Regulation of Targeted Chemotherapy with Cytotoxic Luteinizing Hormone-Releasing Hormone Analogue by Epidermal Growth Factor


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ABSTRACT

Targeting chemotherapy selectively to cancers can reduce the toxic side effects. AN-152, a conjugate of doxorubicin and [d-Lys₆]-luteinizing hormone-releasing hormone (LH-RH), is more potent against LH-RH receptor-bearing cancers and produces less peripheral toxicity than doxorubicin. Many cancers, e.g., 50% of breast cancers, but few normal tissues express these receptors, providing a selective target for this cytotoxic conjugate. In this study, the effectiveness of AN-152 was heightened by receptor up-regulation. The cytotoxic effect of AN-152 can be regulated by the number of active LH-RH receptors on cancer cells. LH-RH receptor-positive (MCF-7) and -negative (UCI-107) cancer cells were treated with epidermal growth factor (EGF) or the somatostatin analogue, RC-160. EGF and RC-160 have been shown previously to regulate LH-RH receptors through phosphorylation. The effect of receptor regulation, by hormone exposure, on the cytotoxicity of AN-152 and doxorubicin and on the cellular uptake of AN-152, [d-Lys₆]LH-RH, or doxorubicin was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and by two-photon laser scanning microscopy. The results demonstrated that the cellular entry of the conjugate was: (a) specific for cancers with LH-RH receptors; (b) up-regulated by EGF; (c) down-regulated by RC-160; and (d) the cytotoxicity of the AN-152 paralleled the efficiency of entry. This study illustrates the potential use of receptor regulation for increasing the efficacy of chemotherapeutic approaches that are directed to cell surface receptors.

INTRODUCTION

Chemotherapy is commonly used in the treatment of many cancers, but it has some distinct limitations and disadvantages (1). A major limitation is that it is not always efficacious because of severe restrictions on the drug dosages that can be used (1). The dose of drug that is required to eradicate some cancers may not be compatible with patient survival. The disadvantage of chemotherapy is mainly related to the toxicity of the drug. The toxicity of the drug makes the patient susceptible to potentially fatal infections, cardiotoxicity, and other side effects. Reducing these side effects by targeting cytotoxic agents more selectively to cancer cells can improve the patients’ chances of a cure and make the quality of life more tolerable to the patient (1).

The targeting of cytotoxic agents linked to hydrophilic peptide hormone analogues could help overcome some of the shortcomings of chemotherapy. AN-152 was developed by coupling the frequently used antineoplastic agent, doxorubicin, to an analogue of the peptide hormone LH-RH.² [d-Lys₆]LH-RH (2). This analogue was chosen to serve as a carrier peptide for targeting because many tumors express receptors for LH-RH (3–5). In addition to gonadotrope cells in the anterior pituitary, the expression of LH-RH receptors has been demonstrated in various human cancers, including breast (6), ovarian (7–9), endometrial (3, 8, 9), prostatic (4, 10, 11), and pancreatic (12, 13). AN-152 is significantly less toxic and more potent against cancers with LH-RH receptors than doxorubicin (2, 14). The decreased toxicity is presumably caused by a slower entry into normal cells than doxorubicin, resulting in a different tissue distribution of the more hydrophilic peptide conjugate from that of the more lipophilic doxorubicin. The enhanced efficacy is likely attributable to the high-affinity binding of AN-152 to receptors for LH-RH (15) on these cancers. Thus, treatment of cancers expressing LH-RH receptors with AN-152 would be more selective and would exert a lower toxicity than conventional therapies.

It has been proposed that the expression of LH-RH receptors in cancers is mediated by the loss of balance in the tyrosine kinase growth control pathway seen in many of these cancers (16, 17). In the hamster cheek pouch carcinoma model of oral cancer, LH-RH receptors appear in a predictable manner during carcinogenesis (16). This change takes place much more rapidly by enhancing selective tyrosine kinase activity through events mediated by EGF stimulation (16). Previous studies using cell membrane preparations have shown that LH-RH receptors can be activated by phosphorylation and inactivated by dephosphorylation (17). LH-RH receptor activation can be dramatically increased by EGF-stimulated tyrosine kinase activity (17). The increase in the tyrosine kinase signal in cancer cells results in activation of latent receptors by phosphorylation and increases the number of functional receptors (17). An analogue of somatostatin, RC-160, which activates tyrosine phosphatase activity, decreases the number of functional receptors through dephosphorylation (17). The use of cell membrane extracts in these LH-RH binding experiments eliminated receptor synthesis or degradation as possible explanations for the modulation of receptor binding in response to EGF and RC-160 (17).

