Effect of Swainsonine on Stimulation and Cell Cycle Progression of Human Lymphocytes

Andrzej Myc, Jolanta E. Kunicka, Myron R. Melamed, and Zbigniew Darzynkiewicz

Sloan-Kettering Institute for Cancer Research, New York, New York 10021

ABSTRACT

The indolizidine alkaloid swainsonine (SW) is an inhibitor of lysosomal α-mannosidase reported to have antimetastatic activity in animal models. The cells grown in its presence develop truncated (hybrid) surface oligosaccharides that may alter their functional properties dependent on interactions of various ligands with membrane receptors. In the present study we observe that SW enhances stimulation of human lymphocytes induced by suboptimal concentration of concanavalin A. The enhancement is manifested by an increased proportion of cells undergoing transition from G0 (G0) to G1 and progressing through the cell cycle (S + G2 + M). In contrast, SW suppresses stimulation of lymphocytes by phytohemagglutinin, and the degree of suppression is greater when measured by the number of cells progressing through the cell cycle (S + G2 + M) than by the proportion of cells entering G1 phase. The suppression remains evident even when SW is added 12 h after phytohemagglutinin, suggesting that SW modifies membrane receptors that develop in G1 and are necessary for cell entrance to S phase. The modification of receptors by SW thus up-regulates stimulation by concanavalin A and down-regulates stimulation by phytohemagglutinin. SW has no effect on lymphocyte stimulation induced by OKT3 monoclonal antibody or on the progression of cells from three leukemic cell lines, HL-60, L1210, and MOLT-4, through the cell cycle. The present data are compatible with the possibility that the reported suppression of the growth of metastatic mouse tumors by SW may be due to the immunomodulatory properties of this alkaloid.

INTRODUCTION

Swainsonine, an indolizidine alkaloid, is a potent inhibitor of lysosomal α-mannosidase and perhaps other mannosidases (Ref. 1; reviewed in Ref. 2). The cells grown in its presence develop truncated (hybrid) surface oligosaccharide chains which contain terminal mannose residues (3) and their functional properties, especially those involving the surface receptor-ligand interactions, are altered as a result of such modifications of the membrane.

It has been reported by Dennis (4) that lymphoid tumor MDAY-D2 and B16F10 melanoma cells were less metastatic when grown in the presence of SW3 for 48 h prior to injection into mice. The treated cells showed a reduction of L-PHA binding glycoprotein in the plasma membrane and a concomitant increase in ability to bind Con A (4). Elbein et al. (2) observed an increase in binding of Con A and a decrease in binding of wheat germ agglutinin but no changes in the cell culture growth rate of B-16 melanoma and other cell lines after treatment with SW. The antimetastatic activity of SW thus may be related to the modification of tumor cell membranes, rendering such cells less able to metastasize because the altered receptors either decrease cell propensity for homing or evoke increased immunological response.

The direct immunomodulatory effect of SW, via its influence on lymphocytes, may play an equally important role in preventing metastases. Namely, it has been reported by White et al. (5) that SW stimulates T-cells in mice. These authors have also observed that SW elicited a 2- to 3-fold increase in splenic NK cell activity, which in turn was correlated with a reduction of metastases of B16F10 melanoma (6). Hino et al. (7) have noted that this alkaloid enhanced incorporation of [3H]thymidine into mouse spleen cells stimulated by Con A. More recently Bowlin et al. (8) have shown that SW augmented proliferation of human lymphocytes induced by suboptimal doses of Con A.

The aim of our present study was to further elucidate the effect of SW on lymphocyte function. To this end the influence of this alkaloid on the stimulation of HPBL by Con A and PHA and their progression through the cell cycle was studied and compared with the response of established leukemic cell lines. Also investigated was the effect of SW on the T3 component of the T-cell receptor.

