Eradiation of Colorectal Xenografts by Combined Radioimmunotherapy and Combiertastatin A-4 3-O-Phosphate


Department of Oncology, Royal Free and University College Medical School, Royal Free Campus, London NW3 2PF [R. B. P., G. M. B., A. A. F., R. B., R. W., J. D., R. H. J. B.], and Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR [S. A. H., D. J. C.], United Kingdom

ABSTRACT

Solid tumors have a heterogeneous pathophysiology, which has a major impact on therapy. Using SW1222 colorectal xenografts grown in nude mice, we have shown that antibody-targeted radioimmunotherapy (RIT) effectively treated the well-perfused tumor rim, producing regressions for ~35 days, but was less effective at the more hypoxic center. By 72 h after RIT, the number of apoptotic cells rose from an overall value of 1% in untreated tumors to 35% at the tumor periphery and 10% at the center. The antivascular agent disodium combretastatin A-4 3-O-phosphate (CA4-P) rapidly reduced tumor blood flow to 62% of control values by 1 h, 23% by 3 h, and between 32–36% from 6 to 24 h after administration. This created central hemorrhagic necrosis, but a peripheral rim of cells continued to grow, and survival was unaffected. Changes in the pattern of perfusion across the tumor over time were zonal. Untreated mice showed perfusion throughout the tumor, with greatest activity at the rim. There was an overall reduction at 1 h, and total cessation of central perfusion from 3 h onward. A narrow peripheral rim of perfusion was always present, which increased in intensity and extent between 6 and 24 h, either through reperfusion or new vessel growth. Combining these two complementary therapies (7.4 MBq 131I-labeled anti-carboxymoebryonic antigen IgG i.v. plus a single 200 mg/kg dose of CA4-P i.p.) produced complete cures in five of six mice for >9 months. Allowing maximal tumor localization of antibody (48 h) before blood flow inhibition by CA4-P increased tumor retention by two to three times control levels by 96 h without altering normal tissue levels, as confirmed by gamma counting and phosphor image analysis. The success of this combined, synergistic therapy was probably the result of several factors: (a) the killing of tumor cells in the outer, radiosensitive region by targeted radiotherapy; (b) enhancement of RIT by entrapment of additional radioantibody after combretastatin-induced vessel collapse; and (c) destruction of the central, more hypoxic and radioresistant region by CA4-P. This work demonstrates the need to consider cancer treatment in a biologically heterogeneous setting, if results are to be effectively translated to the clinic.

INTRODUCTION

Antibody-targeted therapies have significantly improved selective tumor delivery of anticancer agents (1). In RIT,1 radionuclides are targeted to the tumor and deposit their energy over a relatively wide area without requiring either binding to each individual cell or internalization. RIT has produced significant therapeutic effects in model systems and clinical trials (1–3), but the tumors tend to regrow. A major reason for this failure to produce cures is the heterogeneity of tumor pathophysiology (4, 5), particularly antigen distribution and vessel perfusion, which gives an uneven distribution of systemically delivered radioantibodies. Using a range of specific and nonspecific antibodies ranging in size from single-chain Fvs (M, 27,000) to IgGs (M, 150,000), we have shown that specificity, size, and valency are the major factors determining antibody uptake and retention in the tumor (6). Bivalent specific antibodies bind to antigen close to their exit from the circulation, whereas nonspecific antibodies, lacking antigen binding sites, move through the viable tumor regions and are retained in regions of necrosis. Intact 131I-labeled anti-CEA antibody gives the highest tumor uptake, and quantitative phosphor imaging demonstrates that the outer, well-perfused tumor region retains most of the antibody and receives a therapeutic radiation dose. However, the less well-perfused inner region, containing more radioresistant and chemoresistant hypoxic cells, frequently remains relatively untreated and will continue to survive and grow (7).

