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In Vitro Antineoplastic Activity of a Novel Lanthionine-containing Peptide[1]

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ABSTRACT

It has been a long-term goal to discover peptides that can kill tumor cells while sparing normal tissues. Lan-7 is a novel, chemically stable peptide structurally related to somatostatin that contains a lanthionine bridge between the two cysteines in the peptide; TT-232 is a less stable analogue containing a disulfide bridge. The antitumor activity of Lan-7 was examined, relative to that of TT-232 and the clinically used analogue octreotide, against a panel of malignant human tumor cell lines and normal human hematopoietic precursors. Lan-7 was cytotoxic to all four tumor cell lines, with IC_{50} values ranging over a 2-fold range from 16 to 36 μM. The potency of Lan-7 was comparable to that of TT-232, and both of these agents were two to three times more potent than octreotide. At concentrations that were highly cytotoxic to tumor cells, Lan-7 produced no significant toxicity to normal human hematopoietic precursors. Lan-7 induced apoptosis in human ovarian carcinoma 2008 cells over the same concentration range at which it produced cytotoxicity, but it did so without activating G_1, S, or G_2 checkpoints, given that it produced no perturbation of cell cycle phase distribution. Cells engineered to overexpress P-glycoprotein were not more resistant to Lan-7 than isogeneic cells not expressing the *mdr1* gene. These results make Lan-7 of interest as a potential cancer chemotherapeutic agent.

INTRODUCTION

SMS[3] is a tetradecapeptide that exerts a wide variety of biological effects, including inhibition of hormone secretion; antagonism of effects mediated by a variety of growth factors, such as epidermal growth factor and basic fibroblast growth factor, and inhibition of cell proliferation (1). These effects are believed to be mediated via its interaction with one or more of the five SMS receptor subtypes on the surface of the cell that are coupled to several different types of signal transduction pathways, including adenylate cyclase, serine/threonine and tyrosine phosphatases, and ion-conductance channels (2, 3). SMS produces biological effects at nanomolar concentrations, consistent with its affinity for these receptors. SMS is subject to proteolytic digestion and has a very short plasma half-life, and much effort has been expended on the development of analogues with more favorable characteristics. One of these, octreotide, is approved for clinical use to inhibit growth hormone release in patients with acromegaly and for the management of diarrhea and symptoms produced by carcinoid and vasoactive intestinal peptide-secreting tumors arising in the gut (4, 5). Octreotide reduces the release of hormones that mediate these symptoms. However, neither SMS itself nor octreotide directly slows the growth of tumors at clinically tolerable doses, and neither has proven to be of value for treatment of most common types of cancer. Recent efforts have resulted in the discovery of SMS analogues that do have growth-inhibitory effects both *in vitro* and *in vivo* against various endocrine tumors (6–8). A large number of these analogues have been synthesized in an effort to extend the therapeutic spectrum of this class of drugs and enhance selectivity, and in the process several analogues with greater cytotoxicity than the parent compound have been identified, although it is not entirely clear that these produce their biological effects via interaction with the SMS receptors. Among these is TT-232, which has a five-residue ring structure, D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2 (9). TT-232 has no activity as an inhibitor of growth hormone release, but it has antitumor activity in a variety of *in vitro* and *in vivo* tumor models (9–11).

Lan-7 is a lanthionine analogue of TT-232, in which the disulfide bridge between the cysteines has been replaced by a lanthionine bridge; the structures of both of these compounds are shown in Fig. 1. Lan-7 was synthesized with the expectation that it would retain the interesting cytotoxic properties of TT-232 but be more chemically robust based on the greater stability of the lanthionine bridge compared to that of the disulfide bridge. We report here on preclinical studies of the antiproliferative activity of this new analogue against both normal and malignant human cells and on experiments directed at elucidating its mechanism of action and the determinants of sensitivity to this agent.

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[3] The abbreviations used are: SMS, somatostatin; CHO, Chinese hamster ovary; ATRA, all-*trans* retinoic acid; E-BFU, erythroid burst-forming unit; GM-CFU, granulocyte/macrophage colony forming unit; mix-CFU, mixed-lineage colony-forming unit; HPLC, high-performance liquid chromatography; SSTR, cloned SMS receptor.

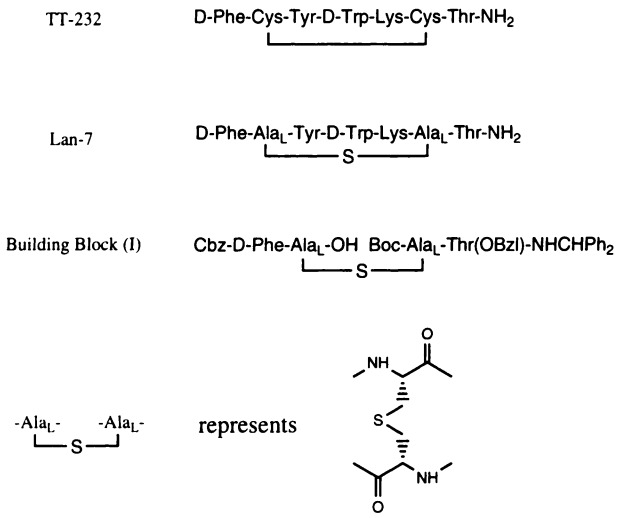

Fig. 1 Structures of Lan-7 and TT-232 and the building block (compound I) from which Lan-7 was constructed.

MATERIALS AND METHODS

Chemicals and Reagents. Cisplatin formulated for clinical use was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Colchicine and sulforhodamine B were obtained from Sigma Chemical Co. (St. Louis, MO). Vinblastine was obtained from Eli Lilly Co. (Indianapolis, IN). RPMI-1640, DMEM, and Iscove's modified Dulbecco's medium were purchased from Irvine Scientific (Irvine, CA). Fetal bovine serum and geneticine were obtained from Life Technologies, Inc. (Gaithersburg, MD).