Lee et al. (18) reported that EGF specifically phosphorylates a tyrosine residue on a Mr 60,000 protein, which corresponds to the LH-RH receptor (19), and that RC-160 dephosphorylates a tyrosine residue on that same protein (18). These findings indicate that the modulation of LH-RH receptor binding in cell membrane preparations in response to EGF and RC-160 are attributable to tyrosine kinase and tyrosine phosphatase stimulation, respectively.

In this study, we show that acute treatment of cancer cells that express LH-RH receptors with EGF or RC-160 can sensitize or desensitize cells to AN-152 chemotherapy. To evaluate AN-152 action on cancer cells and modulation of its uptake by EGF and RC-160, the cytotoxic agent was conjugated to a two-photon fluorophore, C625, used to label AN-152 (AN-152:C625, [d-Lys₆]LH-RH [(d-
Lys\(^6\)LH-RH:C625, and doxorubicin (Dox:C625) (Ref. 20). Optical tracking of the AN-152:C625 or its components, doxorubicin and [\(\alpha\)-Lys\(^6\)]LH-RH:C625, provided valuable information about the mechanism of this new type of hormone-directed chemotherapy. This method of high-resolution optical tracking allowed us to determine the intracellular path of the drug and the effect of receptor regulation on drug entry into cells. We followed the entry through the membrane, into the cytoplasm and the nucleus, by TPLSM. Cytotoxicity assays were performed to examine whether variations in cell sensitivity to AN-152 parallel alterations produced by pretreatment with EGF or RC-160.

MATERIALS AND METHODS

Synthesis of Fluorescent Analogues of AN152, LH-RH, and Doxorubicin. Fluorescent analogues of AN-152, [\(\alpha\)-Lys\(^6\)]LH-RH, and doxorubicin were synthesized as described by Wang et al. (20). The two-photon chromophore C625, a dicarboxylic ester, was coupled covalently to the three-amine group of the doxorubicin portion of AN-152 (2, 21). The same coupling reaction was used to label the \(\epsilon\)-amino group of \(\alpha\)-lysine in [\(\alpha\)-Lys\(^6\)]LH-RH and to label doxorubicin. All final products were purified by high-performance liquid chromatography, and the conjugates were identified by two-dimensional nuclear magnetic resonance and by fast atom bombardment ionization mass spectrometry.

TPLSM. The microscope used was a commercially available, MRC-1024 laser scanning confocal microscope from Bio-Rad, modified for use as a TPLSM. The light source at 800 nm for two-photon microscopy was a mode-locked Ti-Sapphire laser pumped by an Argon ion laser, producing a train of pulses of 80 fs duration at a frequency of 90 MHz. The average power used was 15 mW. The images shown in this report were collected by keeping the aperture fully open, making the system a TPLSM. This enabled us to view biological processes without damaging the living cells. The water immersion objective lens used for all biological imaging was a Nikon Fluor-60X (NA = 1). A scan rate of 4000 nm/s was used, which was found to be safe for the living cells.

Doxorubicin shows some fluorescence with an excitation wavelength of 488 nm. Its entry into cells was monitored by one-photon confocal microscopy using the same equipment described above using an emission filter for 580 nm (band-pass filter, 580/32).

Cell Culture. MCF-7 cells (American Type Culture Collection) were grown in improved MEM supplemented with 10% fetal bovine serum. UCI-MCF-7 cells were derived from MCF-7 cells by Dr. P. Carpenter (University of California, Irvine Medical Center, Irvine, CA) and were grown in improved MEM supplemented with 5% heat-inactivated fetal bovine serum. Both cultures were maintained at 37°C in a humidified atmosphere with 5% CO\(_2\).