Some form of combined complementary or synergistic therapy is thus required to effectively treat the whole tumor. Antivascular therapy is an obvious choice for several reasons: a functional blood supply is crucial to the survival, growth, and spread of solid tumors, and therefore therapy directed at the vasculature has the potential to kill many more tumor cells than direct cell targeting; the target is easily accessible and unlikely to develop resistance to therapy; and finally, the treatment should be applicable to all solid tumor types. Tumor vasculature is abnormal in both growth patterns and morphology and has been selectively targeted in a range of therapies (8). Two major classes of drug, those related to flavone acetate acid and the tubulin-binding agents, have proved effective antivascular agents in model systems (9, 10) and occasionally in humans (11). Although possessing different modes of action, both drugs rapidly create hemorrhagic necrosis and inhibit blood flow to all but the peripheral rim of the tumor (12, 13), which is probably supplied with nutrients by relatively normal vessels in the surrounding tissue (9, 10). It is from here that the tumor continues to grow unabated, although up to 90% of the center may have been destroyed by the antivascular agent. We have commonly found this pattern of destruction in both fast- and slow-growing xenografts, and it has also been reported in orthotopic metastatic deposits of colon carcinoma (13, 14). Because this well-perfused outer zone of the tumor is the region most effectively targeted by our antibody-directed therapies, we are investigating a combined approach to therapy. DMXAA is a potent member of the former group of antivascular agents, the main mechanism of which is immuno-modulatory and at least partially mediated via tumor necrosis factor-α induction. We have reported previously that combining DMXAA (27.5 mg/kg) with both RIT and ADEPT in a colorectal xenograft model system gave significantly enhanced therapy, with no increase in systemic toxicity (9, 15).

The current work was designed to investigate whether the novel tubulin binding agent disodium CA4-P could similarly enhance antibody-targeted therapy. This has a high affinity for tubulin at or near the colchicine binding site, causing destabilization of the tubulin polymers of the cytoskeleton. It appears to target the dividing cells of the tumor endothelium, causing a rapid shutdown of tumor vessels leading to extensive cell death in model systems (10, 16). Several tubulin binding agents in clinical use have demonstrated antivascular effects but only when approaching their maximum tolerated dose (17).

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2To whom requests for reprints should be addressed, at Department of Oncology, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, United Kingdom.

The abbreviations used are: RIT, radioimmunotherapy; CEA, carcinoembryonic antigen; CA4-P, disodium combretastatin A-4 3-O-phosphate; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; ADEPT, antibody-directed enzyme prodrug therapy.

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One of the major advantages of CA4-P is that it causes vascular shutdown at relatively nontoxic doses (12). Both DMXAA and CA4-P are currently producing biological effects in Phase I clinical trials (16, 18).

MATERIALS AND METHODS

Drug Preparation

CA4-P was dissolved in distilled water at 10 mg/ml immediately prior to use and administered i.p. at a dose of 200 mg/kg. Preliminary experiments had shown that this dose gave maximum tumor necrosis with no toxicity in our xenograft model.

Antibody Radiolabeling

A5B7, a monoclonal anti-CEA antibody (19), was labeled with 131I using the chloramine-T method to a specific activity of 180 MBq/1 mg protein and sterilized by passing through a 0.22 μm Gelman filter (Northampton, United Kingdom). This antibody and its fragments are in regular clinical use for RIT and ADEPT in our department (3, 20). The isotype-matched antibody SB10, which recognizes human chorionic gonadotropin, was labeled in the same way and used as a nonspecific control.

Animal Studies

Xenograft. The human colonic adenocarcinoma cell line SW1222 (21) was used to develop a xenograft model in the flanks of female nude mice, which were 2–3 months of age and weighed 20–25 g. Subsequent passaging was by s.c. implantation of small tumor pieces (~1 mm3). This CEA-producing xenograft is organized into well-defined glandular structures around a central lumen and secretes no measurable CEA into the circulation. The hypoxic status of the xenograft was investigated using the in vivo hypoxic marker pimonidazole (60 mg/kg, i.v. into tail vein), which forms protein adducts in cells at oxygen partial pressures <10 mm Hg. Tumors were removed at 3 h, and the relevant immunohistochemistry was performed (22). All experiments were in compliance with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia.

