Different Effect of Paclitaxel on Primary Tumor Mass, Tumor Cell Contents, and Metastases for Four Experimental Human Prostate Tumors Expressing Luciferase

Nadia El Hilali, Nuria Rubio, and Jerónimo Blanco
Dpto. Patología Molecular y Terapéutica (IIBB), Centro de Investigación y Desarrollo (CSIC), Barcelona, Spain

ABSTRACT

Purpose: Primary tumor growth is usually assessed by measuring tumor mass or volume, under the assumption that such variables correlate with the contents of tumor cells. However, tumors are complex interacting mixtures of tumor cells and host components. The different sensitivity of such components to cytostatic agents should be taken into consideration when evaluating the effectiveness of antineoplastic agents. We evaluate the effect of the antineoplastic agent paclitaxel on primary tumors expressing luciferase and their metastases using a sensitive luminescence-based procedure to directly assess the number tumor cells, in comparison with traditionally used tumor mass measurement.

Experimental Design: Nude mice bearing human prostate tumors expressing the luciferase gene, LNCaP.Sluc, DU 145.Sluc, and PC-3.Sluc, i.m. inoculated, and PC-3M.Sluc, orthotopically inoculated, were subjected to a 10-day treatment with either 10 mg/kg/d paclitaxel or saline solution. At the end of the treatment period, primary tumors as well as metastasis target organs were harvested, weighed, and homogenized. The presence of tumor cells in the tissue homogenates was evaluated using a luminometer, following the addition of luciferin. Tumor cell equivalent is defined as the amount of light produced by a single tumor cell in culture.

Results: Paclitaxel had a different effect on the primary tumor mass and the contents of tumor cells for each tumor type. Whereas LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc primary tumor masses were significantly reduced by the action of paclitaxel, their contents in tumor cell equivalents were not significantly affected. In contrast, paclitaxel only reduced significantly the number of tumor cell equivalents in DU 145 primary tumors. In the lymph nodes, paclitaxel reduced the number of DU 145.Sluc metastases significantly, by a factor of 10^3, but had no significant effect on the rest of tumor cells. However, in lungs and muscle, paclitaxel treatment reduced significantly the number of metastatic PC-3.Sluc and PC-3M.Sluc tumor cell equivalents. In the bones, no tumor cell type was significantly affected by paclitaxel.

Conclusions: Some components of tumor stroma seem to be more sensitive to antineoplastic agents than the tumor cells themselves and may also contribute to modulate the response to therapy. Our results caution against the use of a single general variable, such as tumor mass, to evaluate the effectiveness of antineoplastic agents and emphasize the effect of the tumor cell environment in their sensitivity to treatment.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in Western males. Most cases of prostate cancer are diagnosed early when the disease remains confined to the organ (1, 2). It is well known that most patients with cancer do not die because of the primary tumor but rather due to its spreading to other sites. In Europe, ~50% of men with prostate cancer will develop metastatic disease (3). The most important complication in prostate cancer results from tumor progression and generalized metastases to regional and distant sites such as lymph nodes and bones, the main target organs (4, 5). The dissemination of small numbers of cells can begin at very early stages of tumor development and colonizing cells in target organs can remain in a state of dormancy for long periods of time or grow rapidly into metastases. Clinically, these very small numbers of cells known as micrometastases represent the pathophysiologic basis of minimal residual disease that could eventually lead to cancer relapse as overt metastases. However, treatment of the disease at this stage is not generally possible due to methods for detecting such minimal tumor spread not being sufficiently sensitive and specific (6, 7).

In experimental tumor models, as in clinical field, there is a need for very sensitive methods able to detect the smallest number possible of tumor cells present in target organs, which would allow the study of tumor proliferation and spread and of the effect of antineoplastic therapies at early stages.

Counting tumor nodules on the surface of target organ, although laborious and not entirely objective, has represented the basis of a semiquantitative approach for the evaluation of metastasis in highly metastatic tumors (8). Other indicators of metastatic capacity commonly used are survival time of tumor-bearing animals (9) and the evaluation of stained tissue sections (10).

