Antiproliferative Activity in Vitro and in Vivo of the Spicamycin Analogue KRN5500 with Altered Glycoprotein Expression in Vitro

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ABSTRACT

The spicamycin analogue KRN5500 (NSC 650426; SPA) is derived from Streptomyces alanosinicus. The unique structure contains a purine, an aminoheptose sugar, glycine, and a tetradecadiene fatty acid. SPA potently inhibits the growth of certain human tumor cell lines in vitro (IC50 for growth <100 nm) and displays marked activity in vivo in Colo 205 colon carcinoma xenografts. Selective inhibition of labeled precursor incorporation was not evident at 1 or 4 h of exposure to the drug, but at 8 h, [3H]leucine incorporation was inhibited by approximately 40% at or below the IC50 for cell growth. Because of the structural similarity of SPA to inhibitors of glycoprotein processing, we examined the effect of SPA on indicators of glycoprotein synthesis and processing in HL60TB promyelocytic leukemia and Colo 205 colon carcinoma cells. Brief periods of exposure (~30 min) to SPA at the IC50 for growth increased incorporation of [3H]mannose. When examined by Western blotting after prolonged (40–48 h) incubation with lectins that target mannose-containing carbohydrates, Galanthus nivalis agglutinin and concanavalin A, a qualitative change in the pattern of mannose-containing glycoproteins was observed in HL60TB cells. Significant changes in the pattern of surface glycoprotein expression in intact cells were demonstrated by flow cytometry using fluorescence-labeled lectins. An increase in the number of cells binding G. nivalis agglutinin (indicating terminal mannose) was noted, but a decrease in the amount of lectin bound per cell was noted for wheat germ agglutinin (detecting sialic acid and terminal β-N-acetyl glucosamine residues). Electron microscopy revealed loss of microvilli, and the Golgi apparatus appeared inflated. Our findings, therefore, raise the possibility that cells exposed to SPA have altered glycoprotein processing after exposure to low concentrations of drug, prior to the occurrence of overt cytotoxicity. These effects are consistent with a prominent early effect of SPA on the enzymatic machinery or organelles important for proper glycoprotein processing and emphasize the novelty of this agent’s likely mechanism of action.

INTRODUCTION

Spicamycin was originally described as a mixture of unique nucleoside-like components isolated from Streptomyces alanosinicus 879-MT3 (1, 2). The structure of the semisynthetic SPA2 (6-[4-deoxy-4-(2E,4E-tetradecadienoylglycyl)]amino-L-glycero-β-L-mannoheptopyranosyl]amino-9H-purine), shown in Fig. 1, differs from conventional nucleosides in that linkage of the purine to sugar occurs through the purine amino group. All spicamycins contain the common spicamycin amino-nucleoside (L-mannoheptopyranose plus purine; Fig. 1, SAN), linked through glycine (Fig. 1, SAN-gly) to different fatty acid moieties (3). The original spicamycin antibiotic was a mixture of molecules with a common SAN-gly core linked to many different fatty acid components. Subsequent studies with purified semisynthetic spicamycins including KRN5500 have documented antitumor activity in several in vivo models including murine leukemia and human tumor xenografts in athymic mice (4, 5). Based on empirically demonstrated antitumor activity and favorable toxicological and pharmacological properties, SPA will soon be available for Phase I clinical trials in the United States.

The detailed cellular pharmacology of SPA is a matter of current interest and investigation. Kamishohara et al. (5) have described a decrease in protein synthesis following exposure to SPA and related this activity to the hydrolysis of KRN5500 in tumor cells to yield SAN-gly. Although KRN5500 could not inhibit protein synthesis in a rabbit reticuloocyte system, SAN-gly demonstrated comparable potency to puromycin in inhibition of protein synthesis. However, these effects occurred at higher concentrations (IC50 for in vitro protein synthesis, ~2

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2 The abbreviations used are: SPA, spicamycin analogue KRN5500; SAN, spicamycin amino nucleoside; SAN-gly, spicamycin amino nucleoside linked to glycine; NCI, National Cancer Institute; FITC, fluorescein-conjugated; GNA, Galanthus nivalis agglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qd, every day; WGA, wheat germ agglutinin; CFU, colony-forming unit; ConA, concanavalin A; MFI, mean fluorescence intensity.
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target peptide-polyribosome complex as the protein is being processed in the endoplasmic reticulum. Tunicamycin, an inhibitor of the initial stages of oligosaccharide synthesis (transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to dolichol-P), also possesses a fatty acid tail linked through a sugar to the SAN and SAN-gly portions of the molecule (see text).

