

Novel Therapy for Liver Cancer: Direct Intraarterial Injection of a Potent Inhibitor of ATP Production¹

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Abstract

Most types of cancer are difficult to eradicate and some, like liver carcinomas, are almost always fatal. Significantly, we report here that direct intraarterial delivery of 3-bromopyruvate (3-BrPA), a potent inhibitor of cell ATP production, to liver-implanted rabbit tumors, inflicts a rapid, lethal blow to most cancer cells therein. Moreover, systemic delivery of 3-BrPA suppresses “metastatic” tumors that arise in the lungs. In both cases, there is no apparent harm to other organs or to the animals. Thus, intraarterial delivery of agents like 3-BrPA directly to the site of the primary tumor, followed by systemic delivery only when necessary, may represent a powerful new strategy for arresting the growth of liver and other cancers while minimizing toxic side effects.

Introduction

Liver cancer, in particular hepatocellular carcinoma (hepatoma), is one of the most common fatal cancers in the world (1, 2) and soon may reach epidemic levels because of increased viral-induced hepatitis (3). Among its numerous victims are not only those with primary tumors that develop directly in the liver but those with secondary tumors that frequently arise in this critical metabolic organ as a result of metastasis from other tissues, *e.g.*, the colon (4). Unfortunately, traditional treatment options (5–8) are limited by poor response rates, severe toxicities, and high recurrence rates resulting in a mean survival time of ~6 months. To circumvent these multiple shortcomings, we developed a novel strategy based on knowledge that liver tumors, in contrast to liver tissue, are fed primarily by arterial blood (5, 9), and that the inhibition of ATP production in any cell type quickly induces cell death (10). Specifically, this new strategy involves direct intraarterial delivery to liver tumors of the compound 3-BrPA⁴ (Fig. 1, A and B), a strong alkylating agent (11, 12) that abolishes cell ATP production via the inhibition of both glycolysis and oxidative phosphorylation (Ref. 12; Fig. 1C). As described here, we have now demonstrated, using a rabbit model, that this unique approach shows promise as a rapid effective therapy for liver cancer.

Materials and Methods

Tumor Implantation. The rabbit VX2 tumor (5, 12–14) was selected for implantation in the liver because of the similarities of its blood supply to that

of human hepatomas. Other attributes of this tumor include rapid tumor growth, development of a sizable tumor that can be readily identified by X-ray imaging (fluoroscopy; Ref. 5), and a biochemical phenotype (12) characteristic of advanced stage tumors, *i.e.*, high glycolysis and elevated levels of mitochondrial bound hexokinase (15, 16). In addition, the rabbit is large enough that selective manipulation of catheters in the hepatic artery from the common femoral artery for delivery of agents is possible. Adult New Zealand White rabbits (32 total; Robinson Services, Inc.) weighing 3.5–4.2 kg were used. Studies with these animals were approved by the Johns Hopkins University Animal Care and Use Committee and carried out according to their guidelines. For successful implantation of the VX2 tumor into the liver, the tumor was first grown for 2 weeks on the hind leg of a carrier rabbit. Each carrier rabbit was used to supply tumor cells for implantation into the left lobe of the liver of two separate rabbits. All of the animals, carriers and recipients, were anesthetized with a mixture of acepromazine (2.5 mg/kg) and ketamine hydrochloride (44 mg/kg) administered *i.m.*; *i.v.* access was gained via a marginal ear vein, and sodium pentothal was given *i.v.* to maintain anesthesia. The VX2 tumor was then excised from the carrier rabbit and placed in Hanks' solution. Chunks of the tumor were minced in the same solution. Then, the abdomens of the recipient rabbits were shaved and prepped with betadine, after which a midline subxyphoid incision was made. The anterior surface of the liver was exposed and tumor cells (0.1–0.2 ml) from the minced donor tumor were directly implanted onto the left lobe of the liver using the outer cannula of a 21-gauge angiocatheter. This method allows the growth of a single solitary, well-demarcated tumor in the liver of each recipient rabbit. The abdomen was closed in two layers. Proper aseptic technique was rigorously observed during each implantation. After surgery, animals were returned to their cages, kept warm with blankets, and monitored in the animal laboratory under the direct supervision of a physician or a technician until they recovered from anesthesia. Buprenorphine (0.01 mg) was administered for analgesia when the animals were in pain or showed physical distress. The tumors were allowed to grow for another 14 days, at which time they reached an ellipsoidal shape with dimensions of $\sim 1.5 \times 1.8 \times 2.5$ cm.