MATERIALS AND METHODS

Cells. L1210, HL-60, and MOLT-4 cells from the American Tissue Culture Collection (Bethesda, MD) were grown in suspension in culture medium [RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 2 mM l-glutamine, and 10% fetal bovine serum] as described (9). Cells in exponentially growing phase were used for all experiments.

HPBL were isolated by LSM (Litton Bionetics) density gradient centrifugation. Mononuclear leukocytes from interphase were washed 3 times with HBSS and resuspended in culture medium at a concentration of 2 x 10⁶ cells/ml.

Reagents. SW was purchased from Calbiochem (La Jolla, CA) or Sigma (St. Louis, MO), reconstituted in sterile HBSS, and stored at −70°C until used.

Monoclonal antibodies OKT3, OKT11, and OKM5 were purchased from Ortho Diagnostics (Raritan, NJ), MO1 from Coulter Immunology (Hialeah, FL), and anti-IL-2 receptor from Becton Dickinson (Mountain View, CA). OKT3 and OKT11 react with human T-lymphocytes induced by suboptimal doses of Con A. OKM5 and MO1 detect human leukocyte monocytoid and adherent monocytes.

Cell Cultures. Cells were plated in 12-well tissue culture plates (Corning) and PHA (10 µg/ml; Sigma), Con A (20 µg/ml; Sigma), monoclonal antibody OKT3 (25 ng/ml), and/or SW was added. Cells were cultured at 37°C in a humidified 5% CO₂ incubator. Each 24 h, aliquots of cell samples were removed for staining.

Cell Staining. To determine lymphocyte stimulation and analyze their progression through the cell cycle, cells were stained with AO (Polysciences, Inc., Warrington, PA). Details of this method were described previously (10–15). In brief, cell concentration was adjusted to 1 x 10⁶/ml and 0.4 ml of acid detergent (0.1% Triton X-100, 0.08 N HCl, and 0.15 M NaCl) was added to 0.2 ml of culture suspension. Thirty minutes later, 1.2 ml of AO staining solution (6.0 µg of AO, 10⁻³ M EDTA, 0.15 M NaCl, and 0.1 M citrate-phosphate buffer, pH 6) were added to each sample and cells were measured immediately using flow cytometry.

In some cases, direct immunofluorescence staining was used with monoclonal antibodies recognizing human leukocyte surface markers. Aliquots (100 µl) of cell suspension were incubated for 30 min on ice with appropriate monoclonal antibodies conjugated to fluoroscein iso-
thiocyanate. After incubation the cells were washed once and resuspended in 1 ml of HBSS for the surface immunofluorescence analysis.

Fluorescence Measurements. Cell cycle analysis with acridine orange was performed on a FC200 flow cytometer (Ortho Diagnostics, Westwood, MA) as described in detail previously (14, 15). The red (600 to 640 nm) and green (515 to 575 nm) fluorescence emissions from each cell were separated optically and measured simultaneously by separate photomultipliers, and the data were stored in a Compaq Deskpro 386 computer. The number of cells in G₀/G₁, S, and G₂ + M cell cycle compartments was calculated using interactive computer programs as described previously (14, 15). Also, based on differences in RNA content, it was possible to discriminate between G₀ and G₁ cells, as described (18).

Leukocytes stained for surface antigens were analyzed on a Spectrum III Laser Flow Cytometer (Ortho Diagnostics) to determine, in selected subpopulations of lymphocytes, the percentage of positive cells for each particular antibody (16, 17).

RESULTS

SW alone lacks the ability to stimulate lymphocytes to proliferation. When HPBL were cultured with three different concentrations of the alkaloid (0.1, 1.0, or 10.0 μg/ml) for up to 96 h in the absence of PHA, the fraction of proliferating cells (S + G₂ + M) in these cultures was similar to that in negative controls, i.e., in cultures lacking SW, and never was higher than 2.5%. Also the number of cells undergoing transition from G₀ to G₁, as evidenced by an increased RNA content (18), was similar in both sets of cultures and did not exceed 1% (data not shown). In contrast, in positive control cultures of lymphocytes of the same individual containing PHA but not SW, there were over 30% cells in S + G₂ + M by 48 h after addition of mitogen (data not shown).