Oligopeptide Synthesis and Physical and Chemical Characterization. TT-232 was synthesized on an ABD433A synthesizer using MBHA amide resin as solid support and FastMoc 0.1 mmol chemistry. The peptide Boc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp(tBoc)-Lys(tBoc)-Cys(Acm)-Thr(tBu)-MBHA was assembled on the resin followed by oxidation to form the disulfide bond. Final deprotection removed all the peptide from the resin and at the same time cleaved the protecting groups. Lan-7 was synthesized in solution. A tetrapeptide lanthionine building block (Fig. 1, compound I) was synthesized and coupled to the tripeptide Boc-Tyr(ZBr)-D-Trp-Lys(ZCl)-OH. Cyclization following fragment condensation resulted in the fully protected heptapeptide. Cleavage by hydrogen fluoride gas removed all protecting groups. Lan-7 was obtained after HPLC purification. TT-232 and Lan-7 were characterized by mass spectrometry, nuclear magnetic resonance spectroscopy, and analytical HPLC.

Cell Lines and Cell Cultures. The human ovarian adenocarcinoma cell line 2008 (12) and the human head and neck squamous cell carcinoma cell line UMSCC10b (13) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml of penicillin G, 100 μg/ml streptomycin sulfate, and 10% heat-inactivated fetal bovine serum. The human colorectal adenocarcinoma cell line HCT116, which is deficient in DNA mismatch repair due to mutations in both alleles of the *hMLH1* gene, was obtained from American Type Culture Collection (CCL 247); sublines complemented with either chromosome 3 (clone HCT116+ch3) or chromosome 2 (HCT116+ch2) were obtained from Drs. C. R. Boland and M. Koi (14), as was the hMSH2-deficient human endometrial adenocarcinoma cell line HEC59 (15) and a subline complemented with chromosome 2 (HEC59+ch2). Both cell lines were maintained in Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. All of the chromosome-complemented lines were maintained in medium supplemented with geneticine (400 mg/ml for HCT116+ch3 and 600/ml for HEC59+ch2). The absence and presence of expression of hMLH1 in HCT116 and HCT116+ch3 lines as well as expression of hMSH2 in HEC59 and HEC59+ch2 cells were verified by Western immunoblot analysis (data not shown).

The drug-sensitive human epidermoid carcinoma cell line KB-3-1 and a drug-resistant variant, KB-GRC1, derived from KB-3-1 cells by transfection of the *mdr1* gene coupled to a Moloney murine leukemia virus long terminal repeat (16), were maintained in standard DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1% L-glutamine; the KB-GRC1 cell cultures were supplemented with 6 ng/ml colchicine. The mutant *xrs*-6 CHO cell line was derived from parental CHO-K1 cells (17) and is defective in the repair of DNA double-strand breaks due to impaired DNA-dependent protein kinase activity. The xrs-6/Ku80 line was derived by transfection of xrs-6 cells with the expression vector p220LTR, which contains a 2200-bp sequence that encompasses the *Ku80* open reading frame (18); complementation with *Ku80* was demonstrated to restore the DNA double-strand break-rejoining proficiency to the level of the parental CHO-K1 cells (19). Both lines were maintained in DMEM containing 10% fetal bovine serum and 1% L-glutamine; the xrs-6/Ku80 cultures were supplemented with 300 μg/ml hygromycin. All lines tested negative for *Mycoplasma species* and were maintained in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Antitumor Cytotoxicity Assay. Cytotoxicity was assessed using the sulforhodamine B assay according to the procedure reported by Monks *et al.* (20). Cultured tumor cells were seeded into 96-well plates at a density of either 1000 or 2000 cells/well, depending on the plating efficiency of the cell line, in 100 μl of medium and allowed to grow for 24 h. Appropriate concentrations of drugs were added in a final volume of 100 μl of medium per well. Control plates were fixed at the time of drug addition to determine the cellular protein of each well at time 0. Cells were allowed to grow for an additional 48 h in the presence of drug before being fixed by the addition of 25 μl of 50% (w/v) trichloroacetic acid. Cellular protein was stained with sulforhodamine B and measured spectrophometrically with an ELISA plate reader (Molecular Devices, Sunnyvale, CA) (21). The relative growth rate, r, was calculated as reported previously (22). Each experiment was performed in triplicate, and IC_{50} values were estimated by linear interpolation at $r = 0.5$.

Detection of Apoptotic Cells. Cells were trypsinized from culture, washed with PBS, resuspended at 5×10^5 cells/ml, and stained immediately on ice with a mixture of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) prepared

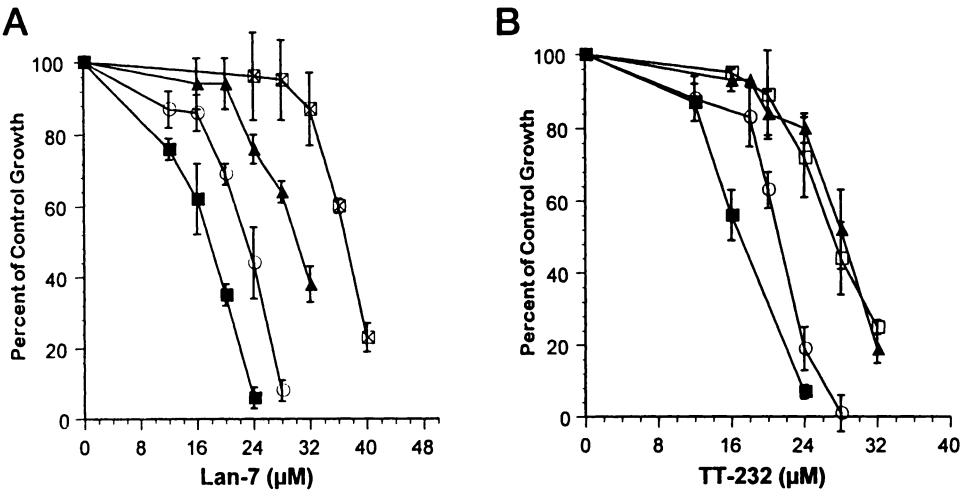

Fig. 2 Growth inhibition of human tumor cell lines by Lan-7 *(A)* and TT-232 *(B)* as measured by the sulforhodamine B assay. ■, 2008 cells; ○, UMSCC10b cells; ▲, HCT116 cells; □ and ⊠, HEC59 cells. Points are means (*bars*, SD) for three to five individual experiments, each conducted with triplicate cultures.

in PBS (23). Stained cells (10 μl) were placed onto a clean microscope slide and examined immediately using a fluorescence microscope; a minimum of 200 cells was scored for early or late apoptotic changes according to standardized criteria (23). Means were determined from triplicate cultures for each data point, and each culture was scored three times using separate aliquots of cells. Each experiment was repeated at least five times. Treatment of 2008 with cells with cisplatin (40 μM) and ATRA (10 μM) for 1 h was included as a positive control; this combination has been validated for the 2008 ovarian cancer cell using other measures of apoptosis (24).