Effect of CA4-P on Tumor Perfusion. To assess the effect of a single optimal dose of CA4-P (200 mg/kg) on functional vascular volume, groups of four mice were given the DNA-binding dye Hoechst 33342 (10 mg/kg) i.v. 1 min before culling at 1, 3, 6, and 24 h after drug. Tumors were snap frozen in isopentane cooled over liquid nitrogen and stored at −80°C. Sections were cut at 10 μm and viewed on a Zeiss Axioskop microscope under UV excitation. Vascular volumes were determined by a random point scoring system (10), and results are expressed as a percentage of scores from untreated mice. In addition, Hoechst 33342 images of whole tumor sections were studied to determine the quantitative changes in perfusion patterns over time. Briefly, individual high resolution microscopy images of neighboring tumor regions were captured by AxioCam digital camera (Zeiss UK, Ltd.). The digitized images were then reconstructed using KS-300 software (Zeiss UK, Ltd.) to form a composite map of perfusion and delineated by drawing a region of interest around the tumor periphery. A shaded three-dimensional surface was then generated to represent the intensities within the tumor.

Effect of CA4-P on Radioantibody Biodistribution. 131I-labeled A5B7 (0.9 MBq/5.0 μg) in 0.1 ml of PBS was administered i.v. to two groups of four mice bearing SW1222 xenografts (0.5–0.75 cm3), which subsequently received either no further treatment or a single i.p. dose of CA4-P (200 mg/kg) at 48 h. Animals were bled at 96 h after antibody (48 h after CA4-P), and then liver, kidney, lung, spleen, colon, muscle, and tumor were removed for comparative activity assessment by gamma counter (Wizard; Pharmacia, Milton Keynes, United Kingdom). The same procedure was carried out for the nonspecific antibody, using 0.9 MBq/5.0 μg 131I-labeled SB10. Results were expressed as the percentage injected dose per gram of tissue (% injected dose/g).

Phosphor Plate Image Analysis Studies. The effect of CA4-P on radioantibody distribution was investigated by phosphor plate image analysis (radioimunography) and histochemistry at 96 h after antibody administration (48 h after CA4-P). Using tumors from biodistribution studies, a series of 4-μm, formalin-fixed tissue sections were prepared from each of the four mice/group. Sections were exposed to phosphor storage plates and scanned with a Storm 860 phosphor image reader (Molecular Dynamics, Seven Oaks, Kent, United Kingdom), and the digitized images of labeled antibody distribution were analyzed using ImageQuant for Windows software (7, 23). 3-Dimensional surface plots (counts/pixel) of radioactivity distribution across these sections were also drawn to illustrate, quantitatively, the heterogeneity of antibody localization. The same tissue sections were subsequently stained with H&E to relate the radioantibody distribution within the tumor, with or without CA4-P treatment, to tumor morphology.

Therapy Studies. Experiments commenced when the xenografts reached 0.1–0.2 cm3, which allowed sufficient time to study relative growth rates after different treatments. There were four groups of six mice. Groups 1 and 2 received CA4-P alone and no treatment, respectively. Group 3 received radio-labeled antibody (7.4 MBq/40 μg 131I-labeled A5B7 in 0.1 ml of PBS) as a single injection via the tail vein. Group 4 was treated with RIT (as above) combined with an i.p. dose of CA4-P (200 mg/kg) at 48 h after radioantibody. Extra control groups received unlabeled specific antibody or radiolabeled nonspecific antibody, with and without CA4-P. Tumors were measured on the day of antibody injection and every subsequent third or fourth day until tumor volume reached 2.0 cm3, when the mice were culled. Tumor volume was estimated as length × weight × height/2 (24). For all studies, the mice were given food and water ad libitum; their weight contained 0.1% potassium iodide to block thyroid uptake of iodine.

Effect of Therapy on Apoptosis. Extra groups of four mice were used to investigate the effect of the above treatments on extent and position of apoptosis within the tumor. Tumors were removed and fixed in formalin at selected times, and the terminal deoxynucleotidyl transferase-mediated nick end labeling assay (Boehringer Mannheim Biochemicals) used for in situ cell death detection, following the manufacturer’s protocol, modified for paraffin-embedded tissue. Five hundred cells/tumor were scored as positive or negative for staining in different regions, permitting the relationship between cellular apoptosis and histological localization to be studied in situ.

Toxicity Studies. Mice in the therapy studies were weighed on the day of treatment and on every subsequent third or fourth day until pretreatment values had been regained. Normal tissues were examined histologically at time points from 1 h to 9 months for signs of morphological change after RIT, CA4-P, and the two treatments combined.