Modulation of AN-152 Uptake by EGF and Somatostatin Analogue RC-160. Cells were pretreated (2 h) with 10 nM EGF (from Sigma) or 10 nM RC-160 or received no pretreatment, followed by addition of 0.6 \(\mu\)M of one of the chromophore-labeled compounds, AN-152:C625, [\(\alpha\)-Lys\(^6\)]LH-RH:C625, or Dox:C625, or 0.6 \(\mu\)M unlabeled doxorubicin. The competition assay was performed by adding 1 or 0.1 \(\mu\)M [\(\alpha\)-Trp\(^{3}\)]LH-RH 10 min prior to addition of AN-152:C625. For each experiment, cells were viewed through the microscope at approximately \(\times600\). The field was viewed initially as soon as the labeled drug was administered for 5 or 10 min, then every 10 min for the first 90 min, and subsequently at 180 and 270 min, and the images were saved. Cell viability was assessed throughout the experiments by visual observation of cell morphology and maintenance of attachment to the Petri dish.

Image Analysis. Image analysis was performed on a PC computer using the public domain Image/J program that was developed at the United States NIH and available on the Internet. The mean gray value (the sum of the gray values of all pixels in the selection divided by the number of pixels in the selected area), a measure of absorbance, was determined by selecting the area occupied by the cell for analysis by the program. The mean gray value of the background was determined and subtracted from that of the cells. The mean gray value was determined for several cells and several background positions.
was compared for cells in each of the three conditions (control, RC-160, and EGF) over 1 h, there was no difference (each condition had a rate of increase in absorbance/min of 0.1 ± 0.03).

Further confirmation of LH-RH receptor mediation of AN-152 action was provided by competition for drug binding sites with [\(\text{[D-Trp}^6\)]LH-RH. Competition for receptors was visualized by AN-152:C625 uptake into MCF-7 cells that had been pretreated with EGF, and then [\(\text{[D-Trp}^6\)]LH-RH was added 10 min prior to addition of AN-152:C625 (Fig. 4A). This was compared with cells under the same conditions in the absence of [\(\text{[D-Trp}^6\)]LH-RH (Fig. 4B). [\(\text{[D-Trp}^6\)]LH-RH effectively competed for receptors for AN-152:C625, and the rate of drug association with the cells was five times faster in the absence of [\(\text{[D-Trp}^6\)]LH-RH (Fig. 1A). The intensity of labeling was significantly reduced by competition with [\(\text{[D-Trp}^6\)]LH-RH, and with higher [\(\text{[D-Trp}^6\)]LH-RH concentrations the entry of the drug decreased, as evidenced by a lower intensity of fluorescence in the cells.

To study the entry of AN-152:C625 into cells that are LH-RH receptor negative, an ovarian cancer line, UCI-107, was selected. Tumors grown from these cells were reported to be less responsive to AN-152 (14). We found that entry into UCI-107 cells was different from entry into MCF-7 cells. Very little entry of the AN-152:C625 was observed over 90 min, and it was unclear whether the drug had entered the nucleus (Fig. 2G). Hormone regulation of drug uptake, as observed in MCF-7 cells, was not seen with the cells lacking LH-RH receptors. Pretreatment of UCI-107 cells with EGF did not alter the rate of AN-152:C625 entry, although RC-160-pretreated cells were more intensely labeled, and the rate of drug entry was greater (Fig. 1A).

**Regulation of AN-152 Efficacy by EGF and Somatostatin Analogue RC-160.** MCF-7 human breast cancer cells, which are reported to express LH-RH receptors (2, 15), were treated with EGF to up-regulate the LH-RH receptors and somatostatin analogue, RC-160, to down-regulate them (17). Although these peptide hormones are widely known to have effects on the proliferation of cells, under our experimental conditions, exposure of MCF-7 cells to this concentration of EGF or RC-160 for 96 h had a relatively small effect on viable cell number (Fig. 5). When these cells are grown in media lacking fetal bovine serum, a greater growth response to EGF and RC-160 has been noted.5

AN-152 sensitivity of MCF-7 cells was regulated by hormonal modulation of receptors. EGF sensitizes cells to AN-152, and RC-160 desensitizes cells to AN-152. The doses of AN-152 or doxorubicin that were effective in killing 50% of MCF-7 cells 96 h after treatment (ED50) were extrapolated from dose-response curves, using cells with no treatment as the baseline condition (Table 1). The cells were more sensitive to AN-152 after exposure to EGF, as reflected by a decrease in the ED50 from 2.4 \(\mu\)M for AN-152 to 0.4 \(\mu\)M for EGF exposure.