Determination of Cell Cycle Phase Distribution. Approximately 10^5 cells were grown in RPMI in T-25 culture flasks, and before reaching confluency, the cells were exposed to Lan-7 at a concentration of 30 μM for 24, 48, or 72 h. Cells were harvested by trypsinization, washed once with ice-cold PBS, and fixed in ice-cold 70% ethanol at a concentration of 10^6 cells/ml. Cells were again washed in PBS and resuspended in 50 μM propidium iodide containing 1000 units/ml RNase A in PBS. After a 30-min incubation at 37°C, the cells were analyzed on a CytoFluorograf (Ortho Diagnostics Systems, Raritan, NJ). Multicycle Cell Cycle software (Phoenix Flow Systems, San Diego, CA) was used to calculate the fraction of cells in each phase of the cell cycle. This program is based on the mathematical model described by Dean and Jett (25), using normal distribution functions (Gaussians) for G_1 and G_2-M phases and a broadened second-degree polynomial for S phase.

Clonal Growth Assay for E-BFUs, Mix-CFUs, and GM-CFUs. Mobilized peripheral blood progenitor cells were collected by leukophoresis from patients undergoing stem cell transplantation at the University of California, San Diego. Progenitor colony formation was performed by mixing cells in premixed methylcellulose medium (Stem Cell Technologies, Vancouver, BC) and plating 5×10^4 to 1×10^5 cells in duplicate cultures in 35-mm dishes (Nunc, Naperville, IL) as described previously (26).

Statistical Analysis. Comparisons between groups were made using the Student's *t* test assuming unequal variance. The 0.05 level of probability was used as the criterion of significance.

Table 1 Relative toxicity of Lan-7, TT-232 and octreotide as a function of exposure duration

Drug	Cell lines	Exposure time (hs)	IC_{50} (μM)[a]
Lan-7	2008	24	17.05 ± 6.61
		48	16.93 ± 1.84
	UMSCC10b	24	33.88 ± 1.98
		48	27.69 ± 2.17
	HCT116	72	29.97 ± 1.19
	HEC59	72	36.81 ± 0.40
TT-232	2008	24	18.34 ± 1.69
		48	17.89 ± 1.15
	UMSCC10b	24	29.89 ± 1.98
		48	25.24 ± 1.19
	HCT116	72	28.02 ± 1.17
	HEC59	72	27.03 ± 2.17
Octreotide	2008	48	45.04 ± 3.64
	UMSCC10b	48	82.23 ± 0.26
	HEC59	72	55.76 ± 6.26

[a] The values represent mean ± SD calculated from at least three individual experiments using triplicate cultures.

RESULTS

Fig. 2 shows the inhibition of growth rate as a function of drug concentration for Lan-7 and TT-232 determined using the sulforhodamine B assay and a panel of four different human cancer cell lines that included lines derived from ovarian, head and neck, endometrial, and colon carcinomas. Table 1 summarizes the IC_{50} values calculated from the growth inhibition curves for all the cell lines and drug exposure durations tested. The IC_{50} values for Lan-7 varied over a 2-fold range from 16 μM for the ovarian carcinoma 2008 cells to 36 μM for the endometrial carcinoma HEC59 cells. The IC_{50} values for Lan-7 were very similar to those for TT-232, and the rank order of sensitivity for the four cell lines was the same for both agents. Thus, Lan-7 has *in vitro* antineoplastic activity quite comparable to that of TT-232. Both Lan-7 and TT-232 were two to three times more cytotoxic than octreotide against the three cancer cell lines, against which the latter drug was also tested (Table 1).

To determine whether the Lan-7-induced growth inhibition

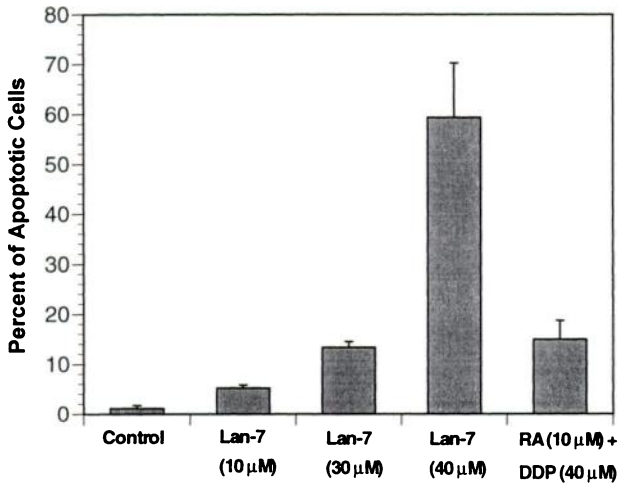

Fig. 3 Induction of apoptosis by Lan-7 in 2008 cells. Values represent the means (*bars*, SD) for 5–10 experiments. The extent of apoptosis produced by each of the Lan-7 concentrations tested, as well as the positive retinoic acid *(RA)*/cisplatin *(DDP)* control, was significantly greater than that observed in untreated cultures ($P < 0.001$).