The effect of SW on stimulation of lymphocytes induced by PHA or Con A was studied in a series of experiments. Addition of SW to HPBL cultures stimulated with PHA at suboptimal concentrations (1 μg/ml) markedly reduced the number of cells undergoing transition from G₀ to G₁ and the number of cells in S, G₂, and M phases of cell cycle (Fig. 1A). The maximal effect was observed after 72 h of stimulation when the number of cells in S + G₂ + M was approximately 4-fold reduced compared to that in the presence of PHA alone and the difference was significant (P < 0.001). The difference was also significant on the second day (P < 0.01). At the optimal concentration of PHA (10 μg/ml), in the presence of a 10 times lower concentration of SW, the number of cells undergoing transition to G₁ was similar to that in cultures lacking SW, but the number of cells progressing through S, G₂, and M was still markedly reduced, as shown in Fig. 1B. Here again, the maximal reduction was seen on day 3, when the number of S + G₂ + M cells was 3-fold lower in the presence of SW (P < 0.001).

Addition of SW at various times before or after PHA had little effect on the degree of suppression of lymphocyte stimulation. Thus, when SW was included in cultures 12 or 1 h before or 1 or 12 h after PHA, the number of cells in S + G₂ + M on the third day of stimulation was reduced, compared to PHA alone (by 60 to 80%), but the degree of reduction was generally similar and not correlated with the time and sequence of SW addition with respect to PHA (data not shown).

In contrast to PHA, stimulation of HPBL by suboptimal concentrations of Con A (1 μg/ml) was markedly enhanced by SW. Thus, whereas Con A alone at that low concentration was a very inefficient mitogen, the combination of Con A and SW resulted in extensive cell stimulation. Both the number of cells entering the cell cycle (G₁) and that of those progressing through S, G₂, and M were increased severalfold when Con A and SW were present in the cultures (Fig. 2). For instance, a 4-fold increase in the proportion of S + G₂ + M cells was observed in cultures containing SW + Con A compared to Con A alone, 48 h after stimulation.

Interestingly, in the cultures stimulated with an optimal dose of Con A (20 μg/ml) SW exhibited cytotoxic effects (Table 1). Thus, between 48 and 96 h, the number of viable cells was reduced by approximately 20% in cultures containing 20 μg/ml Con A and 1 μg/ml SW, in comparison with cultures treated with Con A only. The cytotoxic effect of the Con A + SW combination appears to be selective towards lymphocytes, inasmuch as the remaining viable cells are preferentially labeled with OKM5 and MO1, rather than OKT3 or OKT11 antibodies, and proportionally fewer cells express the IL-2 receptor, compared to cultures containing only Con A (Fig. 3).

The T3 component of the T-cell antigen receptor complex contains two glycoproteins, one of which consists of a 14-kilodalton polypeptide carrying at least three N-linked oligosaccharides (19, 20). To evaluate whether the observed effects of SW on HPBL may be related to modification of the glycoproteins in the T3 component of the T-cell antigen receptor, the ability of cells to respond to OKT3, which is known as a T-
EFFECT OF SW ON HUMAN LYMPHOCYTES

Fig. 2. Cell proliferation in HPBL cultures in the presence of Con A or Con A and SW. Cells were incubated with 1 µg/ml Con A (○) or Con A and SW (10 µg/ml (□)). Results are expressed as a percentage of cells in G₁ (G₁) and S + M + G₂ (P) stages and are the mean values of four experiments.