The introduction of procedures based on the use of reporter genes has recently allowed more sensitive and quantitative measurements of tumor growth and metastasis (11). The
localization of micrometastasis and, in some cases, single tumor cells in histologic sections has become possible by the use of the β-galactosidase gene as a reporter transfected into tumor cells (12). Reporter genes encoding either fluorescent or bioluminescent proteins enable the study of neoplastic disease from minimal to late stages. Green fluorescent protein has been used to detect micrometastasis and even single metastatic cells by histologic procedures (13, 14). Luciferase has been used to detect very small numbers of tumor cells present in whole organs homogenates, allowing an easy quantification of tumor burden (15). Due to the capacity of photons to transverse living tissues, photoprotein reporters have also been used in vivo as internal biological beacons to monitor the development of tumors and the effect of antineoplastic treatments (16–18).

In the case of bioluminescence, firefly (Photinus pyralis) luciferase catalyzes the oxidation of luciferin in the presence of ATP and CoA, producing light and O₂ (19). Recording of the emitted light by sensitive photodetectors, capable of linear response with a range of 6 to 7 orders of magnitude and high quantum efficiency, allows a sensitive quantification of photons from luciferase-expressing cells present in tissue homogenates. Due to the absence in mammalian cells of competing light-producing reactions, labeling of tumor cells with the luciferase reporter has permitted the detection of as few as 5 to 10 tumor cells per murine lymph node and the study of early organ colonization pattern of prostate tumor cells (20).

Traditionally, to measure or monitor tumor growth, the volume of an irregular tumor is calculated by an approximation to that of a regular ovoid having the same major and minor axial dimensions, measured with the aid of a caliper (21). Using this method, it is generally assumed that there is a correlation between tumor volume and the number of tumor cells it contains. However, it is known that tumor cells do not exist in isolation but proliferate in association with stromal tissue and other host factors that support their growth. Cancer cells change the host tissue to establish a permissive and growth supportive environment, tumor stroma (22, 23). Tumor stroma is ill defined, changes continuously during tumor development, and varies between different tumor types. The principal tumor stroma cells are fibroblasts, macrophages, lymphoid cells mast cells, and endothelium. The role of stroma in the malignancy process is largely unknown (24). The different components of tumors may contribute not only to the evolution and spread of tumor cells but, very likely, also influence the responses to antineoplastic treatments. Besides the heterogeneity of tumor cells, a major obstacle to the development of effective treatments could be the heterogeneity of the associated environment in the tumor.

In the present report, using a luminometric procedure, we analyzed tumor growth, metastatic capacity, and response to the antineoplastic agent paclitaxel (Taxol; refs. 25, 26) of four human prostate tumor cell lines, DU 145.Sluc, LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc, expressing the luciferase gene. The comparison of the number of tumor cells, obtained from luminometric data, with the direct measurement of tumor mass indicates that in some primary tumors, a reduction of tumor mass does not correlate with an equivalent reduction on tumor cell load. Thus, in some tumors, paclitaxel seems to have a more significant effect on the reduction of nontumor cell components of the tumor than on the number of tumor cells.

MATERIALS AND METHODS

Cells and Cell Culture. The human prostate cancer cell lines PC-3, DU 145, and LNCaP were purchased from American Type Culture Collection (Rockville, MD) and PC-3M were obtained from Dr. I. Fidler (M.D. Anderson Cancer Center, University of Texas, Houston, TX). The cells were maintained in a 1:1 mixture of DMEM and Ham’s nutrient mixture F12 supplemented with 10% fetal bovine serum.

Cell Transfection. PC-3, PC-3M, DU 145, and LNCaP cells were transfected with the pRc/CMV expression vector (Invitrogen, San Diego, CA) which contains the firefly (P. pyralis) luciferase gene coding region to generate PC-3.Sluc, PC-3M.Sluc, DU 145.Sluc, and LNCaP.Sluc cell sublines, and were cloned as described (11) to generate permanently transfected clones.