Preparation of 3-BrPA Solutions. The solutions of 3-BrPA (Sigma Chemical Co., St. Louis, MO) were prepared in PBS. After adjusting the pH to 7.0 with NaOH the solutions were sterilized via Millipore's Millex GV 0.22 μ m filter unit and used immediately. Freshly made solutions were used in all of the studies reported here.

Intraarterial Injection of 3-BrPA. Administration of anesthesia, *i.v.* access, and sodium pentothal anesthesia were carried out as described above. Transcatheter hepatic artery injection of 3-BrPA was performed under fluoroscopy. The animals were brought to the angiography suite and intubated using a size 3.0-mm endotracheal tube (Mallinkrodt Medical, St. Louis, MO) but not ventilated. Surgical cut-down was performed to gain access into the right common femoral artery, after which a 3 French sheath (Cook Inc., Bloomington, IN) was placed. A specially manufactured 2 French catheter with a tip in the shape of a hockey-stick (JB1 catheter; Cook Inc., Bloomington, IN) was manipulated into the celiac axis, after which a celiac arteriogram was performed to delineate the blood supply to the liver and to confirm the location of the tumor. The tumor could readily be visualized as a region of hypervascular blush located on the left side of the liver near the gastric fundus. The left hepatic artery, which usually provides most of the blood flow to the tumor, was selectively catheterized via the common hepatic artery. When necessary, a steerable guidewire (0.010–0.014 inches Transend wire; Boston Scientific MediTech, Natick, MA) was used to help select the left hepatic

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⁴ The abbreviation used is: 3-BrPA, 3-bromopyruvic acid.