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5 Unpublished observation.
prior to AN-152 treatment. The cells were somewhat less sensitive to AN-152 after exposure to RC-160, although the difference was not statistically significant. The ED$_{50}$ for AN-152 was increased by pretreatment with RC-160 to 3.0 μM. Exposure to the hormones prior to doxorubicin did not have the same regulating effect on the efficacy of the drug. The ED$_{50}$ for doxorubicin was 9.7 μM, and EGF pretreatment did not significantly alter the ED$_{50}$ (9.1 μM). RC-160 increased the sensitivity to doxorubicin, as demonstrated by the lower ED$_{50}$, 5.7 μM. AN-152 was more effective in killing MCF-7 cells than the conventional doxorubicin (P = 0.024), and this could be still significantly enhanced by EGF treatment prior to AN-152 exposure (P = 0.004). AN-152 with RC-160 differed significantly from the doxorubicin exposure (P = 0.016); doxorubicin with RC-160 also differed but not significantly from exposure to doxorubicin alone (P = 0.100).

UCI-107 human ovarian cancer cells, which lack LH-RH receptors, served as a negative control cell line. Hormonal pretreatment of UCI-107 cells had a significant effect on the growth rate of the cells, but no significant change in the efficacy of either agent in response to the hormones could be deduced from the results. This was very different from the hormonal regulation of AN-152 sensitivity in LH-RH receptor-positive cells. EGF increased the proliferation of UCI-107 cells (Fig. 5), which was reflected by an increased number of viable cells treated with AN-152 or doxorubicin. Conversely, RC-160 slowed the growth of the cells (Fig. 5); therefore, upon exposure to AN-152 or doxorubicin, the result was a lower cell number. The ED$_{50}$ for AN-152 or doxorubicin 96 h after treatment were extrapolated from dose-response curves (Table 1). Overall, the ED$_{50}$s are lower in UCI-107 cells than for MCF-7 cells. The ED$_{50}$ for AN-152 was 0.4 μM and was increased by pretreatment with EGF to 2.9 μM. The ED$_{50}$ for AN-152 was decreased by pretreatment with RC-160 to 0.005 μM. The ED$_{50}$ for doxorubicin was 0.4 μM and was increased by EGF pretreatment to 2.0 μM and decreased by RC-160 to 0.001 μM. Killing of UCI-107 cells by AN-152 was not significantly different from the conventional doxorubicin. Treatment with EGF prior to AN-152 exposure resulted in greater cell survival and was significantly different from AN-152 alone (P = 0.002) or doxorubicin alone (P = 0.003). EGF treatment prior to doxorubicin exposure also resulted in greater cell survival and was significantly different from doxorubicin alone (P = 0.014) or AN-152 alone (P = 0.018). AN-152 with RC-160 resulted in fewer surviving cells and differed significantly from the exposure to doxorubicin alone (P = 0.033) and to AN-152 alone (P = 0.027). Similarly, doxorubicin with RC-160 resulted in fewer surviving cells and differed significantly from the exposure to doxorubicin alone (P = 0.026) and to AN-152 alone (P = 0.018).

**DISCUSSION**

TPLSM with its inherent ability for optical sectioning (24, 25) and construction of three-dimensional images allowed the tracking of drug entry into live cells as it occurred and localization of the drug conjugate in the cell. This novel approach to the study of drug action was used to optically investigate the modulation of AN-152 action for the development of strategies to increase the drug effectiveness. Alterations in the time course of drug uptake and differences in relative intensity of labeling of cells in response to EGF and RC-160 pretreatment provided a semiquantitative estimation of changes in AN-152 efficacy through LH-RH receptor regulation.