Experimental Tumorigenesis. Six-week-old BALB/c homozygous male nude mice (nu/nu) were purchased from IFFA-CREDO (L’Arabresle, France) and maintained in a specific pathogen-free environment throughout the experiment. Animals were kept for at least 1 week in the facility before experimental manipulation. To generate i.m. tumors, PC-3.Sluc and DU 145.Sluc (1 × 10⁶ luciferase-expressing tumor cells in 100 μL of culture media without fetal bovine serum) were injected i.m. into each of the mouse thighs. LNCaP.Sluc cells (1 × 10⁵) were inoculated with 0.1 mL of Matrigel (Becton Dickinson Labware, Bedford, MA).

To generate orthotopical tumors, mice were anesthetized with a mixture of 6 mg/kg droperidol (Roche, Basel, Switzerland) and 12 mg/kg midazolam (Rovi S.A., Madrid, Spain) and then inoculated in each prostatic lobe with 5 × 10⁵ luciferase-expressing tumor cells suspended in 50 μL of culture media without fetal bovine serum as described (27).

Mice were sacrificed after the treatment period and necropsies were done. Primary tumors and organs of interest were harvested and weighed.

Cytostatic Treatment. During a 10-day period the mice were i.p. inoculated daily with 10 mg/kg paclitaxel dissolved in dehydrated ethanol and Cremophor (Taxol, Bristol-Myers Squibb, New York, NY) diluted in 0.9% NaCl solution. Control animals were inoculated with 0.9% NaCl solution following the same protocol.

Cell and Tissue Homogenates. Cell lysates were prepared by performing one freeze-thaw cycle in reporter lysis buffer (Promega Corporation, Madison, WI). Whole organs and tissue extracts were prepared by mechanical homogenization of tissue at a ratio 1:1 (w/v) in reporter lysis buffer using an Ultra-Turrax T-25 tissue homogenizer (Janke and Kunkel, Staufen, Germany) followed by centrifugation at 25,000×g for 45 minutes at 4°C to remove insoluble particles. In the case of lymph nodes, tissue homogenates were made in the presence of 10% bovine serum albumin as a protection against proteases.

Luciferase Assay. Luciferase activity in cell extracts or tissue homogenates was measured by chemiluminescence using the standard luciferase assay kit (Promega). The production of light was measured using a Turner Designs luminometer model TD 20/20 after the addition of 100 μL of luciferase assay reagent (luciferin; Promega) to 20 μL of cell lysate or tissue homogenate.
Counting of Tumor Cell Equivalents in an Organ and Detection Sensitivity. All luminometer readings were done using 20 µL aliquots of a sample as described.

Standard Curve. A standard curve is generated for each tissue type analyzed by adding known numbers of the luciferase-expressing tissue cultured tumor cells to 20 µL aliquots of the tissue homogenate, produced as described from an animal devoid of tumor cells. The amount of light generated by the aliquots was determined as described. The slope of the standard curve (relative light units per cell) is the amount of light generated per tumor cell, here defined as one tumor cell equivalent.

Tumor Cell Equivalent Counting. To determine the number of tumor cell equivalents in a tissue homogenate, first we determine the luminometer reading from a blank sample consisting of an aliquot of the same tissue homogenate as that to be tested, but from an animal devoid of luciferase-expressing cells. We determine the luminometer reading from an aliquot of the test tissue homogenate, then we subtract the reading of the blank sample from that of the test sample. We divide the resulting number by the value of the slope of the standard curve (relative light units per cell) for that particular tissue. The resulting value is the number of tumor cell equivalents in the aliquot of tissue homogenate. Finally, the total number of tumor cell equivalents in the tested organ is calculated by proportionally scaling the number of tumor cell equivalents in the test aliquot to the total volume of tissue homogenate.

Tissue homogenates in which the luciferase activity of 20 µL aliquot exceeded detector range (9,999 relative light units/min) were adapted to the counter range by diluting with reporter lysis buffer.

Assay Sensitivity. Minimum assay sensitivity was defined as the number of tumor cell equivalents required to generate a quantity of light equivalent to 2 SD of the background blank sample. Measurements below the defined minimum assay sensitivity were considered equal to zero tumor cell equivalent.

Statistical Analysis. The significance of the difference between treated and control groups was determined by Student’s t test.