Table 1 Cytotoxic effect of swainsonine on HPBL stimulated with Con A

| Growth time (h) | % of viable cells |  |  |
|----------------|------------------|------------------|------------------|------------------|
|                | Con A            | Con A + SW (1 µg/ml) | Con A + SW (10 µg/ml) | Con A + SW (100 µg/ml) |
| 24             | 91 ± 2.6         | 79 ± 4.6         | <0.001           |                      |
| 48             | 87 ± 6.3         | 64 ± 9.7         | <0.001           |                      |
| 72             | 88 ± 9.5         | 66 ± 21.5        | <0.05            |                      |
| 96             | 60 ± 15.5        | 40 ± 14.8        | NS               |                      |

* Calculated on the basis of t test; for details see Sokal et al. (23).
* Mean ± SD.
* NS, not significant.

Fig. 3. Percentage of cells stained with OKT3, OKT11, IL-2 receptor, OKM5, and M01 monoclonal antibodies. Before staining, cells were incubated with Con A (20 µg/ml (○)) or with Con A and SW (1 µg/ml), (□) for 72 h.

Fig. 4. Cell proliferation in HPBL cultures in the presence of OKT3 monoclonal antibody or OKT3 and SW. HPBL were incubated with OKT3 (25 ng/ml) (○) or with OKT3 and SW (1 µg/ml) (□). Results are expressed as a percentage of cells in S + M + G₂.

DISCUSSION

The immunomodulatory role of SW has been studied predominantly in mice and reports on the effect of this alkaloid human lymphocytes are scarce. Furthermore, in all studies in which modulation of lymphocyte response to mitogens by SW was analyzed, the assay of lymphocyte stimulation was based on measurements of [³H]thymidine incorporation in bulk, per
culture. This method has several limitations. Namely, although sensitive, it is rather a crude assay which correlates only with DNA replication; it does not allow one to estimate the intercellular variability in cultures nor does it provide any information about the actual number of responding cells. Furthermore, stimulation of lymphocytes that manifests only as G₀-G₁ transition (e.g., not involving IL-2-related steps and progression through the S phase) cannot be detected by the [³H]thymidine assay, yet there are numerous immunological reactions in which response of lymphocytes does not involve their entrance into S phase. Likewise, the radioactive assay in bulk cannot provide any information about the cell cycle distribution in cultures.

In the present study the flow cytomeric method of analysis of lymphocyte stimulation was used, based on simultaneous measurement of cellular DNA and RNA (18), allowing us to circumvent the methodological limitations of earlier investigations, outlined above. We have examined the effect of SW on stimulation of human lymphocytes induced by suboptimal and optimal doses of PHA and Con A and by a specific T-cell mitogen, the OKT3 antibody. In addition to normal human lymphocytes, sensitivity to SW was studied in three leukemic cell lines, two of them human and one of mouse origin.

Addition of SW alone to cultures of HPBL had no stimulatory effect on either the induction of transition of cells from G₀ to G₁ or their entrance into S phase. Our results thus differ from the observation of White et al. (5), who reported that SW induces proliferation of murine splenocytes, but conform to the data of Hino et al. (7), who showed that addition of SW to murine spleen cell cultures neither increased the cell proliferative activity nor affected their viability. Whereas one may expect a different response of HPBL compared to mouse splenocytes, the reason for the discrepancies in the earlier studies is unclear.