Statistics

For biodistribution studies, different treatment groups were compared using the Mann-Whitney U test. Survival of therapy groups was compared according to the Lee and Desu statistics (25). P < 0.05 was considered significant.

RESULTS

Effect of CA4-P on Tumor Perfusion. When the overall effect of CA4-P on tumor perfusion was plotted over time (Fig. 1), it showed a significant decrease in vascular volume, with a peak effect at 6 h after injection. The decrease was more pronounced in xenografts treated with CA4-P than in control xenografts, as evidenced by the lower vascular volume in the treated group compared to the control group at all time points.

Fig. 1. Time course of relative changes in function vascular volume of SW1222 tumors, as a percentage of untreated tumors, after injection of 200 mg/kg CA4-P. Data are means of four mice/group, bars, SE.

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a rapid reduction to 62% of control values by 1 h, falling to its lowest value of 23% at 3 h and remaining between 33 and 36% from 6 to 24 h after drug administration. The well-perfused nature of an untreated SW1222 tumor is illustrated in Fig. 2A, whereas Fig. 2B shows that perfusion was restricted to an area mainly around the tumor rim by 24 h after CA4-P administration. Fig. 3 shows quantitative three-dimensional surface images of whole tumor sections representing zonal perfusion in a control tumor and in tumors treated with CA4-P. The control tumor (3A) was perfused throughout but with greater activity in the outer region. The pattern of perfusion distribution after CA4-P administration changed over time. There was a general reduction in perfusion throughout the tumor at 1 h (Fig. 3B). Similar
patterns were seen at 3 and 6 h (Fig. 3C), with residual areas of perfusion at the periphery but complete vascular shut-down in the tumor center. By 24 h, perfusion in the peripheral zone had increased, but the center of the tumor remained unperfused (Fig. 3D).

Effect of CA4-P on Radioantibody Biodistribution. Fig. 4 shows the distribution of specific and nonspecific antibodies in tumor and normal tissues. We had shown previously that intact A5B7 reached maximum tumor levels at 24–48 h after administration (9). We therefore allowed 48 h after radioantibody administration (0.9 MBq/5.0 μg 131I-labeled A5B7 or SB10) before giving an optimal dose of CA4-P (200 mg/kg), and studied the effects of the drug on subsequent antibody retention at 96 h after antibody, 48 h after CA4-P. The biodistribution of 131I-labeled A5B7 (Fig. 4A) in all normal tissues remained unaffected by the addition of CA4-P (e.g., liver: \( P \leq 0.6 \)). However, tumors retained a mean value of 90% more radioantibody after CA4-P administration compared with controls receiving antibody alone (42% versus 22% injected dose/g; \( P < 0.01 \)). Phosphor plate image analysis confirmed this increase in antibody retention after CA4-P (Fig. 2, C and D, mean pixel counts of 1790 and 5333, respectively). Results are reflected in the three-dimensional surface plots of radioactivity distribution across these phosphor images (Fig. 5). They demonstrate, quantitatively, the greater activity at the rim than in the center of the tumor after radioantibody alone (Fig. 5A), which became more marked after combined treatment (Fig. 5B), and also the increase in total activity retained in the latter. Histological staining of the same two sections showed that tumors from mice receiving a biodistribution dose of 131I-labeled A5B7 were mainly composed of well-differentiated, viable tissue (Fig. 2E), whereas those also receiving CA4-P exhibited central necrosis with only a thin peripheral rim of viable tumor (Fig. 2F). This outer viable rim, encircling the necrosis, corresponded to the area of greatest radioactivity observed on the phosphor plate image (Fig. 2D). The nonspecific antibody distribution in normal tissues was similar to that of A5B7 (Fig. 4B) and remained unaffected by the addition of CA4-P (e.g., liver: \( P = 1.0 \)). Tumor localization was much lower than for A5B7, but mean values increased by 83% after the addition of CA4-P (1.69% versus 3.11%; \( P = 0.05 \)).