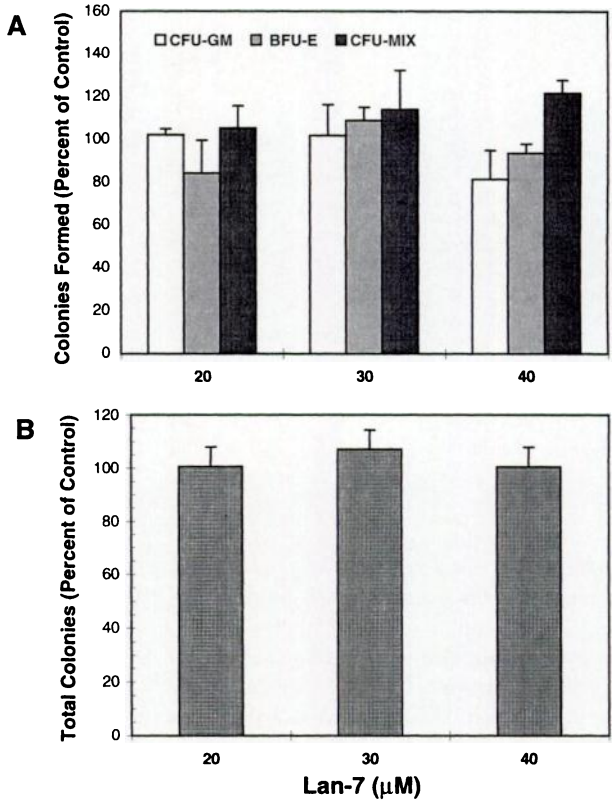

Fig. 4 Cytotoxicity of Lan-7 to normal human hematopoietic progenitor cells. Values represent the means (*bars*, SD) from three independent experiments performed with duplicate cultures. *A*, effect on colony subtypes; *B*, effect on total colony numbers.

was correlated with its ability to induce apoptosis in cancer cells, the fraction of apoptotic 2008 cells was determined following a 24-h exposure to increasing concentrations of Lan-7. A 1-h exposure to cisplatin 40 μM in combination with 10 μM ATRA was used as a positive control for the methodology employed in determining the fraction of apoptotic cells (24, 27). The cisplatin/ATRA combination resulted in 15% apoptotic cells at 24 h. As shown in Fig. 3, Lan-7 produced a concentration-dependent increase in the percentage of apoptotic cells measured after 24 h of exposure that reached 60% at a concentration of 40 μM. Thus, there was good agreement between the ability of Lan-7 to inhibit proliferation as measured by the sulforhodamine B assay and its ability to induce apoptosis for the 2008 cell line.

If Lan-7 is to be useful as a chemotherapeutic agent, then it must demonstrate selective toxicity for malignant cells relative to that for dose-limiting normal tissues. At the present time, the dose-limiting toxicity of Lan-7 *in vivo* is not known. However, hematopoeitic progenitor cells and their proliferating progeny in the bone marrow are often the dose-limiting normal cells for cytotoxic agents. Fig. 4 shows the effects of continuous exposure to Lan-7 at concentrations of 20, 30, and 40 μM on the ability of the GM-CFUs, E-BFUs, and mix-CFUs present in the population of peripheral blood hematopoeitic stem cells to proliferate over a period of 14 days to form colonies in methylcellulose in a total of three experiments performed with triplicate cultures for each drug concentration. Even at a concentration of 40 μM, Lan-7 produced only a small reduction in GM-CFUs and no significant effect on E-BFUs or mix-CFUs. The number of colonies formed expressed as a percentage of those in the untreated control cultures was 81.4 ± 10.3 (mean ± SD) for the GM-CFUs, 93.6 ± 18.3 for the E-BFUs, 121 ± 6.1 for the mix-CFUs, and 100.5 ± 7.3 for total colonies of all types. One additional experiment was carried out at a Lan-7 concentration of 80 μM. This concentration of Lan-7 reduced GM-CFU colonies to 74.8% of control, and mix-CFU and total colonies to 71.7 and 70.8% of control, respectively. To exclude the possibility that Lan-7 was inactivated by binding to methylcellulose, 20 μM Lan-7 was incubated with methylcellose under the conditions used for the hematopoietic progenitor culture and free drug concentration was measured by HPLC following separation from the methylcellulose by ultrafiltration. Lan-7 binds nonspecifically to the ultrafiltration membrane, and in the absence of methylcellulose, the concentration in the ultrafiltrate was 9.7 ± 6.7% of that in the unfiltered standard. In contrast, when methylcellulose was present, the concentration in the ultrafiltrate was increased an average of 5.5-fold to 65.2 ± 10.2% of that in the unfiltered standard. Thus, rather than sequestering Lan-7, the methylcellulose increased its free concentration by displacing it from nonspecific binding sites. Despite the somewhat different types of assays used (growth rate *versus* clonogenic), these data indicate that Lan-7 is very much less toxic to hematopoeitic precursors than to the four tumor cell lines tested.

As a first step toward elucidation of the mechanism by which Lan-7 inhibits growth and causes apoptosis, its effect on cell cycle phase distribution was examined in 2008 cells. Fig. 5 shows that exposure to 30 μM Lan-7, a concentration 1.8 times the IC_{50}, had no detectable effect on the cell cycle phase distribution with up to 72 h of exposure. Similarly, TT-232 also

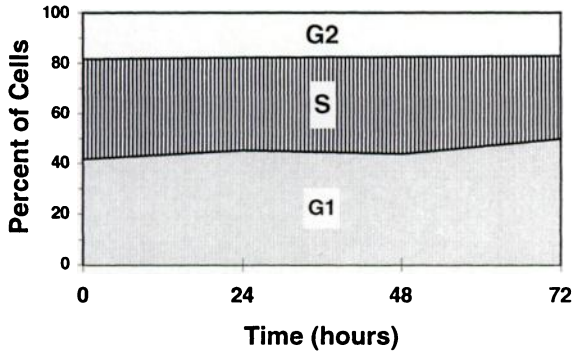

Fig. 5 Effect of Lan-7 on the cell cycle phase distribution. Human ovarian carcinoma 2008 cells were exposed to Lan-7 at 30 μM, and cells were harvested at 24, 48, and 72 h. Duplicate cultures were analyzed for each time point.

did not have an effect on the cell cycle phase distribution of 2008 cells (data not shown). These results suggest that neither drug triggers the G_1, S, or G_2 phase checkpoints activated commonly by other types of chemotherapeutic agents that cause cellular injury.

