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Advances in Brief

2-Deoxy-D-glucose Increases the Efficacy of Adriamycin and Paclitaxel in Human Osteosarcoma and Non-Small Cell Lung Cancers *In Vivo*

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Abstract

Slow-growing cell populations located within solid tumors are difficult to target selectively because most cells in normal tissues also have low replication rates. However, a distinguishing feature between slow-growing normal and tumor cells is the hypoxic microenvironment of the latter, which makes them extraordinarily dependent on anaerobic glycolysis for survival. Previously, we have shown that hypoxic tumor cells exhibit increased sensitivity to inhibitors of glycolysis in three distinct *in vitro* models. Based on these results, we predicted that combination therapy of a chemotherapeutic agent to target rapidly dividing cells and a glycolytic inhibitor to target slow-growing tumor cells would have better efficacy than either agent alone. Here, we test this strategy *in vivo* using the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) in combination with Adriamycin (ADR) or paclitaxel in nude mouse xenograft models of human osteosarcoma and non-small cell lung cancer. Nude mice implanted with osteosarcoma cells were divided into four groups as follows: (*a*) untreated controls; (*b*) mice treated with ADR alone; (*c*) mice treated with 2-DG alone; or (*d*) mice treated with a combination of ADR + 2-DG. Treatment began when tumors were either 50 or 300 mm^3 in volume. Starting with small or large tumors, the ADR + 2-DG combination treatment resulted in significantly slower tumor growth (and therefore longer survival) than the control, 2-DG, or ADR treatments ($P < 0.0001$). Similar beneficial effects of combination treatment were found with 2-DG and paclitaxel in the MV522 non-small cell lung cancer xenograft model. In summary, the treatment of tumors with both the glycolytic inhibitor 2-DG and ADR or paclitaxel results in a significant reduction in tumor growth compared with either agent alone. Overall, these results, combined with our *in vitro* data, provide a rationale for initiating clinical trials using glycolytic inhibitors in combination with chemotherapeutic agents to increase their therapeutic effectiveness.

Introduction

In 1930, Warburg (1) proposed that all tumor cells undergo accelerated aerobic glycolysis, but it was not until the advent of molecular biology, particularly the identification of oncogenes, that the essential underlying mechanisms began to be understood. In 1987, it was reported that Ras or Src oncogene-mediated transformation of fibroblasts led to up-regulation of glucose transport protein and messenger RNA (2). In the early 1990s, hypoxia-inducible factor (HIF) was identified as a transcription factor that, under hypoxic conditions, up-regulates genes encoding glucose transporters, glycolytic enzymes, and vascular endothelial growth factor, among others (3–5). It is well known that most, if not all, solid tumors contain regions of varying degrees of hypoxia. Thus, the accelerated glycolysis observed by Warburg (1) can now be explained by at least two mechanisms, one driven by malignant transformation, and the other driven by hypoxia.

For decades, screening and selection of anticancer agents were based on the more rapid division of tumor cells relative to normal cells. As a result, radiation therapy and most chemotherapy currently used to treat cancer target the rapidly dividing cells of a tumor as well as the most rapidly dividing normal cells that reside in the bone marrow, gastrointestinal regions, and hair follicles. Thus, the main selectivity of these treatments is not between malignant and normal cells but between rapidly and slowly dividing cells. Solid tumors, however, contain regions of slowly proliferating cells and because most normal cells are either resting in G_0 or are dividing slowly, one of the most difficult populations to selectively target are the slow-growing malignant cells. A distinguishing feature between slow-growing tumor and normal cells is that the microenvironment of the former is hypoxic, which contributes to reduced growth rate and drug resistance (6–8). Under hypoxia, tumor cells must metabolize glucose anaerobically to generate ATP, thereby providing a window of selectivity that can be exploited with inhibitors of glycolysis (9–11).

In a recent series of papers using three *in vitro* models of simulated hypoxia (9–11), we have shown that cells under hypoxic conditions are more sensitive than cells under aerobic conditions to agents that inhibit glycolysis, such as 2-deoxy-D-glucose (2-DG) and oxamate. The basis for this increased sensitivity is that a tumor cell growing under low or no oxygen must rely primarily on glycolysis to produce ATP. Thus, when challenged with a glycolytic inhibitor, ATP synthesis is shut off, and the cell succumbs to this treatment. However, in the presence of oxygen, a cell can use alternative sources of energy, such as fats and proteins, to synthesize ATP and hence survive inhibition of glycolysis. Because the slowly proliferating tumor population can be selectively killed with glycolytic inhibitors, combining such agents with chemotherapeutic drugs, which target the rapidly dividing aerobic cells, should raise the overall efficacies of these treatments (9–11). Here, we have designed experiments to test this strategy *in vivo* using 2-DG in combination with either Adriamycin (ADR) or paclitaxel in two different human tumor types, osteosarcoma and non-small cell lung cancer (NSCLC), growing in nude mice.