RESULTS

Tumor Implantation and Metastasis. To compare the proliferation and metastatic capacity of tumor cells in nude mice as well as their response to the antineoplastic agent paclitaxel, we used the tumor cell lines PC-3, PC-3M, DU 145, and LNCaP, labeled by permanent transfection with the P. pyralis luciferase gene, as models of human prostate tumors. Groups of eight animals were inoculated i.m. with $1 \times 10^6$ luciferase-expressing cells of each type, suspended in 100 µL medium without serum. The same number of PC-3M.Sluc cells was inoculated orthotopically in the prostate gland of a further group of mice. Following a growth period allowed for tumor development, mimicking a clinical situation, each group of animals was randomly divided into two, treated and control groups, and each half was subjected to a 10-day treatment period with either 10 mg/kg/d paclitaxel or 100 µL 0.9% NaCl solution, respectively. At the indicated times, the animals from each test group were sacrificed and the primary tumors plus the indicated target organs were harvested, homogenized, and processed as described in MATERIALS AND METHODS for analysis of light emission capacity. Following addition of luciferin, the content in luciferase-expressing tumor cells in an aliquot of the clear lysate supernatant was determined using a luminometer, also as described in MATERIALS AND METHODS.

Table 1 summarizes the experimental variables for each tumor type, including duration of experimental tumor growth, tumorigenicity, metastatic capacity, and response to paclitaxel treatment. Before the treatment, different tumor development periods were allowed to reduce size disparities between tumor types, which result from differences in tumor growth rates. I.m. and orthotopic inoculation resulted in tumor implantation in all but one of the cases (97%). Although the animals were observed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Tumor growth and metastases of luciferase-labeled human prostate tumor cells: DU 145.Sluc, LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days postinoculation*</td>
<td>43</td>
</tr>
<tr>
<td>Mean tumor weight (mg)</td>
<td>513.25</td>
</tr>
<tr>
<td>Significance ($P$)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Metastasis</td>
<td>Yes</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>17/23</td>
</tr>
<tr>
<td>Bones</td>
<td>12/12</td>
</tr>
<tr>
<td>Sexual organs</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>14/16</td>
</tr>
</tbody>
</table>

NOTE. (+) PC-3M.Sluc cells were othotopically implanted, primary tumor growth in prostate gland. Lymph nodes include axillary, inguinal, mesenteric, renal, lumbar, knee, and tail lymph nodes. Others include liver, brain, spleen, and kidney.

*Postinoculation days include 10 days of treatment period (paclitaxel).
†Significance of difference on tumor weight control / treated was calculated using Student’s t test.
‡Inclusion criterium of metastasis was determined from the number of tumor cell equivalents present in each studied organ.

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daily, no evident changes in appearance or behavior due to the paclitaxel treatment were detected in any of the animals.

As shown in the table, mean tumor weights for control animals were similar for LNCaP.Sluc and PC-3.Sluc whereas that of DU 145.Sluc was the smallest. In the case of PC-3M.Sluc, the mean tumor weight includes that of the prostate gland, which was impossible to separate cleanly from the rest of tumor tissue. However, the mass of prostatic gland, which in nude mice varies between 30 and 40 mg, represents a small fraction (~10%) of the total tumor mass.

As can also be observed from Table 1, the presence of tumor cells was general and all the tumor types produced metastatic cells in all the organs assayed at the end of the experiment.

**Tumor Mass versus Number of Tumor Cells.** At the end of the treatment period, primary tumors harvested from control and paclitaxel-treated mice were weighed, homogenized, and analyzed for contents of light-emitting tumor cells using a luminometer as described in Materials And Methods.

Figure 1 is a graph showing tumor mass versus the number of tumor cell equivalents in the same primary tumors; each data point represents a single animal. Compared with control tumors, paclitaxel treatment resulted in a significant ($P < 0.05$) reduction in the weight of PC-3M.Sluc, PC-3.Sluc, and LNCaP.Sluc tumors ($A$, $B$, and $C$, respectively), but not in that of DU 145.Sluc tumors ($D$, $P > 0.05$). However, paclitaxel treatment resulted in a significant ($P < 0.05$) reduction in the number of tumor cell equivalents for DU 145.Sluc tumors only but had no significant effect ($P > 0.05$) on the number of tumor cell equivalents in PC-3.Sluc, PC-3M.Sluc, or LNCaP.Sluc tumors.