Mutant cell lines with specific deficiencies in the ability to repair DNA were used to determine whether Lan-7 and TT-232 toxicity was mediated by DNA damage. Loss of the ability to carry out DNA double-strand break repair results in hypersensitivity to γ-radiation and radiomimetic drugs such as bleomycin, whereas loss of DNA mismatch repair results in resistance to some types of platinating (28, 29) and methylating agents (30) as well as 6-thioguanine (31). xrs-6 cells have a defect in the *Ku80* gene that impairs repair of double-strand breaks in DNA; these cells are hypersensitive to γ-radiation. Complementation of the genetic defect by insertion of a wild-type copy of Ku80 on an expression vector restores radiation sensitivity to normal (32). Fig. 6 shows a comparison of the sensitivity of xrs-6 and xrs-6/Ku80 cells to Lan-7 and TT-232 and demonstrates that there was no difference in the survival curves, suggesting that these agents do not cause double-strand DNA breaks. Human colon carcinoma HCT116 cells are deficient in DNA mismatch repair due to mutations in both alleles of *hMLH1*; mismatch repair proficiency is restored in the HCT116+ch3 subline complemented by insertion of an extra chromosome 3 carrying a wild-type copy of *hMLH1*. Likewise, human HEC59 endometrial carcinoma cells are mismatch repair deficient due to mutations in both alleles of *hMSH2*, and proficiency is restored in the HEC59+ch2 subline into which an extra copy of chromosome 2 has been inserted carrying a wild-type copy of *hMSH2*. Fig. 7 shows that, whereas Lan-7 and TT-232 demonstrate somewhat different potencies against the HCT116 versus HEC59 cells, there was no difference in sensitivity to either agent as a function of mismatch repair proficiency *versus* deficiency, suggesting that these drugs do not cause adduct formation in DNA recognized by the mismatch repair system.

P-glycoprotein serves to extrude many types of chemotherapeutic agents and some types of small peptides from the plasma membrane and cytosol (33), and over-expression of P-glycoprotein is a major determinant of sensitivity to many types of drugs (34). Wild-type KB-3-1 human cervical carcinoma cells express essentially no P-glycoprotein, whereas cells of the KB-GRC1 subline, into which an expression vector containing the *mdr1* gene has been inserted, express high levels and are highly resistant to drugs that serve as substrates for the P-glycoprotein pump (35). Fig. 8 shows the survival curves for the KB-3-1 and KB-GRC1 cells following exposure to increasing concentrations of Lan-7. The cytotoxic concentration range for Lan-7 was quite narrow (32–40 μM), and the KB-GRC-1 cells were slightly more sensitive than KB-3-1 cells to growth inhibition by Lan-7; the IC_{50} was 37.0 ± 0.4 μM for the KB-3-1 cells and 34.0 ± 0.6 μM for the KB-GRC1 cells. These results are consistent with the hypothesis that Lan-7 is not a substrate for the P-glycoprotein pump. A separate experiment was performed to document persistence of resistance to vinblastine in the KB-GRC1 cells, and >250-fold resistance was again observed, consistent with previous experiments from this laboratory (data not shown; Ref. 35).

DISCUSSION

Lan-7 is a novel type of structural SMS analogue based on the use of a lanthionine rather than a disulfide intramolecular bridge. The results reported here indicate that Lan-7 shares with TT-232 the ability to inhibit the proliferation of cell lines representative of several different common types of human malignancies with greater potency than octreotide (11). As has been established previously for TT-232 (36), Lan-7 kills cells via apoptosis. In the human ovarian carcinoma 2008 cell line, it produced increasing degrees of apoptosis over the same concentration range required to reduce cell proliferation measured in the sulforhodamine B assay, suggesting that Lan-7 is capable of activating the caspases responsible for executing the apoptotic program (37).

A central question regarding Lan-7 and TT-232 is whether their cytotoxicity is mediated via interaction with one or more of the known SSTRs or whether it is mediated either through some other cell surface receptor system or interaction with an intracellular target. Five SMS receptor subtypes, $SSTR_1$–$SSTR_5$, have thus far been identified (2), and they differ with respect to their ability to mediate antiproliferative effects in response to exposure to various SMS analogues (38). It was demonstrated recently that at least one of these receptors does mediate a change in cell proliferation: expression of $SSTR_2$ in NIH 3T3 cells resulted in growth inhibition due to the activation of SMS secretion that then bound to the $SSTR_2$ on the cell surface and slowed proliferation via an autocrine loop (39). Expression of $SSTR_1$, $SSTR_2$, and $SSTR_3$ mRNA has recently been demonstrated in a variety of primary human tumors by *in situ* hybridization. Among the 55 tumors studied, 22% expressed $SSTR_1$ mRNA, 60% expressed $SSTR_2$, and 25% expressed $SSTR_3$ mRNA (40). Thus, it is possible that the cell lines used in the studies reported here expressed SSTRs and that the effects of Lan-7 and TT-232 were mediated by binding to these receptors. However, the concentrations of Lan-7 and TT-232 required to inhibit cell proliferation were 2–3 orders of magnitude higher than the K_d for the binding of SMS to these receptors, suggesting that if one or more of the SSTRs are involved in

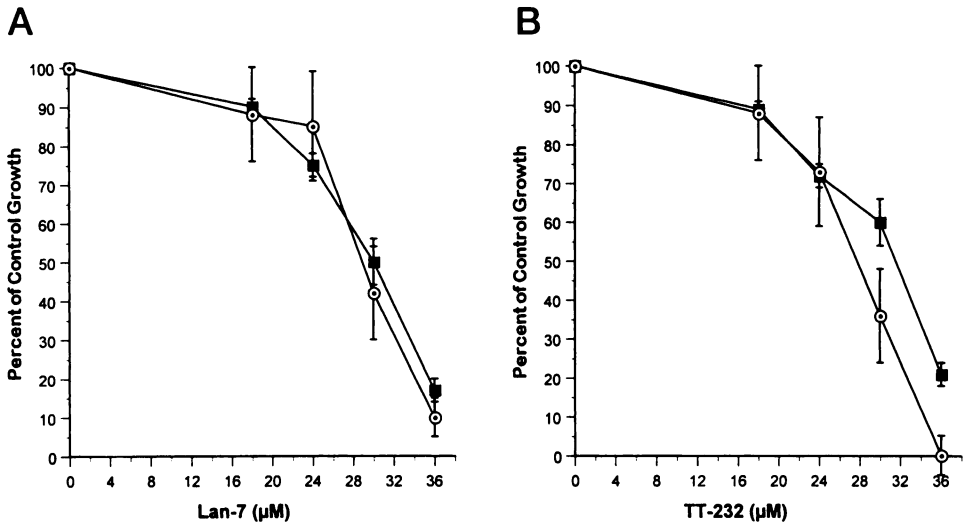

Fig. 6 Cytotoxicity of Lan-7 *(A)* and TT-232 *(B)* to cells defective in Ku80-mediated DNA repair. ■, xrs-6 cells; ○, xsr-6/Ku80 cells. Data points represent the mean (*bars*, SD) for three individual experiments, each conducted with triplicate cultures.