As glycoprotein processing proceeds, mannose and other sugars are added in a stepwise fashion to the lipid carrier, dolichol phosphate, to form an oligosaccharide donor. The mannose residue is contributed to the oligosaccharide donor via a UDP carrier. The oligosaccharide unit is then transferred to the target peptide-polyribosome complex as the protein is being processed in the endoplasmic reticulum. Tunicamycin, an inhibitor of the initial stages of oligosaccharide synthesis (transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to dolichyl-P), also possesses a fatty acid tail linked through a sugar to uracil (6–8). Because the structure of SPA is reminiscent (although clearly distinct) of tunicamycin, we addressed the hypothesis that SPA could affect glycoprotein processing at concentrations relevant to human tumor cell growth inhibition. We thus clearly distinct) of tunicamycin, we addressed the hypothesis that SPA could affect glycoprotein processing at concentrations relevant to human tumor cell growth inhibition. We found that SPA does indeed alter glycoprotein expression at concentrations that approximate those with antiproliferative effects in vitro; therefore, an effect on glycoprotein synthesis must be considered an early consequence of SPA action, potentially relevant to its antiproliferative mechanism.

MATERIALS AND METHODS

Drugs and Reagents. SPA (KRN5500; NSC 650426; Fig. 1) was provided by Kirin Brewery Co., Ltd. (Tokyo, Japan) to the Developmental Therapeutics Program, NCI. Other drugs were acquired from the Drug Synthesis and Chemistry Branch, NCI. Stock solutions were prepared in DMSO (American Burdick and The Jackson Laboratory, Muskegan, MI). Fluorescein-labeled lectins were obtained from Vector Laboratories, Inc. (Burlingame, CA), and digoxigenin-labeled lectins were from Boehringer Mannheim Co. (Indianapolis, IN). [2-3H]omannose (specific activity, 25 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA); [3H]thymidine (specific activity, 85 Ci/mmol), [3H]uridine (specific activity, 46 Ci/mmol), and [3H]leucine (specific activity, 153 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). Cell culture media and supplements were obtained from Life Technologies, Inc. (Gaithersburg, MD) and Costar Co. (Cambridge, MA); all other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise.

To synthesize the FITC lectin, GNA-FITC, 5 mg of lectin from Galanthus nivalis were dissolved in 0.15 M sodium chloride solution to a final concentration of 10 mg/ml. Thirty-five µl of 1 M NaHCO3/Na2CO3 buffer (pH 9.5) and 1.25 mg of FITC-Celite were added, and the mixture was incubated for 1 h at room temperature. The solution was centrifuged at 10,000 rpm for 3 min, and the supernatant was dialyzed to remove excess FITC-Celite.

Antiproliferative Activity in Vitro. The NCI examines natural products and synthetic compounds in a human tumor cell line screen (9). Quantitation of antiproliferative effect uses an in vitro protein-based sulfonamide B protein stain for viable cell mass, described previously in detail (10). The Colo 205 human colon carcinoma cell line, HL60TB (clone TB human promyelocytic leukemia line), and the W1-38 and MRC-5 human fibroblast cell lines were obtained from the Frederick Cancer Research Tumor Repository (Frederick, MD) and cultured in RPMI 1640 supplemented with 2 mM glutamine and 10% FCS. HL60TB cells grew as a suspension; all others were monolayers. The effects of SPA on tumor cell growth were also assessed using MTT conversion to formazan, as described previously (11).

The soft agar colony-forming assay was performed, as described elsewhere (12), with the Colo 205 and HL60TB cell lines. Cells were plated in agar in 35-mm plates (104 cells/plate); SPA was added (0.1–1000 µM) in separate experiments at 1 or 4 days after plating. Six days after the addition of SPA, the number and size of viable colonies were recorded following visualization of the colonies. Compound efficacy was assessed by:

\[
\%T/C = \frac{\text{Median tumor weight of treated}}{\text{Median tumor weight of control}} \times 100
\]