Materials and Methods

2-DG + ADR in Human Osteosarcoma Cells in Nude Mice. Nude mice, strain CD1, approximately 5–6 weeks of age and weighing approximately 30 g, received s.c. implantation with 100 μl of human osteosarcoma cell line 143b at 10^7 cells/ml. When tumors were approximately 50 mm^3 in size (9 days later), the animals were pair-matched into four groups (8 mice/group) as

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follows: saline-treated control; 2-DG alone; ADR alone; and ADR + 2-DG. At day 0, the 2-DG alone and ADR + 2-DG groups received 0.2 ml of 2-DG i.p. at 75 mg/ml (500 mg/kg), which was repeated 3 × per week (Monday, Wednesday, and Friday) for the duration of the experiment. On day 1, the ADR and ADR + 2-DG groups received 0.3 ml of ADR i.v. at 0.6 mg/ml (6 mg/kg), which was repeated once per week for a total of three treatments (18 mg/kg). This experiment was then repeated ($n = 4$/group), but treatment was delayed until the tumors were approximately 300 mm^3. Mice were weighed, and tumor measurements were taken by caliper three times weekly. Tumor measurements were converted to tumor volume by using the formula $W \times L^2/2$. Mice were killed when either W or L exceeded 15 mm. At sacrifice, mice were weighed, and tumors were excised and checked histologically for verification of tumor growth.

2-DG + Paclitaxel in Human MV522 Lung Tumor Carcinoma Cells in Nude Mice. Nude female mice, approximately 5–6 weeks of age and weighing approximately 20 g, received s.c. implant by trocar with fragments of human MV522 lung tumor carcinoma harvested from s.c. tumors growing in nude mice hosts. When tumors were approximately 70 mm^3 in size (11 days later), the animals were pair-matched into five groups (10 mice/group) as follows: vehicle-treated control; paclitaxel alone; paclitaxel + 2-DG (500 mg/kg); paclitaxel + 2-DG (1000 mg/kg); and paclitaxel + 2-DG (2000 mg/kg). An aqueous solution of 2-DG was delivered twice daily by oral gavage. Doses of 500 and 1000 mg/kg were given for the duration of the experiment, whereas the 2000 mg/kg 2-DG dose was given twice daily for only 10 days. Paclitaxel was given by i.p. injection at 16 mg/kg once a day for 5 days, beginning 5 days after the first day of 2-DG treatment. Mice were weighed, and tumor measurements were taken by caliper twice weekly. Tumor measurements were converted to tumor volume using the formula $W \times L^2/2$. Mice were killed when their tumor volume exceeded 1000 mm^3. At sacrifice, mice were weighed, and tumors were excised and weighed.

Statistical Analysis. For the osteosarcoma studies, linear regression was used to determine the rate of the log of tumor growth over time for each treatment using the following equation: $\text{Log}_e(\text{tumor volume} + 1) = \alpha_i + \beta_i(\text{time}) + \varepsilon_{ik}$, where $i = 1,2,3,4$ indicates treatment group, and k is an index for each mouse ($k = 1, \ldots, n_i$). Faster growth of tumor is represented by larger slopes (β) in the regression equation, which in turn represent greater rates of disease progression. This model was used to estimate the number of days required to reach specific tumor volumes of 400, 800, and 1200 mm^3. Although the mice in some of the treatment groups survived for more than 4 weeks, the analyses of tumor growth rates were truncated at the time when the control animals began to reach the maximum tumor volume allowed. Multiple comparisons were made between the various treatment groups to determine whether the rates of tumor growth, *i.e.*, slopes, differed across treatment groups.

For the NSCLC study, the time for each individual tumor to grow to 600 mm^3 was determined, and a one-way ANOVA repeated measures test was done to determine whether there was a significant difference in time for any of the groups to reach a tumor size of 600 mm^3. When a significant difference was found ($P < 0.050$, a Dunnett's *post hoc* comparison was performed to determine whether the combined treatment was significantly different from paclitaxel alone.