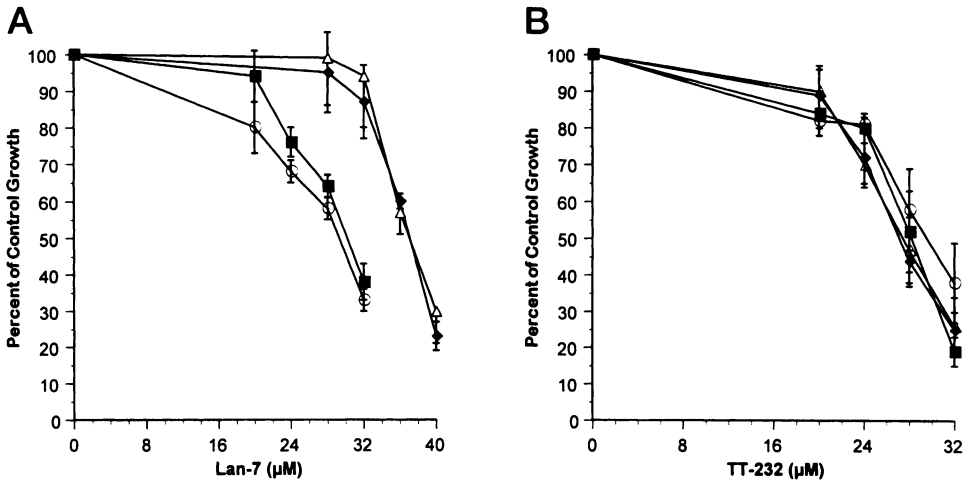

Fig. 7 Cytotoxicity of Lan-7 and TT-232 to cell lines with defects in DNA mismatch repair. ■, HCT116 cells; ○, HCT116/3–6 cells; ◆, HEC59 cells; △, HEC59/2–4 cells. Data points represent the means (*bars*, SD) of three or four individual experiments, each conducted with triplicate cultures.

triggering apoptosis, their affinities for Lan-7 and TT-232 are well below those for SMS. Preliminary studies have in fact confirmed that the binding affinities for Lan-7 to all of the cloned SSTRs is >1 μM.[4] Additional studies are needed to better define the binding affinity of Lan-7 for each of the SSTR subtypes.

A high degree of selectivity for effects on tumor cells is an essential requirement for an anticancer drug. Because myelosuppression is the dose-limiting toxicity for many antiproliferative chemotherapeutic agents, we undertook to assess the cytotoxicity of Lan-7 to human hematopoeitic precursors and found that it produced minimal effects at concentrations that were quite cytotoxic to all four human tumor cell lines included in this study. This is encouraging, in particular because to be scored, a single stem cell must divide through at least five generations in the presence of the drug to be scored in the colony-formation assay used in this study. The lack of toxicity of Lan-7 to human hematopoietic precursors is consistent with the lack of toxicity of TT-232 in mice (11). However, some caution is appropriate, because this type of *in vitro* comparison has only limited value in assessing therapeutic ratio *in vivo*.

It is of some interest that Lan-7 induced apoptosis without producing disruption of cell cycle phase distribution. The cellular injury produced by the great majority of cytotoxic drugs that work by triggering apoptosis also results in activation of one or more cell cycle checkpoints that mediate arrest of progression in G_1, S, G_2, or M phase. Lan-7 appears to be unique in that it can activate the apoptotic mechanism without concurrently activating the major cell cycle checkpoints. This is consistent with our observation that cells with two types of deficits in DNA repair do not demonstrate hypersensitivity to Lan-7. Although none of these experiments establish definitively that Lan-7 does not work by

[4] T. Reisine, personal communication.

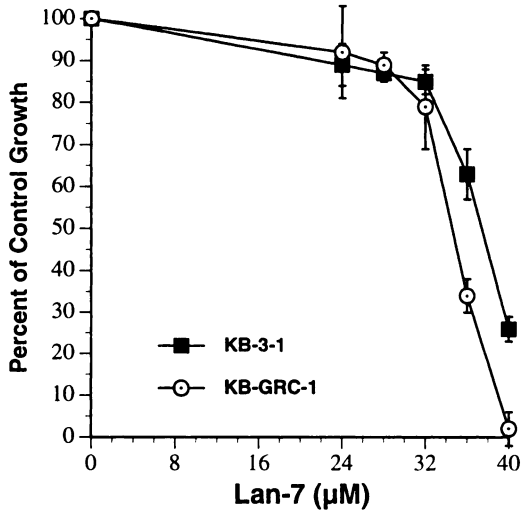

Fig. 8 Effect of overexpression of P-glycoprotein on the cytotoxicity of Lan-7. ■, parental KB-3-1 cells; ○, P-glycoprotein-overexpressing KB-GRC-1 cells. Data points represent the means (*bars*, SD) for three or four individual experiments, each conducted with triplicate cultures.

altering some aspect of DNA function or metabolism, the fact that most drugs that damage DNA also trigger cell cycle checkpoints, and the lack of an effect of loss of two types of DNA repair mitigates against this possibility.

Ku was originally identified as a nuclear autoantigen complex detected by antibodies found in the serum of patients with rheumatoid arthritis and collagen vascular diseases (41). One of its functions is to bind to broken or gapped DNA (42, 43), but a recent study has also, somewhat enigmatically, identified the M_r 86,000 subunit of Ku as a receptor to which SMS and its analogues, such as octreotide, bind at nonamolar concentrations (44, 45). There was no significant difference in the growth inhibition pattern produced by Lan-7 or by TT-232 in the xrs-6 *versus* xrs-6/Ku80 cells, and thus, it may be concluded that Ku80, although shown to be bound specifically by SMS analogues, may not be a physiological molecular target for Lan-7.