with a %T/C (where T is treated and C is control) of less than 40, indicative of tumor growth suppression. Compound treatments were initiated when the median tumor weights were 150 ± 50 mg. NSC 650426 was suspended in saline containing 0.05% Tween 80. The compound suspension was administered i.p. either as five daily doses (qd x 5) or as three doses spaced 4 days apart (qd x 3) to groups of six mice. The control group of 20 mice was vehicle (saline/0.05% Tween 80) treated qd x 5 only.
Macromolecular Synthesis and Radioactive Precursor Uptake. DNA, RNA, and protein synthesis were studied by following the incorporation of a 2-h pulse of $[^{3}H]$thymidine, uridine, or leucine, respectively, into Colo 205 and HL60TB cells after exposure to SPA for 1, 4, and 8 h, followed by collection of cells on glass fiber filter mats, and automated scintillation counting (14). The measurement of the ATP content in cells using a bioluminescence photometer (Analytical Luminescence Labs, San Diego, CA) was accomplished by the firefly luciferase method, as described in detail elsewhere (14, 15), which was used previously to detect a specific effect of tyrosin AG17 on tumor cell mitochondria (16).

Incorporation of $[^{3}H]$mannose was studied, as described previously (17). In brief, log phase growing HL60TB and Colo 205 cells were cultured in 162-cm$^2$ flasks (10$^7$ cells/flask). To ensure removal of all traces of glucose and no effect on cell surface glycoproteins by trypsin, adherent cells were detached by scraping, washed with glucose-free RPMI, counted, and stained as follows. Cells were incubated with 1 µg/10$^6$ cells of fluoresceinated lectin for 15 min at 4$^\circ$C, washed in HBSS containing 0.1% BSA, 0.1% sodium azide, and 20,000 events were collected on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). The percentage of cells with positive staining was that with a MFI exceeding the negative controls (unstained cells).

Electron Microscopy. Colo 205 cells were exposed to SPA (50 and 500 nM) for 40 h and harvested by gently scraping. Cells (10$^7$) were washed twice with PBS and fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer. Cell preparation for electron microscopic ultrastructural studies was performed, as described elsewhere (22), and samples were examined using a Hitachi H-7000 electron microscope.

RESULTS

Tumor Cell Growth Inhibition. Of 68 cell lines tested in the NCI in vitro human tumor cell line screen, six (HL60TB, and SR in the leukemia/lymphoma subpanel; colon carcinoma Colo 205; glioma U251; ovarian carcinoma OVCAR-5; and renal carcinoma 786-0) demonstrated striking sensitivity to SPA (IC$_{50}$, <5 nM; IC$_{100}$, <100 nM) in the routine 48-h assessment of growth inhibition. Seventeen of the 68 tumor lines tested were not sensitive to concentrations as high as 10 µM. The remaining cell lines had intermediate degrees of sensitivity. The pattern of the activity of SPA in the screen was unique, not suggesting similarity to any current clinically used agent or to any investigational agent available to NCi. The pattern in the cell screen, thus, suggests that SPA may possess a novel mechanism of growth inhibition (23). To confirm the striking growth inhibitory potency of SPA in certain cell lines and to delineate the mode(s) of action of SPA, we further studied the SPA-sensitive, solid tumor-derived Colo 205 and the promyelocytic leukemia HL60TB cells in vitro. In a 6-day MTT assay, the IC$_{50}$ for Colo 205 was 30 ± 10 nM (SE), and the IC$_{100}$ was 300 ± 95 nM. In the HL60TB line, the IC$_{50}$ was 15 ± 5.6 nM, and the IC$_{100}$ was 52 ± 8.2 nM. Interestingly, two human fibroblast lines, WI-38 and MRC-5, were insensitive to the growth-inhibitory effects of SPA when tested at concentrations up to 20 µM.

In a soft agar colony-forming assay, SPA potently inhibited the colony-forming ability of both HL60TB and Colo 205 (Fig. 2) cells. When following the cumulative volume of CFUs >60 µm, IC$_{50}$ and IC$_{100}$ of SPA for HL60TB were ~10 and ~100 nm, respectively. The IC$_{50}$ and IC$_{100}$ for Colo 205 were ~50 and ~500 nm, respectively. Colony counts decreased in a similar manner with increasing concentrations of SPA.

The detailed behavior of SPA in the NCI drug screen is available upon request.
manner after initial slight stimulation at low concentrations of drug in the case of Colo 205. Subsequent experiments reported in this paper were performed between 10 and 500 nM of drug in these cell types to vary the concentration of exposure between no and complete growth inhibition for each cell type.