Therapy Studies. Fig. 6 shows the mean tumor growth over time following different treatments. CA4-P alone (group 1) had no effect on tumor growth compared with untreated controls (group 2). RIT (group 3) produced significant tumor growth inhibition of ~35 days, but all tumors subsequently regrew. Combined administration of RIT and CA4-P (group 4) gave a significantly greater therapeutic effect than RIT alone; tumors were eradicated in 85% of mice, with a single tumor regrowing at 97 days. By the termination of the experiment, at greater than 9 months after treatment, there was no histological evidence of residual tumor cells in the cured mice. RIT using radiolabeled nonspecific antibody produced a small reduction in tumor growth, all mice being culled within 5 days of the untreated controls. This was extended to 10 days by the addition of CA4-P, but all tumors continued to grow. Unlabeled A5B7 had no effect on tumor growth, with or without CA4-P (not shown).

Toxicity Studies. Fig. 7 illustrates therapy toxicity (groups 1–4) by weight change over time; the mean values of six mice/group were expressed as a percentage of their pretreatment values until these were regained or the group had been culled. Groups 1 and 2 gained weight throughout the experiment and were culled prior to day 14 because of their tumor size. After RIT (group 3), mean weight fell to 95% of
pretreatment values at day 4, rising to 98% by day 7 and surpassing the original weight by day 14. A slightly greater weight loss (91% at day 4) was observed after combined therapy (group 4). This rose to 95% by day 7 and again surpassed the pretreatment value by day 14. Comparative therapies using unlabeled A5B7 and labeled nonspecific antibody produced no weight loss. Histology revealed no evidence of normal tissue damage after any of the treatment regimes.

**Effect of Therapy on Apoptosis.** Terminal deoxynucleotidyl transferase-mediated nick end labeling assays on tumor sections from untreated mice (group 2) showed staining of scattered cells, comprising 1% of the total counted and consistent with naturally occurring apoptosis (Fig. 2G). After RIT alone (Group 3), the tumors exhibited a distinct pattern of multiple apoptotic cells at the rim. These were evident at 24 h but most intense at 72 h and comprised 35% of total cells in that region (Fig. 2H). This reflected the position of major radioantibody localization at the well perfused outer zone of the tumor (Fig. 2, C and D), which contained few hypoxic cells (Fig. 2I). The center of the tumor contained occasional scattered apoptotic cells (10% of total), whereas the intensity of hypoxic staining increased progressively from the outside inward (Fig. 2, I and J). At 72 h after CA4-P alone (group 1), apoptotic cells comprised 2.1% of the total in the surviving periphery, whereas insufficient cells remained after combined therapy (group 4) for accurate analysis (not shown).

**DISCUSSION**

This study has shown that combining RIT, which targets tumor cells, with the antivascular agent CA4-P can convert tumor growth inhibition into tumor eradication. An optimal dose of the drug alone had no effect on subsequent tumor growth. RIT inhibited tumor growth for ~35 days after which all tumors regrew, but combining the two therapies produced cures in 85% of the mice. This mirrored the effect of a second class of antivascular agent, DMXAA, which has a different mode of action but produced similar morphological and therapeutic end results when combined with RIT (9). The advantage of CA4-P is that it is effective at doses significantly below the maximum tolerated dose. Using these agents to enhance antibody-targeted therapies such as RIT and ADEPT is an ideal combination; antibody-directed therapies target the outer well-perfused area of the tumor (7), which is sensitive to treatment, whereas the antivascular agents destroy the hypoxic center, which is more resistant to radio- and chemotherapy (9, 15). The combined treatment is therefore capable of successfully treating the entire tumor mass. The specificity of this therapy was demonstrated by comparative studies using a matched antibody that did not react with colorectal tumor antigens. This gave significantly reduced tumor localization and therapeutic efficacy than 131I-labeled A5B7, and survival was only extended by 5 days when RIT was combined with CA4-P.