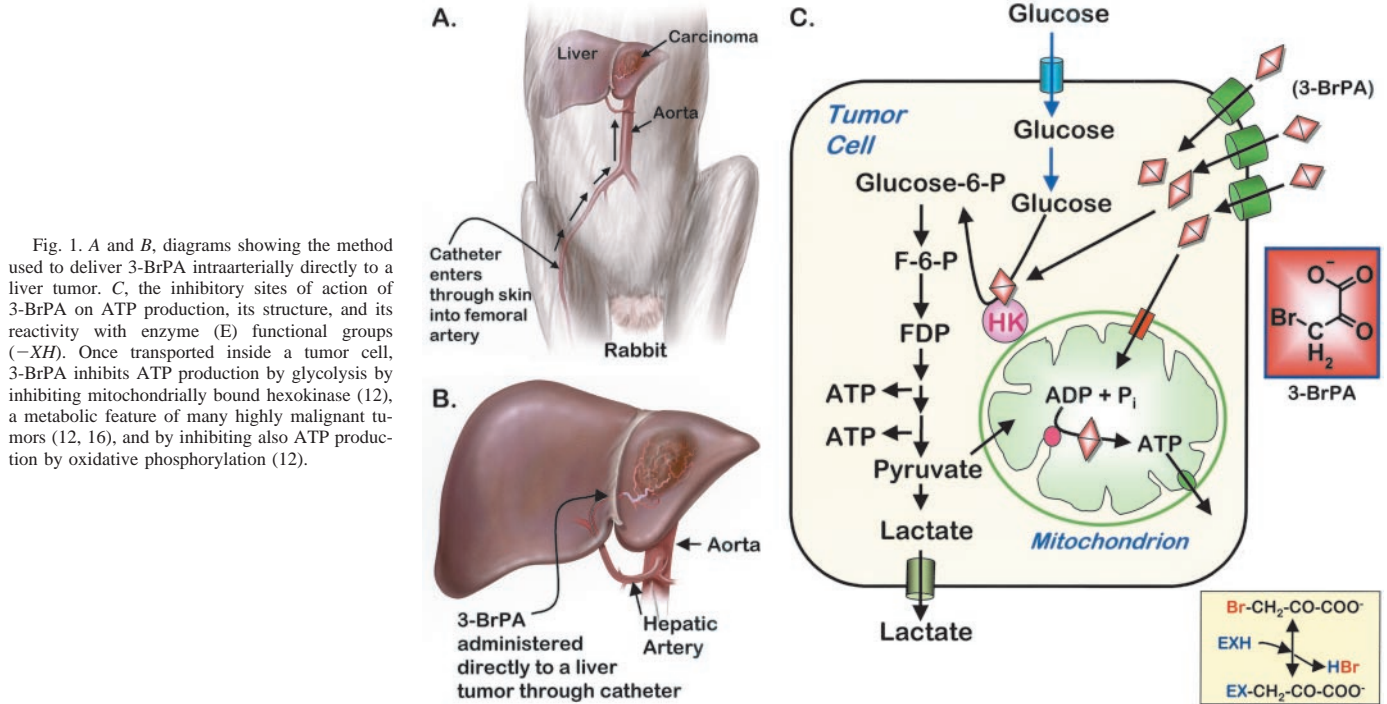


Fig. 1. A and B, diagrams showing the method used to deliver 3-BrPA intraarterially directly to a liver tumor. C, the inhibitory sites of action of 3-BrPA on ATP production, its structure, and its reactivity with enzyme (E) functional groups (-XH). Once transported inside a tumor cell, 3-BrPA inhibits ATP production by glycolysis by inhibiting mitochondrially bound hexokinase (12), a metabolic feature of many highly malignant tumors (12, 16), and by inhibiting also ATP production by oxidative phosphorylation (12).

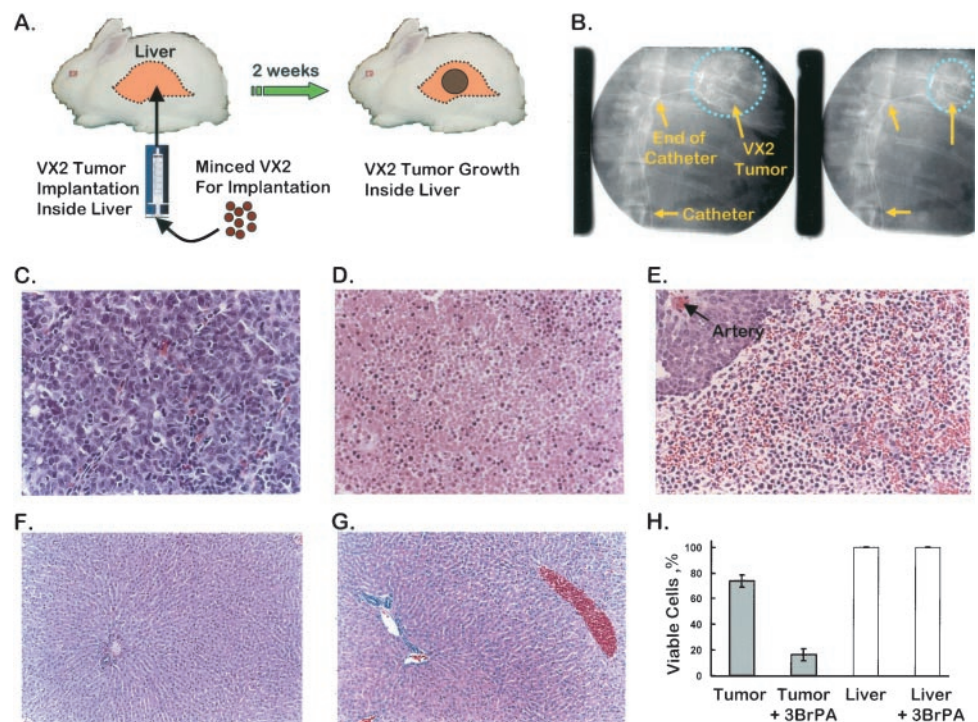
artery. After having adequately positioned the catheter within the left hepatic artery, the 3-BrPA solution was infused directly into the artery. The animals were monitored after the procedure and given analgesics when they showed signs of physical distress.