To compare the effects of paclitaxel on the primary tumor mass and on the number of tumor cell equivalents obtained from luminometric analysis, which is a measure of the number of tumor cells, we calculated the ratio “total primary tumor cell equivalents/primary tumor mass,” which is a measure of the proportion of tumor cells contributing to each primary tumor. For such ratio, we only found a significant difference between treated and control tumors in the case of DU 145.Sluc ($P < 0.05$). For the other three tumors, the differences between treated and controls were not significant (LNCaP.Sluc, $P > 0.1$; PC-3.Sluc, $P > 0.1$; and PC-3M.Sluc, $P > 0.1$). These results indicate that the observed reductions in the mass of LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc tumors subsequent to the treatment are due to the effect of paclitaxel on tumor components other than tumor cells.

**Metastasis and Response to Paclitaxel.** Metastasis was defined as the presence of tumor cell equivalents in the target organs, which were detected as indicated in Materials And Methods by luminescence of organ homogenates following luciferin addition. Table 1 also summarizes the findings of metastasis in target organs. As can be observed from the table, tumor cell spread was generalized and cells from the four tumor types, independently of tumor inoculation site, were detected in all the organs assayed except blood where tumor cells were not detected at any time.

In spite of this generalized spread of tumor cells, no visible metastatic nodules were observed in any of the animals. The first group of organs including the lymph nodes, bones, and lungs are typical targets of prostate tumor cells. The bones are important metastatic targets for human prostate cancer and in our experiments were found heavily colonized independently from tumor cell type. Moreover, there was no difference between treated and control animals in the number of bone metastasis from any of the cell types. In other organs such as the seminal vesicles, testis, scrotum, liver, brain, spleen, and kidney, which are rarely reported as targets for macro metastases, we also found metastatic cells.

Figure 2 is a histogram showing the distribution of the different tumor cell types in four target organs with high and low metastatic propensity. The effect of paclitaxel on metastasis seemed to depend not only on tumor cell type but also on the host organ. The first four columns from the left represent the total number of metastatic tumor cell equivalents found in all the harvested organs listed in Table 1 for each cell type. The lymph nodes and bones, second and third column groups, respectively, were the highest metastasis targets for the four cell types. The number of DU 145.Sluc cells accumulated in lymph nodes...
DISCUSSION

A significant difference between treated and control animals was observed in the case of DU 145.Sluc cells, where there was a significant reduction in the number of tumor cells by 3 to 4 orders of magnitude ($P < 0.05$) for total metastasis. However, most of the effect of paclitaxel took place in the lymph nodes where the drug reduced the number of tumor cells by 3 to 4 orders of magnitude ($P < 0.05$), but there was no significant effect in the rest of organs analyzed. The rest of the cell types, LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc, were considerably more resistant to paclitaxel. LNCaP.Sluc metastases were not significantly affected by paclitaxel in any of the target organs and only PC-3.Sluc and PC-3M.Sluc tumor cells colonizing the lungs were significantly reduced by the treatment.

As shown in Fig. 2, in spite of the lack of visible macrometastatic nodules, the luminometric measure of tumor cells in the homogenates reveals a high incidence of tumor cells in some organs. Moreover, the detection system was very sensitive and versatile, allowing the quantification of very large and also very small number of tumor cells using the same assay conditions; as few as 10 cells were detected in lungs and muscle.

Primary tumors were assessed by two independent procedures: by measuring tumor mass and by determining the amount of light generated by tumor cells present in tumor homogenates, a quantifiable measure of the number of tumor cells. Metastatic capacity was evaluated exclusively by the light generation capacity of target organ homogenates, due to secondary, extractable macrometastatic nodules or tumors not being observed. In the present report, “metastatic cell” refers to tumor cells present in tissues other than the primary tumor.