It is known that the hybrid type of oligosaccharide, which is synthesized in the presence of SW (3), has diminished L-PHA binding affinity (4, 22). This may explain the suppressive activity of SW on lymphocyte stimulation induced by suboptimal concentrations of PHA, as presently observed. However, higher doses of PHA (10 µg/ml) appear to compensate for this effect and the number of cells undergoing transition from G₀ to G₁ was similar to that observed in cultures lacking SW. In the presence of a concentration of SW as low as 1 µg/ml, the number of cells entering S phase even at high PHA concentration was still reduced. Thus, the effect of SW in suppressing the early events of lymphocyte stimulation by PHA can be overcome. The late effect (entrance to S phase) is still suppressed. The late events involve development of IL-2 receptors, which occurs prior to entrance to S phase and at least 12 h after stimulation with the mitogen and is essential for entry into the S phase (13). The data thus suggested that SW could affect development of the IL-2 receptor, making it less effective in binding this growth factor. The experiments demonstrating that SW was equally potent in suppressing the appearance of cells in S + G₂ + M when added 12 h after PHA as when included prior to PHA provide additional support for the possibility that late developing receptors may play a role in the observed phenomenon. It is unclear why SW does not induce similar suppression of HPBL stimulation by Con A. Perhaps in the latter case the reported increase of IL-2 receptor expression (8) is a secondary effect of SW. The more direct effect of SW on cells stimulated with Con A is the considerable increase in the binding of Con A, which could obscure any decrease in IL-2 receptor expression.

It was reported by Bowlin et al. (8) that SW augmented HPBL proliferation ([³H]thymidine assay) in response to suboptimal (1 µg/ml) Con A. Our results are consistent with their observation. The number of cells entering the cell cycle (G₁) and those in S + G₂ + M after 48-h incubation were 6 and 4 times higher, respectively, in cultures containing SW than in cultures lacking this alkaloid. SW thus appears to modify the Con A receptors in such a way that their affinity for binding the ligand is increased. This modification, however, does not affect lymphocyte stimulation by OKT3. Even at SW concentrations as high as 10 µg/ml, we could not see any significant effect of this alkaloid on T-cell proliferation or entrance to G₁ following stimulation by OKT3 (data not shown).

It was unexpected to find that SW at the low concentration of 1 µg/ml, combined with optimal concentrations of Con A, was cytotoxic. The cytotoxicity was selective towards lymphocytes; the surviving cells were characterized by the immunofluorescence properties typical of the monocyte/macrophage population. A fraction of the surviving cells were proliferating. It is possible that these proliferating cells represent a minor subpopulation of the OKT11 IL-2-positive cells.

The results presented in this paper show that SW has multiple effects on mitogen-stimulated cells. This alkaloid appears to affect the initial steps of lymphocyte stimulation (depending on the nature of the mitogen), as well as their entrance into the cell cycle. Most likely, SW exerts both effects by modulating expression (affinity) of the lymphocyte receptors. Whether these immunomodulating properties of SW may find use in clinical applications remains to be seen.

ACKNOWLEDGMENTS

We thank Paula DeAngelis for excellent technical assistance.

REFERENCES

1. Dorling, P. R., Huxtable, C. R., and Colegate, S. M. Inhibition of lysosomal α-mannosidase by swainsonine, an indolizidine alkaloid isolated from Swain-

---

Table 2 Lack of an effect of swainsonine on the exponential growth of leukemic cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Time of treatment with SW (h)</th>
<th>Control</th>
<th>SW 0.1 µg/ml</th>
<th>SW 1.0 µg/ml</th>
<th>SW 10.0 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>24</td>
<td>G₀ 49</td>
<td>S 28</td>
<td>G₀ 46</td>
<td>S 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G₂ + M 23</td>
<td>21</td>
<td>G₂ + M 45</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>47</td>
<td>38</td>
<td>15</td>
<td>46</td>
</tr>
<tr>
<td>L1210</td>
<td>24</td>
<td>53</td>
<td>22</td>
<td>54</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>17</td>
<td>48</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>51</td>
<td>22</td>
<td>59</td>
<td>23</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>24</td>
<td>55</td>
<td>25</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>30</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>57</td>
<td>26</td>
<td>61</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>20</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>59</td>
<td>25</td>
</tr>
</tbody>
</table>

* Not tested.
EFFECT OF SW ON HUMAN LYMPHOCYTES


Effect of Swainsonine on Stimulation and Cell Cycle Progression of Human Lymphocytes

Andrzej Myc, Jolanta E. Kunicka, Myron R. Melamed, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/11/2879

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.