Embolization. This procedure was performed in a manner similar to the technique described above for 3-BrPA and as described earlier in detail (5). However, instead of using 3-BrPA, a mixture of Ethiodol and embolic material (polyvinyl alcohol; Target Incorporated, Fremont, CA) was injected into the left hepatic artery. The procedure was considered successful when forward

flow was no longer demonstrated within the left hepatic artery. In addition, an intense tumor stain was identified in each case, which suggested a successful embolization procedure.

Histopathology. Normal tissues and tumors were fixed in 10% formalin, sliced at 5-mm intervals for gross examination, and then embedded completely in paraffin, after which 4- μ m sections were stained with H&E. Tumor viability was estimated by visual inspection and expressed as a percentage of viable tumor area for each slice. The overall percentage of viable tumor in each rabbit was calculated.

Fig. 2. Experimental setup and effect of intraarterial injection of 3-BrPA on liver tumors. A, tumor implantation and growth. B, two representative hepatic arteriograms. Each shows the hepatic artery leading into a highly vascularized tumor (circled) located within the left lobe. C, histological section of a control "untreated" liver implanted tumor isolated 4 days after intraarterial injection of only a saline solution (see "Materials and Methods"). This section, obtained from a region of the tumor located outside of the necrotic tumor core, shows almost all viable cells. ($\times 640$). D, sections of a liver-implanted tumor isolated 4 days after intraarterial injection of 3-BrPA (see "Materials and Methods"). This section obtained from the same location of the tumor as the control, shows no viable cells. ($\times 640$). E, sections from a 3-BrPA-treated tumor identical to D but showing a region near an artery (arrow) where a tiny cluster of cells remains viable. ($\times 640$) F and G, sections from the liver of a control untreated animal and from the liver tissue surrounding an implanted tumor into which 3-BrPA had been injected intraarterially. In both, all of the cells are viable. ($\times 120$). H, bar graph summarizing the killing efficacy of intraarterial 3-BrPA on liver tumors. Data are plotted as the mean \pm SD. For the liver samples, there was no SD because all of the cells tested viable.