The *mdr1* gene product P-glycoprotein is a plasma membrane protein that functions as a multidrug transporter capable of effluxing a variety of antineoplastic agents, such as vinblastine and paclitaxel (46). P-glycoprotein can also transport small peptides out of the cell (33), and this was the basis for determining whether high-level expression of P-glycoprotein could mediate resistance to Lan-7. The results indicated that the KB-GRC1 cells, which express a high level of P-glycoprotein, were no more resistant to Lan-7 than the KB-3-1 cells that do not express P-glycoprotein at all. Thus, assuming that Lan-7 must enter the cell or the plasma membrane to cause injury, it appears that Lan-7 is not a substrate for the P-glycoprotein efflux pump. This increases the attractiveness of Lan-7 as a potential chemotherapeutic agent, because it would be expected to circumvent clinical resistance mediated by P-glycoprotein.

A number of important issues remain to be addressed regarding the potential of Lan-7 as a chemotherapeutic agent; however, the information available at this time is encouraging and mandates further commitment to the development of this class of drugs.

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REFERENCES

1. Lewin, M. J. M. The somatostatin receptor in the GI tract. Annu. Rev. Physiol., *54:* 455–468, 1992.

2. Reisine, T. Somatostatin receptors. Am. J. Physiol., *269:* G813–G820, 1995.

3. Law, S. F., Woulfe, D., and Reisine, T. Somatostatin receptor activation of cellular effector systems. Cell. Signalling, *7:* 1–8, 1995.

4. Bauer, W., Briner, U., Doepfner, W., Haller, R., Huguenin, R., Marbach, P., Petcher, T. J., and Pless, J. SMS-201-995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. Life Sci., *21:* 1133–1140, 1982.

5. Anthony, L., Johnson, D., Hande, K., Shaffer, M., Winn, S., Krozely, M., and Oates, J. Somatostatin analogue Phase I trials in neuroendocrine neoplasms. Acta Oncol., *32:* 217–223, 1993.

6. Setyono-Han, B., Henkelman, M. S., Foekens, J. A., and Klijn, J. G. M. Direct inhibility effects of somatostatin (analogues) on the growth of human breast cancer cells. Cancer Res., *47:* 1566–1570, 1987.

7. Liebow, C., Reilly, M., Serrano, M., and Schally, A. V. Somatostatin analogues inhibit growth of pancreatic cancer by stimulating tyrosine phosphatase. Proc. Natl. Acad. Sci. USA, *86:* 2003–2007, 1989.

8. Bogden, A. E., Taylor, J. E., Moreau, J. P., Coy, D. H., and LePage, D. J. Response of human lung tumor xenografts to treatment with a somatostatin analogue (somatuline). Cancer Res., *50:* 4360–4365, 1990.

9. Keri, G., Mezo, I., Nadasz, Z., Horvath, A., Idei, M., Vantus, T., Balogh, A., Bokonyi, G., Bajor, T., Teplan, I., Tams, J., Mak, M., Horvath, J., and Csuka, O. Structure activity relationship studies of novel somatostatin analogs with antitumor activity. Pept. Res. *6:* 281–288, 1993.

10. Keri, G., Mezo, I., Horvath, A., Vadasz, Z., Balogh, A., Idei, M., Vantus, T., Teplan, I., Mak, M., Horvath, J., Pal, K., and Csuka, O. Novel somatostatin analogs with tyrosine kinase inhibitory and antitumor activity. Biochem. Biophys. Res. Commun., *191:* 681–687, 1993.

11. Keri, G., Erchegyi, J., Horvath, A., Mezo, I., Idei, M., Vantus, T., Balogh, A., Vadasz, Z., Bokonyi, G., Seprodi, J., Teplan, I., Csuka, O., Tejeda, M., Gaal, D. Szegedi, Z., Szende, B., Roze, C., Kalthoff, H., and Ullrich, A. A tumor-selective somatostatin analog (TT-232) with strong *in vitro* and *in vivo* antitumor activity. Proc. Natl. Acad. Sci. USA, *93:* 12513–12518, 1996.

12. Disaia, P. J., Sinkovics, J. G., Rutledge, F. N., and Smith, J. P. Cell-mediated immunity to human malignant cells. Am. J. Obstet. Gynecol., *114:* 979–989, 1972.

13. Krause, C. J., Carey, T. E., Ott, R. W., Hurbis, C., McClatchey, K. D., and Regezi, J. A. Human squamous cell carcinoma: establishment and characterization of new permanent cells lines. Arch. Otolaryngol., *107:* 703–710, 1981.

14. Koi, M., Umar, A., Chaudan, D. P., Cherian, S. P., Carethers, J. M., Kunkel, T. A., and Boland, C. R. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N'*-nitro-*N*-nitroguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res., *54:* 4308–4312, 1994.

15. Umar, A., Boyer, J. C., Thomas, D. C., Nguyen, D. C., Risinger, J. I., Boyd, J., Ionov, Y., Perucho, M., and Kunkel, T. A. Defective

mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J. Biol. Chem., *269:* 14367–14370, 1994.

16. Choi, K., Chen, C., Kriegler, M., and Roninson, I. An altered pattern of cross-resistance in multi-drug resistant human cell results from spontaneous mutation in the *mdr1* (P-glycoprotein) gene. Cell, *53:* 519–529, 1989.

17. Kemp, L. M., Sedwick, S. G., and Jeggo, P. A. X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. Mutat. Res., *132:* 189–196, 1984.

18. Taccioloi, G., Gottlieb, T. M., Blunt, T., Priestly, A., Demengeot, J., Mizuta, R., Lehmann, A., Alt, F. W., Jackson, S. P., and Jeggo, P. A. Ku80: product of the *XRCC5* gene and its role in DNA repair and V(D)J recombination. Science (Washington DC), *265:* 1442–1445, 1994.

19. Ross, G. M., Eady, J. J., Mithal, N. P., Bush, C., Steel, G. G., Jeggo, P. A., and McMillan, T. J. DNA strand break rejoining defect in *xrs*-6 is complemented by transfection with the human *Ku80* gene. Cancer Res., *55:* 1235–1238, 1995.

20. Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., and Boyd, M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst., *83:* 757–765, 1991.

21. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst., *82:* 1107–1112, 1990.

22. Picketts, D. J., Mayanil, C. S., and Gupta, R. S. Molecular cloning of a Chinese hamster mitochondrial related to the "chaperonin" family of bacterial and proteins. J. Biol. Chem., *264:* 12001–12008, 1989.