Other groups have reported significant therapeutic enhancement or cures by combining antitumor cell therapy with treatment against established or neovasculature (e.g., Refs. 26–28). Obtaining optimal results requires an understanding of how both moieties function in different tumor areas. The onset of CA4-P effects on tumor blood vessel perfusion was very rapid, with minimum perfusion at 3 h (Fig. 1). By 1 h after CA4-P, we also observed early signs of tumor vessel hemorrhage, which progressed with time until all the outer rim was necrotic by 24 h (Fig. 2F). The zonal patterns of perfusion across the tumor after CA4-P changed over time (Fig. 3). Although untreated mice showed perfusion throughout the tumor with the greatest activity at the rim, by 1 h after CA4-P an overall reduction was observed.
From 3 h onward, the center of the tumor remained unperfused and subsequently became necrotic, but a narrow rim of perfusion remained at the periphery. The intensity and extent of this zone increased between 6 and 24 h, either as a result of reopening of existing vessels affected previously by the antivascular agent or the development of new vessels, and tumor growth continued from this region. We have observed a similar time course in other tumor models using radiolabeled iodoanitriprine, magnetic resonance imaging, and spectroscopy (12, 17, 29).

Untreated SW1222 xenografts contained occasional scattered apoptotic cells, conforming to natural programmed cell death (Fig. 2G). By 72 h after RIT alone, there was an accumulation of DNA damage leading to apoptosis, the effect being more pronounced in the outer rim of the tumor than in the center (Fig. 2H). Using the in vivo hypoxic marker pimonidazole, we found that this outer tumor area was well oxygenated, with regions of hypoxia developing progressively toward the center (Fig. 2I). This suggests that RIT exerts a localized effect because of prolonged antibody retention in the tumor periphery (Fig. 2, C and D), coupled with the greater radiosensitivity of cells at the rim compared with those in the more hypoxic center. Early apoptosis of endothelial cells has been found with CA4-P in vitro (30), and therefore we also investigated whether CA4-P induced apoptosis of tumor cells. This was impossible to assess in the center of the tumor because of extensive drug-induced tissue destruction (Fig. 2F), but the level of apoptosis found in the surviving rim at 24 h was only slightly higher than control values. Tissue destruction after combined therapy was too extensive for accurate analysis of apoptosis.

The relative time of administration for combined therapy is vitally important. Giving DMXAA before antibody reduced the effectiveness of RIT and ADEPT by inhibiting their tumor uptake. Conversely, by allowing antibody localization prior to vessel destruction, we trapped the antibody while at its maximum level in the tumor periphery without affecting normal tissue levels (9, 15). In the present study, this was demonstrated for CA4-P by gamma counting (Fig. 4) and by phosphor image analysis (Fig. 2, C and D; Fig. 5). We hypothesize that this region contained areas of both vascular shut-down and perfusion after CA4-P administration, which trapped the prelocalized antibody and also allowed continued tumor growth. This is supported by the changes observed in zonal perfusion over time after the antivascular agent (Fig. 3).

It is possible that the difference in tumor size used for biodistribution and therapy studies could affect physiology and structure and influence these results. However, we have found similar patterns of perfusion, antigen distribution, and peripheral antibody localization in both small and large tumors. Interstitial fluid pressure, which could affect the pattern of antibody distribution, has been found in colorectal xenografts by day 10, at the time and size when our therapies commenced, reaching maximum levels at a depth of only 0.2 mm from the tumor surface (31, 32). It would therefore be similar for both our studies. Levels of hypoxia were also comparable in small and large tumors, in agreement with other authors (33). We therefore believe that similar zonal heterogeneity will influence both biodistribution and therapy.

A potential problem of combined therapies is increased systemic toxicity. This was not seen when DMXAA was combined with RIT (9) or ADEPT (15). The current study showed no toxicity from CA4-P alone and only a slight increase in weight loss over RIT alone when given as a combined treatment (Fig. 7).

In conclusion, CA4-P can significantly potentiate the antitumor action of RIT and produce long-term cures. The current work has established a factual basis to support our hypothesis that antivascular and antibody-directed therapies are working, in a synergistic and complementary manner, on different tumor regions. There are three possible mechanisms: (a) a simple additive killing of the more hypoxic tumor center by CA4-P and the radiosensitive rim by RIT; (b) enhancement of the RIT effect by increased retention of radioantibody after CA4-P-induced vessel collapse; or (c) a combination of (a) and (b), with option (c) being the most probable. This work demonstrates the need to consider cancer treatment in a biologically heterogeneous setting. A greater understanding of the effect of tumor biology on the outcome of different therapies will allow us to translate our findings into optimized clinical trials.

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R. Barbara Pedley, Sally A. Hill, Geoffrey M. Boxer, et al.


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