Data are plotted for each group up until the day when no animals have been sacrificed due to tumor burden. For animals that died prematurely, tumor volumes were set to 1000 mm^3 for all subsequent time points.

Results

The Effect of 2-DG and Adriamycin in Osteosarcoma Tumors Growing in Nude Mice. Fig. 1, *A* and *B*, summarize tumor growth rates for mice treated with control vehicle (saline), 2-DG, ADR, or ADR + 2-DG, and treatment began when the tumors were an average of 50 or 300 mm^3, respectively. In Fig. 1, *A* and *B*, it can be seen that ADR + 2-DG is clearly more effective in reducing tumor volume as compared with treatment with either ADR alone, 2-DG alone, or control.

In the experiment where treatment began when tumors were 50 mm^3 ($n = 8$/mice/group), three mice in the ADR + 2-DG group had apparent cures (tumors regressed slowly with continued treatment and were undetectable at 38–80 days). No cures were observed in any of the other groups. In Fig. 1*A*, where cures are excluded, the ADR + 2-DG treatment is still shown to be more effective than control, 2-DG, or ADR alone. A statistical analysis of the rate of tumor growth for each treatment type was used so that the cures could be included. This analysis showed that the group treated with ADR + 2-DG had tumors that grew significantly more slowly than the 2-DG, ADR, or control groups ($P < 0.0001$ for each comparison). When the time to achieve tumor volumes of 400, 800, or 1200 mm^3 is estimated by linear regression, the longest delay in tumor growth again is in the group of animals treated with ADR + 2-DG for all three tumor volumes (Table 1). Specifically, the combination of ADR + 2-DG requires 45 days for tumor volume to reach 400 mm^3, whereas this volume is reached in 19 days with control, 20 days with 2-DG alone, and 25 days with ADR alone. Similar beneficial results for the combined treatment group are observed for tumor volumes of 800 and 1200 mm^3 (Table 1). All treatments were well tolerated, as evidenced by similar body weights for all groups throughout the treatment period.

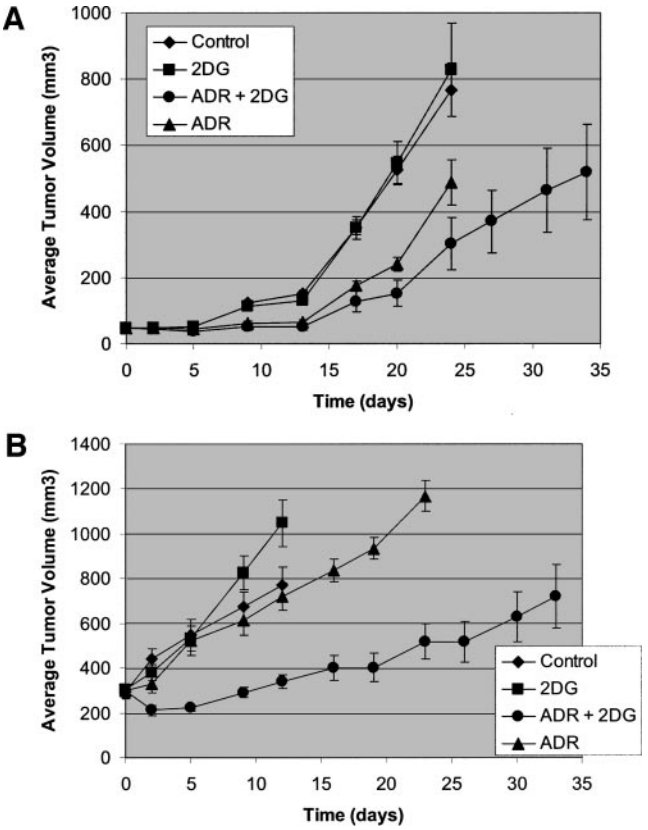

Fig. 1. Effect of 2-DG and ADR, alone and in combination, on growth of human osteosarcoma xenografts in nude mice. Average tumor volumes are shown as a function of time after initiation of treatment, when treatment was started when tumors were (*A*) 50 mm^3 or (*B*) 300 mm^3. On day 0, the control animals received saline (i.p.), and animals in the 2-DG alone and ADR + 2-DG groups received 500 mg/kg, 2-DG (i.p.), which was repeated 3 × per week (Monday, Wednesday, and Friday) for the duration of the experiment. On day 1, the ADR alone and ADR + 2-DG groups received 6 mg/kg ADR (i.v.), which was repeated once per week for a total of three treatments (18 mg/kg). Note the enhanced efficacy of the combined treatment compared with either agent alone.

