity assay. In this way, the NK activity was determined per animal rather than per spleen cell. In these experiments, swainsonine was found to cause a reproducible increase in spleen cell number $[32.0 \pm 22.2\%$ increase in 2 days in 8 independent experiments ($P < 0.05$); see legend to Fig. 5]. Therefore, this approach actually resulted in a higher number of spleen cells being added to the incubation mixture for swainsonine-treated mice. From the data presented in Fig. 5a, it is clear that spleen cell preparations obtained from swainsonine-treated mice 2 days after administration of the drug possessed 2 to 3 times the NK activity of spleen cell preparations from mice receiving unsupplemented drinking water ($26.7 \text{LU}_{50}/10^6$ for control mice and $6.19 \text{LU}_{50}/10^6$ for swainsonine-treated mice). This result was consistently obtained in 6 independent experiments. Interestingly, the swainsonine-induced increase in NK cell activity peaked after 2 days and had declined by 3 days. This result is consistent with the time course profile of NK cell stimulation achieved by administration of previously characterized biological response modifiers (33–36). The increased killing observed

<table>
<thead>
<tr>
<th>Table 1 Examination of the effect of B16-F10 cell inoculum size on the antitumoral activity of swainsonine in anti-asialo-GM$_1$, antibody-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice were depleted of NK activity by i.v. injection of anti-asialo-GM$_1$ antibody 2 days before tumor cell injection. Swainsonine (3 $\mu$g/ml in drinking water) was administered for the 24-h period immediately preceding tumor cell injection. Statistical significance was estimated using the Mann-Whitney U test.</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>SW</td>
</tr>
<tr>
<td>Anti-aGM$_1$</td>
</tr>
<tr>
<td>Anti-aGM$_1$/SW</td>
</tr>
<tr>
<td>Anti-aGM$_1$</td>
</tr>
<tr>
<td>Anti-aGM$_1$/SW</td>
</tr>
<tr>
<td>Anti-aGM$_1$</td>
</tr>
<tr>
<td>Anti-aGM$_1$/SW</td>
</tr>
</tbody>
</table>

* Mean ± SD.

* SW, swainsonine; anti-aGM$_1$, anti-asialo-GM$_1$; NS, not significant.

<table>
<thead>
<tr>
<th>Table 2 Effect of swainsonine on activation of NK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice were provided with either unsupplemented drinking water or drinking water containing 3 $\mu$g/ml of swainsonine for 24 h prior to sacrifice. Spleen cells were then prepared from each group of mice and tested for their ability to lyse $^{51}$Cr-labeled YAC-1 cells at the indicated effector:target cell ratios.</td>
</tr>
<tr>
<td>Effectortarget cell ratio</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Swainsonine</td>
</tr>
</tbody>
</table>

* Mean ± SD.

[Image 12x5 to 600x787]
swainsonine is able to interfere with the pulmonary colonization of B16-F10 cells after only a short, systemic administration to mice; (b) the kinetics of inhibition of both experimental metastasis and pulmonary retention of injected tumor cells is consistent with swainsonine mediating its biological effects 1 to 3 days after administration; (c) NK cells appear to be required for swainsonine function in vivo, since mice with depleted NK cell activity are unable to respond to swainsonine; and (d) direct evidence is presented to show that swainsonine is able to stimulate spleen cell proliferation and that this activity may be one mechanism through which the antitumor effects of the drug are manifested.

Currently, the biochemical mechanisms of swainsonine-mediated enhancement of NK cell activity and inhibition of experimental metastasis are unknown. In the future, it will be particularly instructive to examine whether these effects are related to the well-characterized ability of swainsonine to function as an inhibitor of glycoprotein processing (12, 13, 22, 56–59), or whether the drug has other as yet undiscovered activities. It will also be important to test the antitumoral and antitumor activity of swainsonine in combination with other chemotherapeutic and/or immunotherapeutic agents. Since swainsonine exhibits activity after only transient exposure of animals to microgram levels of the drug, a dosage unlikely to produce side effects, it is conceivable that combination therapy with swainsonine may help alleviate some of the toxicity obtained with other biological response modifiers.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. I. J. Fidler for B16-F10 cells and Dr. Kenneth M. Yamada for critical reviewing of the manuscript.

REFERENCES

13. Colegate, S. M., Dorling, P. R., and Huxtable, C. R. A spectroscopic investigation of swainsonine: an α-mannosidase inhibitor isolated from

*S. L. White et al., manuscript submitted for publication.
ENHANCEMENT OF NK CELL ACTIVITY BY SWAINSONINE


Augmentation of Murine Natural Killer Cell Activity by Swainsonine, a New Antimetastatic Immunomodulator

Martin J. Humphries, Kazue Matsumoto, Sandra L. White, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/6/1410

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.