Confirmation of in vivo activity was pursued with the Colo 205 xenograft. As shown in Fig. 3, treatment with SPA markedly inhibited Colo 205 tumor growth at several of the dose levels evaluated in the study. The only group in which drug related toxicity occurred was the 50 mg/kg/injection q4d group in which two of six mice died. The remaining dose levels did not produce lethal toxicity nor a significant weight loss in SPA-treated as compared to control mice.

Macromolecular Synthesis. With potent in vitro growth inhibition and marked in vivo antitumor effect, a greater understanding of the molecular mechanism of action of SPA is of importance. The effect of SPA on protein, RNA, and DNA synthesis was evaluated in Colo 205 and HL6OTB cells. For Colo 205, after 1 h of exposure to drug, incorporation of metabolite was not selectively affected at <1 μM drug (Fig. 4A); at 4 h, there was equivalent effect on incorporation of all three precursors, with IC50 between 0.1 and 1 μM and no apparent selectivity for effect on metabolite incorporation (Fig. 4B). However, at 8 h of drug exposure, Fig. 4C indicates more marked inhibition of incorporation of leucine at <50 nM of drug, consistent with the observation of Kamishihara et al. (5) that protein synthesis was preferentially affected by SPA. However, at >50 nM SPA, there was pronounced inhibition of incorporation of all three labeled precursors. Viability of the cells was confirmed by the fact that no changes were observed in intracellular levels of ATP when comparing control and SPA-treated cells exposed to the IC50 for growth for 24 h in either the Colo 205 or HL6OTB cell lines, with continued exclusion of trypan blue (data not shown).

2-[3H]Mannose Incorporation and Protein Glycosylation. SPA has certain structural features that recall tunicamycin, an inhibitor of oligosaccharide synthesis prior to addition of the nascent oligosaccharide to peptides (6–8). Specifically, both compounds possess lipid "tails" linked to unusual nucleoside-like entities. Therefore, mannose incorporation was studied to investigate the potential effects of SPA on glycoprotein processing. Following exposure to SPA for 20 min in the absence and 15 additional min in the presence of label, both HL6OTB and Colo 205 showed a significant (P < 0.05) increase in mannose uptake (Fig. 5); approximately a 50% increase in HL6OTB cells and approximately 100% increase in Colo 205 cells were observed at either IC50 or IC100 concentrations for growth inhibition. At both of these concentrations and durations of exposure, there was no effect of the drug on incorporation of labeled precursors into DNA, RNA, or protein (compare with Fig. 4). In contrast, mannose uptake was inhibited by the glycoprotein processing inhibitors nojirimycin, in both HL6OTB and Colo 205 cells, and by tunicamycin in the concentration used here in HL6OTB cells. As expected, the well-established protein synthesis inhibitor, cycloheximide (4), inhibited mannose uptake in HL6OTB cells. These results are consistent with reported effects of these compounds on mannose incorporation (6–8, 24–26).

These data, therefore, raise the possibility that SPA can potently affect the normal processing of glycoproteins, perhaps by interrupting the glycoprotein synthesis pathway where the normal removal and processing of mannose from immature precursors to mature glycoproteins occurs. To address this possibility directly, the effects of SPA on glycoproteins in whole-cell lysates were examined with the lectins GNA, WGA, and ConA after exposure of HL6OTB cells for prolonged periods to permit treated cells to synthesize glycoproteins in the presence of SPA. Exposure of HL6OTB cells to concentrations of SPA, which inhibit cell growth, causes an increase in terminal and high mannose structures seen in HL6OTB cells (Fig. 6A), consistent with the effect of the drug on incorporation of [3H]mannose after short-term exposures (Fig. 5). A Western blot from HL6OTB cells demonstrates a species at approximately Mr 63,000 that binds GNA, a mannose-specific lectin. This band is not present in lysates from untreated controls or from HL6OTB cells treated with 1 nM SPA, yet is seen after exposure to 10 nM SPA and is even more intense after treatment with 100 nM SPA (the IC100); a similar observation was made for a diffuse, presumptively high mannose-containing structure of molecular weight (Fig. 6A). ConA staining of HL6OTB glycoproteins leads to an analogous conclusion (Fig. 6B). Exposure to SPA induces an increase in the binding of ConA, a probe for high mannose structures, to at least two novel species. Treatment with the dolichololigo-saccharide transferase inhibitor, tunicamycin, caused the opposite effect, with a decrease in the intensity of the material migrating at Mr = 200,000. Exposure of cells to tunicamycin for 48 h led to total loss of ConA-binding structures in the lysates (data not shown). Of interest, when blots of SPA-treated Colo 205 protein were probed with WGA, which binds sialic acid residues, a decrease in lectin binding was observed after exposure to SPA, similar to the effect of tunicamycin (Fig. 6C). These results are of interest because they
suggest that not all carbohydrate structures are increased in relative expression after SPA treatment. SPA may, therefore, selectively increase mannose-containing structures.