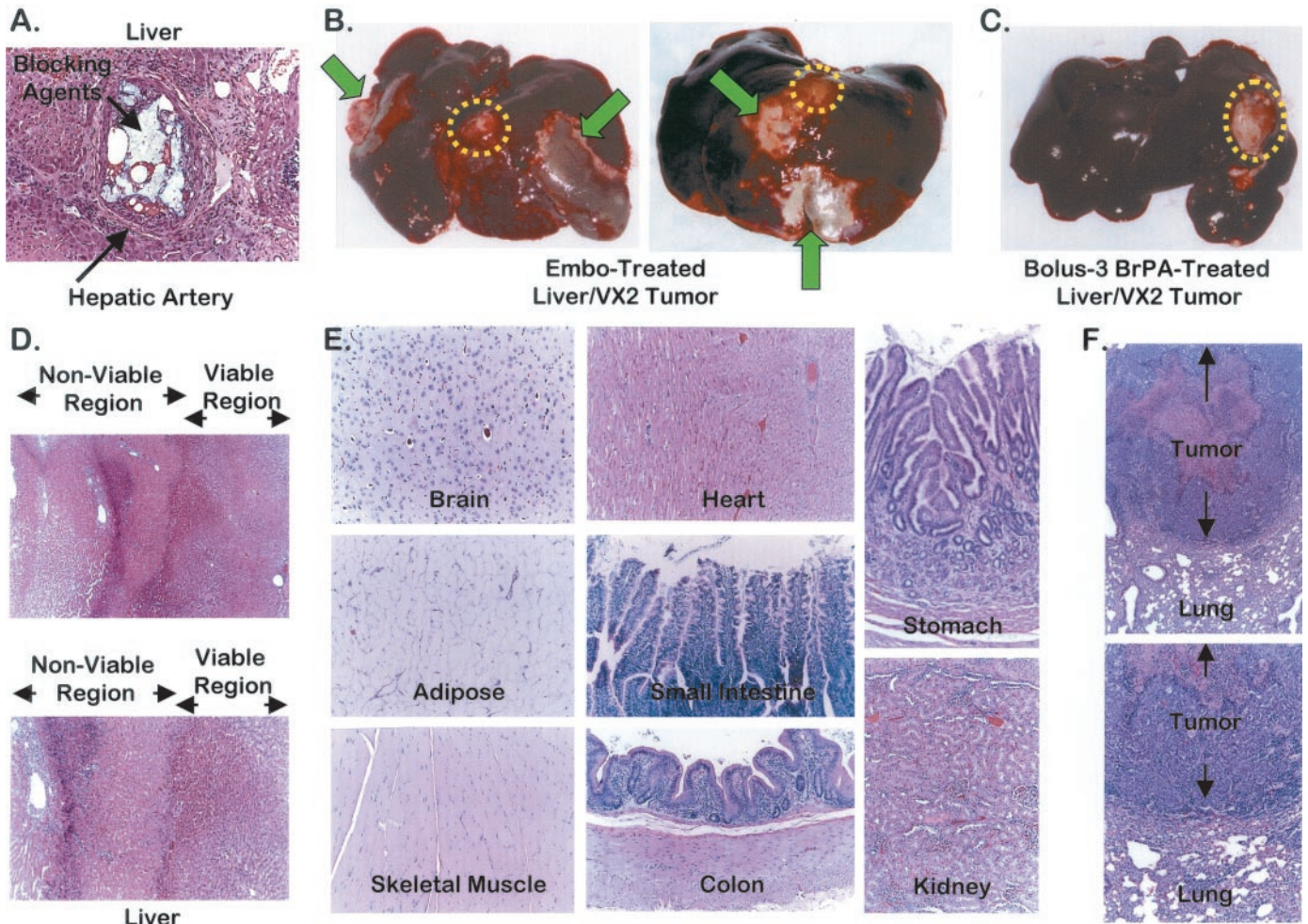


Fig. 3. Evidence for the benefits of intraarterial therapy for liver cancer using 3-BrPA over present therapy using embolization. *A*, view of the left hepatic artery observed microscopically after injection of embolization material (polyvinyl alcohol) and Ethiodol to block blood flow to the liver (5). ($\times 120$). *B*, embolized livers harboring VX2-implanted tumors (circles). Arrows, damage 4 days after embolization. *C*, liver isolated 4 days after its implanted VX2 tumor (circle) received a single injection of 3-BrPA. There is no sign of liver damage. *D*, histological sections from those regions of livers shown in *B* that had been affected by embolization. Some tissue has suffered severe damage (nonviable region) and some has remained viable. ($\times 120$). *E*, sections of eight tissues from an animal harboring a liver-implanted VX2 tumor treated by intraarterial injection of 3-BrPA. All of the tissues exhibit a normal staining pattern. ($\times 120$). *F*, sections derived from the same animal showing metastatic lung tumors. ($\times 120$).

Statistical Analysis. The mean fractions of tumor necrosis \pm SD were compared using the unpaired Student *t* test for between-group comparisons. Differences were considered statistically significant for $P < 0.05$.