23. McGahon, A. J., Martin, S. J., Bissonnette, R. P., Mahboubi, A., Shi, Y., Mogil, R., Nishioka, W. K., and Green, D. R. The end of the cell line: methods for the study of apoptosis in vitro. *In:* L. M. Schwartz and B. A. Osborne (eds.), Methods in Cell Biology, pp. 153–181, 1995.

24. Gercel-Taylor, C., Gibb, R. K., and Taylor, D. D. Effect of all-*trans* retinoic acid on the apoptosis induced by cisplatin and taxol in ovarian cancer cell lines. Proc. Am. Assoc. Cancer Res., *37:* 23, 1996.

25. Dean, P. N., and Jett, J. H. Mathematical analysis of DNA distributions derived from flow microfluorometry. J. Cell Biol., *60:* 523–527, 1974.

26. Lane, T. A., Law, P., Maruyama, M., Young, D., Burgess, J., Mullen, M., Mealiffe, M., Terstappen, L. W., Hardwick, A., Moubayed, M., Oldham, F., Corringham, R. E. T., and Ho., A. D. Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF: potential role in allogeneic marrow transplantation. Blood, *85:* 275–282, 1995.

27. Jozan, S., Lafon, C., Mathieu, C., Caliaro, M. J., Barboule, N., Bugat, R., and Valette, A. Enhancement of cisplatin cytotoxicity by all *trans* retinoic acid in a human ovarian carcinoma cell line (OVCCRI) is characterized by apoptosis. Proc. Am. Assoc. Cancer Res., *37:* 414, 1996.

28. Aebi, S. P., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Christen, R., Boland, C. R., Koi, M., Fishel, R., and Howell, S. B. Loss of DNA mismatch repair in acquired resistance to cisplatin. Cancer Res., *56:* 3087–3090, 1996.

29. Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R., and Howell, S. The role of DNA mismatch repair in platinum drug resistance. Cancer Res., *56:* 4881–4886, 1996.

30. Kat, A., Thilly, W. G., Fang, W., Longley, M. J., Li, G., and Modrich, P. An alkylation-toleration, mutator human cell line is deficient in strand-specific mismatch repair. Proc. Natl. Acad. Sci. USA, *90:* 6424–6428, 1995.

31. Aquilina, G., Biondo, R., Dogliotti, E., and Bignami, M. Genetic consequences of tolerance to methylation DNA damage in mammalian cells. Carcinogenesis (Lond.), *14:* 2097–2103, 1993.

32. Boubnov, N. V., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamin, D. M., Pulaski, C. R., Band, H., Reeves, W., and Hendrickson, E. A. Complementation of the ionizing radiation sensitivity, DNA end binding, and V(D)J recombination defects of double-strand break repair mutants by the p86 Ku autoantigen. Proc. Natl. Acad. Sci. USA, *92:* 890–894, 1995.

33. Sharma, R. C., Inoue, S., Roitelman, J., Schimke, R. T., and Simoni, R. D. Peptide transport by the multidrug resistance pump. J. Biol. Chem., *267:* 5731–5734, 1992.

34. Euda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. Expression of a full-length cDNA for the human *"MDR1"* gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc. Natl. Acad. Sci. USA, *84:* 3004–3008, 1987.

35. Shalinsky, D. R., Heath, D. D., Jekunen, A. P., Alcaraz, J. E., and Howell, S. B. Selective modulation of vinblastine sensitivity by 1,9-dideoxyforskolin and related diterpenes in multidrug resistant tumor cells. Br. J. Cancer, *67:* 471–479, 1993.

36. Keri, G., Mezo, I., Horvath, A., Vadasz, Z., Balogh, A., Idei, M., Vantus, T., Teplan, I., Horvath, J., and Szende, B. Novel somatostatin analogs with antitumor activity. *In:* N. Yanaihara (ed.), Peptide Chemistry 1992, pp. 334–336. Leiden: ESCOM Science Publishers BV, 1993.

37. Fraser, A., and Evan, G. A license to kill. Cell, *85:* 781–784, 1996.

38. Buscail, L., Esteve, J-P., Saint-Laurent, N., Bertrand, V., Reisine, T., O'Carroll, A-M., Bells, G. I., Schally, A. V., Vaysse, N., and Susini, C. Inhibition of cell proliferation by the somatostatin analogue RC-160 is mediated by somatostatin receptor subtypes SSTR2 and SSTR5 through different mechanisms. Proc. Natl. Acad. Sci. USA, *92:* 1580–1584, 1995.

39. Rauly, I., Saint-Laurent, N., Delesque, N., Buscail, L., Esteve, J. P., Vaysse, N., Susini, C. Induction of a negative autocrine loop by expression of Sst2 somatostatin receptor in NIH 3T3 cells. J. Clin. Invest., *97:* 1874–1881, 1996.

40. Reubi, J. C., Schaer, J. C., Waser, B., and Mengod, G. Expression and localization of somatostatin receptor SSTR2 and SSTR3 messenger RNAs in primary human tumors using *in situ* hybridization. Cancer Res., *54:* 3455–3459, 1994.

41. Reeves, W. H. Antibodies to the p70/p80 (Ku) antigens in systemic lupus erythematosus. Annu. Rev. Physiol., *54:* 455–468, 1992.

42. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Mol. Cell. Biol., *12:* 5041–5049, 1992.

43. Bannister, A. J., Gottlieb, T. M., Kouzarides, T., and Jackson, S. P. c-Jun is phosphorylated by the DNA-dependent protein kinase *in vitro*: definition of the minimal kinase recognition motif. Nucleic Acids Res., *12:* 1289–1295, 1993.

44. Le Romancer, M., Cherifi, Y., Reyl-Desmars, F., and Lewin, M. J. Somastostatin specifically binds p86 subunit of the autoantigen Ku. C. R. Acad. Sci., *316:* 1283–1285, 1993.

45. Le Romancer, M., Reyl-Desmars, F., Cherifi, Y., Pigeon, C., Bottari, S., Meyer, O., and Lewin, M. J. M. The 86-kDa subunit of autoantigen Ku is a somatostatin receptor regulating protein phosphatase-2A activity. J. Biol. Chem., *269:* 17464–17468, 1994.

46. Choi, K., Chen, C., Kriegler, M., and Roninson, I. B. An altered pattern of cross-resistance in multi-drug resistant human cells results from spontaneous mutation in the *MDR*1 (P-glycoprotein) gene. Cell, *53:* 519–529, 1988.