Up-regulation of LH-RH receptors by EGF, through tyrosine phosphorylation of receptors, has been demonstrated previously (17, 18). In this study, we used EGF stimulated up-regulation of LH-RH receptors to enhance the uptake and efficacy of the targeted chemotherapeutic agent, AN-152, in responsive cancer cells. Pretreatment with EGF resulted in more rapid cellular entry of AN-152:C625 and more intense labeling in cancer cells that express LH-RH receptors, such as the human MCF-7 breast cancer cells. Other LH-RH receptor-positive cells under current investigation by our laboratory, KB and HPCP (human and hamster oral carcinoma cells, respectively), responded in a similar manner to hormone-induced receptor regulation.6 The enhanced uptake of AN-152 coincided with the increased binding activity of LH-RH receptors (17). The increased entry of AN-152:C625 after pretreatment with EGF, seen by TPLSM, mirrors the heightened sensitivity of these cells. Our results showed that the rate of association with the cells was 50% faster (Fig. 1A), and the ED$_{50}$ decreased by 83% (Table 1). The rate of AN-152:C625 association

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**Table 1 Effect of peptide hormones on AN-152 and doxorubicin dose-response in MCF-7 and UCI-107 cells**

The doses of AN-152 or doxorubicin that were effective in killing 50% of MCF-7 cells (LH-RH receptor positive) or UCI-107 cells (LH-RH receptor negative) 96 h after treatment (ED$_{50}$) were extrapolated from dose-response curves, using cells with no treatment as the baseline condition. Dose-response curves were generated using the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay for cells exposed to 1 nM to 10 μM AN-152 or doxorubicin alone (control), or AN-152 or doxorubicin preceded by 10 nM EGF or 10 nM RC-160 exposure.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>ED$_{50}$ for AN-152 (μM)</th>
<th>ED$_{50}$ for doxorubicin (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4$^{a}$</td>
<td>9.7$^{b}$</td>
</tr>
<tr>
<td>EGF</td>
<td>0.4$^{a-d}$</td>
<td>9.1$^{b}$</td>
</tr>
<tr>
<td>RC-160</td>
<td>3.0$^{a,b}$</td>
<td>5.7$^{b}$</td>
</tr>
<tr>
<td>UCI-107</td>
<td>Control</td>
<td>0.4</td>
</tr>
<tr>
<td>EGF</td>
<td>2.9$^{a-b}$</td>
<td>2.0$^{a-d}$</td>
</tr>
<tr>
<td>RC-160</td>
<td>0.005$^{a-d}$</td>
<td>0.001$^{a-d}$</td>
</tr>
</tbody>
</table>

$^a$ Versus doxorubicin control P ≤ 0.05.
$^b$ Versus AN-152 control P ≤ 0.05.
$^c$ Versus doxorubicin control P ≤ 0.01.
$^d$ Versus AN-152 control P ≤ 0.01.

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6 Unpublished data.
with the cells was 50% faster than the rate of doxorubicin entry into cells, and the entry of doxorubicin was not affected by EGF pretreatment. Accordingly, AN-152 was more effective than doxorubicin in killing these cells (75% lower ED_{50}), and the efficacy of doxorubicin was not significantly enhanced by EGF pretreatment, as the AN-152 was (Table 1). This indicates that simply altering the mitotic rate with EGF cannot account for the increased efficacy seen in response to EGF during AN-152 treatment. These results are consistent with the regulation of activity of LH-RH receptors by EGF-mediated tyrosine kinase phosphorylation (16–18, 26) to increase the functional binding sites for AN-152.

Pretreatment of MCF-7 cells with RC-160 resulted in slower entry of AN-152:C625 and reduced cytotoxicity of the AN-152. The rate that the agent associated with the cells was 20% slower (Fig. 1A), and the cytotoxicity decreased by 20% but did not represent a statistically significant decrease (Table 1). RC-160 has been shown to down-regulate LH-RH receptors through stimulation of tyrosine dephosphorylation (17, 18). It follows that pretreatment of cells with RC-160 decreases the functional binding sites for AN-152. Conversely, RC-160 decreased the doxorubicin ED_{50} by 41%. This corroborates the findings of Lee et al. (27) and Weckbecker et al. (28) that somatostatin analogues enhance the entry and cytotoxicity of doxorubicin.