Flow Cytometric Analysis of Lectin Binding. Because alterations were observed in the incorporation of mannose and in the pattern of lectin binding to whole-cell lysates following exposure to SPA, we investigated potential changes in the binding of various lectins to the surface of viable HL60TB and Colo 205 tumor cells. Using flow cytometry and following the binding of fluoresceinated lectins before and after SPA treatment, prominent changes were observed after 24 h of exposure to 1IC100 concentrations for growth (Fig. 7). SPA-treated HL60TB cells bound the lectin GNA (which binds terminal 1-3, 1-6, 1-2 linked mannose) more intensely, with 40% more integrated GNA binding in SPA-treated cells compared to vehicle-treated controls (Fig. 7A). Colo 205 cells showed the same effect with respect to GNA binding (>25%; data not shown). The pattern of GNA binding following treatment with standard antitumor agents, fluorouracil (50 μM) or 1,3-bis(2-chloroethyl)-1-nitrosourea (100 μM), did not differ from control cells (data not shown). A second type of SPA-induced alteration in lectin-labeling was seen in the binding

Fig. 3. Effect of SPA on growth of Colo 205 in athymic mouse xenografts. S.c. implanted Colo 205 colon carcinoma xenografts were treated with the following schedules: ■, vehicle control; ○, 13.5 mg/kg qd×5; △, 33.5 mg/kg qd×3; ●, 22.4 mg/kg qd×3; ○, 20 mg/kg qd×5; ×, 30 mg/kg qd×5; □, 50 mg/kg qd×3 (P values of SPA-treated groups in comparison to vehicle-treated groups were calculated using the Mann-Whitney test: 13.5 mg/kg qd×5, P = 0.36; 20 mg/kg qd×5, P = 0.015; 30 mg/kg qd×5, P = 0.005; 22.4 mg/kg qd×3, P = 0.028; 33.5 mg/kg qd×3, P = 0.26; and 50 mg/kg qd×3, P = 0.015). All treatments were intraperitoneal using saline with 0.05% Tween 80 as vehicle. Twenty mice were in the control group, vehicle treated, and six each were in each SPA-treated group.

Fig. 4. Effect of SPA on macromolecular synthesis in Colo 205 cells. Cells were incubated for: A, 1 h; B, 4 h; and C, 8 h with the indicated concentration of SPA, and then incorporation of: ●, [3H]thymidine; ■, [3H]uridine; and △, [3H]leucine assessed as described in "Materials and Methods." Each point is mean (bars, SE) of six determinations, and the figure is representative of three experiments. Control incorporations were (cpm): 1 h: thymidine, 881 ± 79; uridine, 554 ± 15; leucine, 304 ± 16; 4 h: thymidine, 1624 ± 104; uridine, 1021 ± 47; leucine, 310 ± 15; and 8 h: thymidine, 2351 ± 53; uridine, 1228 ± 45; and leucine, 403 ± 25. In C, the effect of 0.001 and 0.01 μM SPA on leucine incorporation is statistically different (P < 0.01) compared to uridine and thymidine, by Student's t test.
Treatment of the lectin WGA, a probe for terminal N-acetylglucosamine or sialic acid residues, where there was a 50% decrease in the amount of lectin bound per cell, resulting in a decrease in the MFI (Fig. 7B). The MFI of WGA-labeled Colo 205 cells decreased by 30% (data not shown). This result is concordant with the decrease in WGA-detectable material observed by Western blot.