Results and Discussion

Direct Intraarterial Injection of 3-BrPA into Liver-Implanted VX2 Tumors Selectively Inhibits the Viability of Cells Therein without Altering the Viability of Surrounding Liver Tissue.

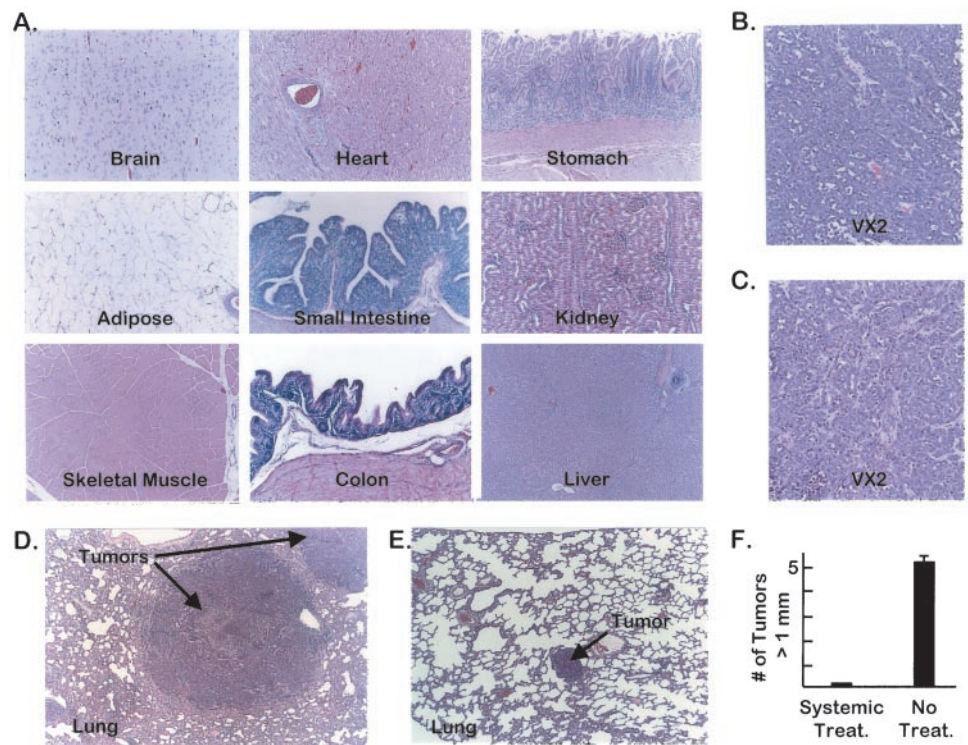
To test our hypothesis that direct intraarterial injection of a potent inhibitor of cell ATP production (3-BrPA) may selectively inhibit the viability of cells within the tumor, we employed the established VX2 tumor model for reasons described under "Materials and Methods." Small chunks of a donor VX2 tumor were minced, surgically implanted in the livers of six rabbits/experiment, and allowed to grow for 14 days (Fig. 2*A*). At this time, the single well-delineated tumor that developed in each liver exhibited a high degree of arterial vascularization because of the onset of angiogenesis. After fasting the animals for 24 h and administering anesthesia, a catheter was carefully inserted into the femoral artery and guided by fluoroscopy into the hepatic artery to a position near the tumor site (Fig. 2*B*). Then, a single bolus injection of 3-BrPA was delivered in ~ 2 min directly into the artery. Animals treated identically, but not receiving 3-BrPA, served

as controls. Optimal results were obtained by delivering 25 ml of 0.5 mM 3-BrPA, waiting 4 days, and then excising and subjecting each tumor and the surrounding liver tissue to histological analysis.