Treatment protocol started a period of time after tumor cell inoculation, which varied depending on the growth rate of each tumor type (Table 1) to minimize size disparities between tumor types. After 10 days of paclitaxel treatment, there was a significant reduction ($P < 0.05$) in the mass of all but the DU 145.Sluc primary tumors, as well as the smaller ones, in comparison with saline controls. Surprisingly, in the case of LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc tumors, the actual number of tumor cells (tumor cell equivalent), measured by their light production capacity, was not significantly affected by the treatment. Only in the DU 145.Sluc tumors the number of tumor cells was significantly reduced ($P < 0.05$) by the treatment. Thus, it could be concluded that the action of paclitaxel in reducing tumor mass must have resulted from the reduction of nontumor cell components of the tumor (i.e., host cells or stroma) and not by killing tumor cells. This effect of paclitaxel was observed for i.m. as well as for the intraprostate PC-3M.Sluc tumors.

Metastatic cells from the four tumor types appeared in all the organs tested. However, large differences in cell number were found between the recognized metastasis target organs (e.g., average of $\sim 10^7$ cells in lymph nodes) and other lower-frequency targets (e.g., average of $10^2$ to $10^3$ cells in muscle). Moreover, it was possible to observe a predisposition of each tumor cell type for colonization of specific organs. Whereas LNCaP.Sluc cells were the main colonizers of lymph nodes, DU 145.Sluc cells were the most predominantly colonizers of bones and lymph nodes, and PC-3M.Sluc cells of muscle tissue.

The effect of paclitaxel on metastases from the different tumor types tested depended on the specific tissue environment in which the tumors were embedded. Thus, whereas only DU 145.Sluc cells colonizing the lymph nodes were highly sensitive to the treatment ($P < 0.05$), LNCaP.Sluc, PC-3.Sluc, and PC-3M.Sluc cells responded poorly. However, in the lungs the situation was reversed, the number of PC-3.Sluc and PC-3M.Sluc cells, the most abundant cell types, was significantly reduced by paclitaxel but DU 145.Sluc cells responded poorly. LNCaP.Sluc cells were the most resistant in all the situations. There was no difference in the sensitivity to paclitaxel of PC-3M.Sluc and PC-3.Sluc cells from prostate and i.m. tumors, respectively.

It is well known that solid tumors are heterogeneous, resulting from complex and poorly understood interactions between tumor cells and host components, and constitute a dynamically changing environment that promote tumor development. It should be expected that such interactions also participate in modulating the sensitivity of the tumor cells to paclitaxel.
treatment with antineoplastic agents. Moreover, as our data show, the physical changes taking place in tumors as a result of antineoplastic treatments do not necessarily reflect changes in the actual number of the tumor cells they contain but also result from changes in host-derived and possibly more sensitive constituents.

As it happens with other antitumoral agents, it has been reported that paclitaxel is not very effective as a single agent against prostate cancer. However, from our results it seems that the capacity of paclitaxel to kill tumor cells does not only depend on cell type but also to a large extent on other factors, such as the tissue environment in which the cells are embedded, as indicated by the observed differential cell killing capacity of paclitaxel in the different environments of primary tumors and metastases.

Probably due to the lack of procedures to accurately account for the effects of antineoplastic agents over the different components of solid tumors, there is a scant information in the literature on the subject. To our knowledge, this is a first report directly relating tumor cell number, tumor mass, and their changes in response to paclitaxel treatment.

In these experiments, the measurement of the same tumor by two different procedures revealed the existence of thus far poorly documented interactions between tumor cells, stroma, and antineoplastic agents that may determine the outcome of therapies. We used a highly sensitive luciferase-based procedure to directly measure the number of tumor cells and its change in response to the antitumor agent paclitaxel. We show that treatment of prostate experimental tumors with paclitaxel may result in a reduction of the overall tumor mass that results from a reduction in tumor components besides the number of tumor cells themselves. However, the same tumor cells, refractory to the agent in the primary tumors, may become sensitive to killing when embedded in a different physiologic environment, such as that of some metastases. It could be argued that proapoptotic factors, absence of supporting growth factors, or merely physical determinants may be responsible for the observed differences in tumor cell sensitivity to the treatment. Regardless of the mechanisms responsible for the observed effects, the present observations are relevant in the context of evaluating the clinical effectiveness of antineoplastic agents in vivo and emphasize the importance of the interactions between tumor cells and host that likely modulate the effectiveness of antineoplastic drugs.

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