**Electron Microscopy.** Ultrastructural analysis was performed to determine if the compartments involved in post-translational modifications of proteins and glycoprotein processing, which include the Golgi apparatus and the endoplasmic reticulum, are altered following exposure to SPA at IC50 and IC100 doses. To mimic the duration of exposure that demonstrates alteration in lectin-binding components (Figs. 6 and 7), cells were exposed to SPA for 40 h. Control Colo-205 cells (Fig. 8A) and SPA-treated cells (Fig. 8, B and C) possessed a heterogeneous cytoplasm with mitochondria, rough endoplasmic reticulum, small vacuoles, and unidentified osmiophilic granules. The Golgi apparatus in SPA-exposed cells appeared more prominent, and the Golgi cisternae were more dilated than those in the control cells (Fig. 8, inserts, B and C). Higher SPA concentrations caused increasing abnormalities of the Golgi compartment, ranging from dilation at 50 nM (Fig. 8B) to disruption of cisternae at the 500 nM (Fig. 8C). Altered Golgi morphology was present in all cells treated for indicated duration and concentration. In addition, there was a decrease in number of microvilli (mv) most appreciated at 500 nM.

![Figure 5](image)

**Fig. 5** [2,3H]Mannose uptake by SPA-treated HL6OTB and Colo 205 cells. A, incorporation of [2,3H]mannose by HL6OTB cells was determined in untreated cells and after exposure to 0.01 and 0.1 μM SPA, 25 μM tunicamycin (TM), 25 μM nojirimycin (NM), and 10 μM cycloheximide (CHX). Cells were exposed to drug for 20 min and pulsed for an additional 15 min with the label. B, incorporation of [2,3H]mannose by Colo 205 cells in the presence of 0.05 and 0.5 μM SPA, 25 μM TM, and 25 μM NM. Depicted in A and B are cpm of treated versus control (C) cells as mean (n = 3; bars, SE) representative of at least three independent experiments. * significant at the level of P < 0.05 (Student’s t test).

![Figure 6](image)

**Fig. 6** Effect SPA on glycoproteins after prolonged exposure to drug. A, HL6OTB(TB) cells were exposed to SPA for 48 h, and mannose-containing glycoproteins were detected by digoxigenin-labeled lectin GNA. Lane 1, control glycoprotein, carboxypeptidase Y (M, 63,000 marker); Lane 2, vehicle; Lane 3, 0.001 μM; Lane 4, 0.01 μM; Lane 5, 0.1 μM SPA. B, mannose-containing glycoproteins were detected after 24 h exposure to drugs but using digoxigenin-labeled ConA. Lane 1, control glycoproteins, transferrin (M, 79,500), and carboxypeptidase Y (M, 63,000); Lane 2, vehicle; Lane 3, 0.01 μM; Lane 4, 0.1 μM SPA; Lane 5, 25 μM tunicamycin. The arrows indicate two novel species that presumptively contain mannose. C, sialic acid content after 24 h exposure to drugs in Colo 205 cell lysates probed with WGA. Lane 1, untreated control cells; Lane 2, 0.01 μM SPA; Lane 3, 0.05 μM SPA; Lane 4, 25 μM tunicamycin.
Fig. 7 Flow cytometric analysis of HL6O cell surface glycoproteins. HL6O cells were exposed to vehicle or 0.1 μM SPA for 24 h and then stained with FITC lectins. A, GNA, and B, WGA, prior to flow cytometry. —, SPA treated; --, vehicle treated.

DISCUSSION

The data presented in this paper allow the following conclusions. SPA possesses potent antiproliferative effects as assessed in certain cell types in vitro and also shows activity in vivo. Although at concentrations ≥IC₅₀ for growth, incorporation of all labeled precursors diminished after 8 h of exposure, at low concentrations of drug (<IC₅₀) there was at best a modest selectivity for inhibition of protein synthesis without a decrease in measures of cell viability. After brief exposures to drug, several features related to the glycosylation of proteins were greatly altered, with an increase in the mass of mannose-containing glycoproteins as assessed by lectin-labeling, flow cytometry, and incorporation of labeled mannose. There is a concomitant decrease in sialic acid and complex carbohydrate-containing structures. Ultrastructural studies revealed an alteration in the appearance of the Golgi apparatus in cells exposed to SPA at concentrations similar to the IC₅₀ for growth after 40 h.