The results obtained from this novel approach proved to be quite dramatic. Compared with control "untreated" tumors, where representative sections (seven slides/tumor) obtained outside the central core region revealed nearly 100% viable cells (Fig. 2*C*), similarly located sections obtained from tumors treated with 3-BrPA (Fig. 2*D*) contained almost all nonviable cells (nearly 100% necrosis). Viable tumor cells were detected only in small areas near arteries feeding the tumors (Fig. 2*E*), and at the tumor periphery, where sinusoidal blood is available. This may reflect more active mitochondria in these oxygen-rich environments that are not completely debilitated at the concentrations of 3-BrPA used. Significantly, no damage occurred to liver tissue surrounding tumors that had been treated with 3-BrPA (Fig. 2*F* and *G*).

These results, reproduced in a number of experiments, were subjected to statistical evaluation. Tumors untreated with 3-BrPA (controls) contain $74 \pm 5\%$ viable cells in the entire population (Fig. 2*H*, column 1). The remaining cells, located within the hypoxic tumor core, have already become nonviable, a common feature of rapidly growing solid tumors. Treatment with a single intraarterial injection of

Fig. 4. Effect of systemic delivery of 3-BrPA on animals harboring the liver-implanted VX2 tumor. *A*, histological sections of nine different tissues isolated 4 days after injecting 3-BrPA (25 ml, 0.5 mm) into a marginal ear vein. No damage to these tissues is evident. ($\times 120$). *B*, section from a liver-implanted VX2 tumor isolated from a control animal not receiving 3-BrPA. *C*, comparable sample from an animal receiving 3-BrPA systemically. Cells in both appear completely viable. ($\times 120$). *D*, section of lung tissue isolated from an animal in which the liver harbored a VX2 tumor after 14 days of growth. *E*, comparable section isolated from the lung of an identical animal 4 days after receiving a systemic injection of 3-BrPA. ($\times 64$). The growth of metastatic tumors has been markedly suppressed. *F*, bar graph emphasizing that, of the total number of metastatic lung tumors counted (>27) in comparable histological sections, five were >1 mm in diameter in untreated (*no treat.*) animals harboring a liver-implanted VX2 tumor, and none were >1 mm in identical animals that received 3-BrPA systemically (*systemic treat.*) (Animals evaluated = 4).



3-BrPA decreases the number of viable cells to $16 \pm 5\%$ (Fig. 2*H*, column 2), thus increasing the total number of nonviable cells in the population to $84 \pm 5\%$ ($P < 0.05$). The maximal number of nonviable cells observed in any one experiment was 90%. In sharp contrast, the surrounding liver tissue remained completely viable in all of the cases examined (Fig. 2*H*, columns 3 and 4).

In data not presented, the portal veins, sinusoids, and bile ducts remained completely intact, with the only apparent damage occurring occasionally in the peribiliary arteriolar complexes at much higher concentrations of 3-BrPA (5 mM). These and the above findings suggest that most of the 3-BrPA, injected directly into the tumor, remained therein, and if any leakage occurred, most was neutralized by natural reducing agents (*e.g.*, glutathione) present in the surrounding tissue (17, 18).

In Contrast to Direct Intraarterial Injection of 3-BrPA, Conventional Therapy for Advanced-Stage Liver Tumors Using Embolization Results in Significant Damage to Surrounding Liver Tissue. We next inquired how this new strategy compares with the approach, called “embolization” or “chemoembolization,” that is currently used to treat advanced stage liver cancer in humans (5, 6, 8, 19, 20). Embolization involves blocking the hepatic artery feeding the tumor with a resin-like material mixed with an oil base (*e.g.*, polyvinyl alcohol in Ethiodol), thus depriving the tumor of its oxygen and nutrient sources. Chemoembolization refers to the same procedure but with the inclusion of one or more anticancer agents. Using the same rabbit model, we found that embolization alone of the hepatic artery (Fig. 3*A*) leading into the VX2 tumor causes such severe damage to the surrounding liver tissue that it is visually evident (Fig. 3*B*). This is in sharp contrast to the normal-appearing liver tissue surrounding VX2 tumors that were not embolized but instead were subjected to direct intraarterial injection of 3-BrPA (Fig. 3*C*). These findings were further substantiated by histological analyses that revealed extensive nonviable liver tissue surrounding tumors treated by embolization (Fig. 3*D*), as opposed to only viable tissue surrounding the tumors treated by intraarterial injection of 3-BrPA (Fig. 2, *F* and *G*).