UCI-107 human ovarian cancer cells, which are devoid of LH-RH receptors (14), were studied as a control cancer. These cells showed little uptake of AN-152:C625, and pretreatment with EGF or somatostatin did not appreciably alter the rate of uptake (Fig. 1A). In cytotoxicity studies, AN-152 and doxorubicin were almost equally effective. Because of the lack of LH-RH receptors, internalization of AN-152 does not occur, and in time doxorubicin is freed from the peptide carrier through hydrolysis by serum carboxyl esterases (EC.3.1.1.1) present in the medium. The short, 10-min half-life of AN-152 in the serum of female nude mice supports this view that explains a similar effect of AN-152 and doxorubicin in UCI-107 cells (14). In the case of AN-152:C625, it is doxorubicin:C625, which is insoluble in aqueous media, is cleaved by the enzymes, and thus the entry of the labeled doxorubicin into cells does not occur (Fig. 2G).

Our hormonal pretreatment results are also consistent with an absence of LH-RH receptors. UCI-107 cells that had been treated with EGF prior to AN-152 or doxorubicin displayed a decrease in cytotoxicity (Table 1), likely accounted for by the increase in the rate of growth in response to the EGF (Fig. 5). RC-160 exposure prior to AN-152 or doxorubicin resulted in increased cytotoxicity (Table 1). RC-160 exposure alone in this cell line resulted in decreased cell survival; therefore, this result could be, at least in part, attributable to the inherent antiproliferative properties of RC-160 in this cell line (Fig. 5).

The lack of regulation of drug uptake in this LH-RH receptor-negative cell line supports that the action of AN-152 in LH-RH receptor-positive cells is mediated by LH-RH receptor activity, as suggested previously in cytotoxicity assays (2). Optical tracking of AN-152, [D-Lys^6]LH-RH, and doxorubicin, in combination with hormonal alteration of LH-RH receptors, provided important information about this new type of targeted chemotherapy. The localization capabilities afforded by this technique enabled us to determine that the labeled drug enters the cell and is later concentrated within the nucleus. Prior to this investigation, it was theorized that the agent associated with the membrane receptors and acted from outside of the cell (2), possibly through the generation of cytotoxic free radicals. We now know that there is opportunity for nuclear interaction as well in LH-RH receptor-bearing cells. AN-152, like doxorubicin (29), the cytotoxic base of AN-152, is likely to have several cytotoxic mechanisms of action. These could include membrane interactions, free radical production, topoisomerase II inhibition, apoptosis induction, and multiple interferences with DNA.

Our experiments tracking the entry of the drug and its components helped to clarify the cellular trafficking of AN-152 and the modulation of its effectiveness by hormonal pretreatment. The predictable pattern of uptake of [D-Lys^6]LH-RH:C625 in response to EGF and RC-160 provides further evidence that action of AN-152 is indeed receptor mediated. Addition of [D-Trp^6]LH-RH prior to AN-152:C625 administration demonstrated competition with AN-152:C625 for LH-RH binding sites. Labeling of cells with AN-152:C625 was significantly decreased by the presence of [D-Trp^6]LH-RH, even after pretreatment with EGF. These results strongly support the hypothesis that LH-RH receptors mediate entry of AN-152.

Evaluation of doxorubicin uptake demonstrated that in contrast, the mechanism of doxorubicin action does not involve LH-RH receptors or binding to membrane receptors. The regulation of entry of AN-152 by hormonal modulation in MCF-7 cells, but not in UCI-107 cells, and the lack of regulation of doxorubicin uptake in either cell line lend additional support for the hypothesis that the sensitivity of MCF-7 cells to AN-152 is related to LH-RH receptor regulation.

Our results reinforce the view that the increase in the efficacy of a targeted antineoplastic drug could help to circumvent the disadvantages and limitations of conventional chemotherapy for some cancers. These results also suggest that the elevated levels of EGF seen in certain cancers may further facilitate a higher uptake of targeted cytotoxic LH-RH analogues. Previous studies indicate that this occurs by EGF-mediated tyrosine phosphorylation and activation of LH-RH receptors. LH-RH receptors are present in ~50% of breast cancers (6), ~80% of ovarian and endometrial cancers (8), and 86% of prostate cancers (10), making these cancers excellent candidates for this type of chemotherapy.

REFERENCES


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