There is no question that the metabolite of SPA (SAN-Gly) without the lipid “tail” can act as an inhibitor of protein synthesis in a cell-free system (5). In addition, Lee et al. (27) have recently demonstrated that SPA-resistant cells showed decreased generation of SAN-gly from radioactive drug, and that permeabilization of the resistant cells to SAN-gly was associated with inhibition of cell growth. However, although any hypothesis for the basis of the action of SPA must take into account the capacity of the metabolite SAN-gly to act as an inhibitor of protein synthesis, agents which clearly inhibit glycoprotein processing as their primary target, e.g., tunicamycin, can also in certain instances, inhibit the incorporation of leucine into protein (6). Elbein had pointed out that this effect could be caused by a disruption of a regulatory link coupling glycosylation to protein synthesis (6, 28). Thus, when glycosylation is blocked or altered, the synthesis of the protein portion of the glycoprotein might also be abnormal (26). An extension of this reasoning might posit that selective inhibition of protein synthesis in a compartment where glycosylation is simultaneously occurring might lead to detectable abnormalities in glycoprotein synthesis or processing.

In this respect, it is intriguing that SPA clearly alters the morphological appearance of the Golgi apparatus, a site of known glycoprotein processing at a step of the synthesis pathway that is closely linked to their eventual migration to the cell surface with or without subsequent secretion. Therefore, our results call for a need to address the capacity of SPA to alter glycoprotein processing. As reviewed recently by Goss et al. (8), sialic acid and complex carbohydrate residues are added to growing glycoprotein chains after mannose-containing core structures have been trimmed in a pathway involving the sequential action of glucosidases, mannosidases, and glycosyl transferases in the Golgi compartment. Thus, SPA disruption of the Golgi-N-linked carbohydrate processing, either through a direct or an indirect mechanism, could account for altered distribution of glycoproteins observed after SPA exposure, as described above and specifically indicated by high mannose accumulation. Thus, SPA might be expected to act after the enzyme or compartment that allows the addition of mannose but then does not allow further progression through the processing pathway.

In comparison to known modulators of glycoprotein processing, SPA may be distinct. One classic inhibitor of glycoprotein processing is tunicamycin. Although there are clear structural similarities between SPA and tunicamycin, functionally SPA appears to induce effects different from those of tunicamycin, an inhibitor of dolichol-oligosaccharide transferase. Tunicamycin prevents initial glycosylation events of asparagine residues in many peptides undergoing glycoprotein assembly. In accordence with previous reports (6, 20), tunicamycin caused a decrease in mannose incorporation. Also, when compared with controls, the binding of GNA to proteins isolated from tunicamycin-treated cells was decreased. SPA induces the opposite effects with an increase in radiolabeled mannose incorporation and an increase in GNA binding on Western blots. Swainsonine is a known antitumor and antimetastatic agent and inhibitor of Golgi-mannosidase II that, therefore, induces a defective Golgi-N-linked carbohydrate pathway. Accumulation of oligomannosides also described for SPA here was used as a clinical marker in swainsonine Phase I trials (8). Additional studies must clarify in a detailed way the similarity of SPA to swainsonine and other inhibitors of glycoprotein processing using purified enzymes and subcellular compartments. Nonetheless, our results do raise the possibility that alteration of expressed glycoproteins could serve as a pharmacodynamic marker of the effect of SPA in vivo, as was done during the Phase I trials with swainsonine (reviewed in Ref. 8).
Fig. 8 SPA-induced ultrastructural modifications. A. Colo 205 cells were exposed to vehicle control; B, 50 nM; C, 500 nM SPA. mv, microvilli; g, Golgi apparatus; vc, vesicular compartment. The insert in each panel shows details of Golgi apparatus; bar, 0.5 μm.
The basis for the differential cytotoxicity of SPA is not yet apparent, because approximately 25% of the cell types in NCI's anticancer drug screen from a variety of histological types were resistant to the action of SPA, whereas in approximately 10% of cell types, there was exquisite sensitivity, with \( IC_{50} \) for cell growth <100 nm. Clarification of cell surface glycoprotein pattern after exposure to drug and of the extent to which the different cell types metabolize SPA to SAN-Gly will be key issues in understanding this differential sensitivity. The molecular mechanism for this drug’s effect remains to be clarified. However, the experiments presented here suggest that the machinery for glycoprotein processing should be examined in detail after exposure to this agent. Reconstitution of these effects in subcellular fractions will be important in further clarifying the action of the drug. In addition, the effect of SPA on specific glycoproteins and their processing will be of interest to examine.

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