The Major Tissues of Animals Bearing 3-BrPA-Treated Liver Tumors Show No Apparent Damage, but the Lungs of these Animals and Identical Animals Not Receiving 3-BrPA Show Metastatic Tumors. Despite the promising results obtained in support of direct intraarterial injection of 3-BrPA as a therapy for liver cancer, the possibility still existed that 3-BrPA may be damaging other organs. For this reason, nine major tissues were isolated from animals harboring liver-implanted VX2 tumors 4 days after receiving a single intraarterial injection of 3-BrPA. In no case was there evidence for damage to these tissues (Fig. 3, *E* and *F*). However, the unexpected discovery was made that secondary tumors had developed in the lungs (Fig. 3*F*), a finding observed also in animals bearing liver-implanted tumors that had not been treated with 3-BrPA. Because this was a consistent finding ($n =$ six animals), and because there was no evidence of such tumors in the eight other major tissues examined, these distant lesions are most likely the result of metastatic spread of the VX2 tumor from the liver to the lung.

Systemic Delivery of 3-BrPA Has No Noticeable Effect on the Animals’ Health or Behavior and No Effect on Liver-implanted VX2 Tumors, but Does Markedly Suppress the Growth of the Metastatic Lung Nodules. Finally, it was important to examine the effect of 3-BrPA when delivered systemically (*i.e.*, via the general circulation) on both animal toxicity and its capacity to damage liver-implanted tumors. After delivery of 3-BrPA (25 ml, 0.5 mM) via a marginal ear vein, rabbits that had been harboring liver-implanted VX2 tumors for 14 days exhibited normal behavior and, on sacrifice, histological examination of nine major tissues revealed no obvious damage (Fig. 4*A*). Moreover, there was no killing effect on liver-implanted VX2 tumors (Fig. 4*B* and *C*) as we had observed earlier after direct intraarterial delivery of 3-BrPA (Fig. 2, *C* and *D*), thus adding further support for this targeted approach as a preferred therapy for liver cancer. However, in sharp contrast to the failure of systemic delivery of 3-BrPA to be therapeutic for liver-implanted VX2 tumors (Fig. 4, *B* and *C*), it was found to be therapeutic for secondary tumors that had developed in the lungs. Interestingly,

animals bearing the liver-implanted VX2 tumors developed numerous “metastatic” nodules in their lungs, the largest of which were several mm in diameter (Fig. 4D). Most striking in these animals after systemic treatment with 3-BrPA was the finding of only very small tumors (Fig. 4E), and the almost complete disappearance of those with a diameter >1 mm (Fig. 4F).

In summary, we commenced this study with the objective of testing a novel strategy for the treatment of liver cancer, a strategy that envisioned direct intraarterial injection of 3-BrPA, a potent inhibitor of cell ATP production. We have shown that this strategy is highly effective, reducing in a single injection the total number of viable cells in liver-implanted rabbit tumors to as low as 10% without doing any apparent harm to the animals or their major tissues. As an unexpected extension of our original objective, we have shown also that systemic delivery of 3-BrPA to the same animals bearing the liver-implanted tumors, also does no apparent harm to the animals or their major tissues, but suppresses secondary metastatic tumors that appear in the lungs. Thus, it is possible with a single, carefully selected known chemical agent, and a combination of intraarterial and systemic delivery methods, to inflict extensive damage on both a primary tumor and a secondary metastatic tumor within the same host without doing noticeable harm to the host. Future studies will focus on how the animal's natural defense mechanisms are able to cope with such a reactive alkylating agent as 3-BrPA whereas the liver and lung tumors studied are highly sensitive to this agent.

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