In the second experiment, in which treatment began when the tumors were larger (300 mm^3), the group receiving the ADR + 2-DG combination treatment again showed smaller average tumor volumes than the 2-DG, ADR, and control groups at all time points (Fig. 1*B*). One of the mice in the combination therapy group had a tumor that regressed slowly, starting at 303 mm^3 and shrinking to 56 mm^3 by day 44. At day 34, all of the animals in the combined treatment group had

Table 1 *Estimated time in days to reach tumor volumes of 400, 800, or 1200 mm^3*

Tumor volume (mm^3)[a]	Control	2-DG	ADR	ADR + 2-DG
400	19	20	25	45
800	25	25	32	58
1200	28	29	36	66

[a] Log_e (tumor volume + 1) = $\alpha + \beta \times$ time; $P < 0.0001$ for comparisons of the rate of tumor growth in the ADR + 2-DG group to each of the other groups (control, 2-DG, and ADR). ADR, Adriamycin; 2-DG, 2-deoxy-D-glucose.

Table 2 *Estimated time in days to reach tumor volumes of 400, 800, or 1200 mm^3*

Tumor volume (mm^3)[a]	Control	2-DG	ADR	ADR + 2-DG
400	2	3	4	20
800	13	10	14	43
1200	19	15	20	57

[a] Log_e (tumor volume + 1) = $\alpha + \beta \times$ time; $P < 0.0001$ for comparisons of the rate of tumor growth in the ADR + 2-DG group to each of the other groups (control, 2-DG, and ADR). ADR, Adriamycin; 2-DG, 2-deoxy-D-glucose.

tumor volumes below that which would require sacrifice, whereas all of the animals in the control, ADR, and 2-DG groups had already been sacrificed due to tumor burden.

When the time to achieve tumor volumes of 400, 800, and 1200 mm^3 was estimated by linear regression in this experiment, the longest delay in tumor growth was again found in the group of animals treated with ADR + 2-DG for all three tumor volumes (Table 2). Specifically, this group of animals required approximately 20 days for tumor volume to reach 400 mm^3, whereas this volume was reached in approximately 2 days with control, 3 days with 2-DG alone, and 4 days with ADR alone. Similar advantageous results for the combination treatment of 2-DG + ADR were observed when time to reach 800 and 1200 mm^3 tumor volumes were estimated. It should be noted that this second experiment was a more stringent test of the effect of the various treatment regimens because larger tumors are typically more difficult to treat. The data from both studies are consistent in demonstrating that beginning with small or large tumors, there is significant advantage in combination treatment with 2-DG + ADR as compared with treatment with either agent alone. In addition to slowing tumor growth, cures were found or significant tumor shrinkage was observed only in the ADR + 2-DG group.

The Effect of 2-DG and Paclitaxel in NSCLC Tumors Growing in Nude Mice. Tumor growth for NSCLC xenografts in nude mice treated with control, paclitaxel, or paclitaxel + 2-DG (500, 1000, or 2000 mg/kg 2-DG) are shown in Fig. 2. As observed for the combination of ADR + 2-DG, paclitaxel + 2-DG was more effective in reducing tumor growth in human NSCLC tumors than was paclitaxel alone. Average tumor volume and SE are plotted in Fig. 2 only for groups where no animals had been sacrificed. There were two premature deaths, however, at relatively small tumor volume, one in the paclitaxel group on day 14, and one in the paclitaxel + 2-DG (500 mg/kg) group on day 19. These animals' tumor volumes were set to 1000 mm^3 for all subsequent time points. All treatment regimens were well tolerated, with a maximum body weight loss of 8% that was of short duration.

The time to reach a tumor volume of 600 mm^3 was determined for each individual mouse. The average time to 600 mm^3 was 21.0, 22.1, 29.0, and 33.7 days for the paclitaxel, paclitaxel + 2-DG (500 mg/kg), paclitaxel + 2-DG (1000 mg/kg), and paclitaxel + 2-DG (2000 mg/kg) groups, respectively. An ANOVA analysis showed that the time for the paclitaxel + 2-DG (1000 mg/kg) and paclitaxel + 2-DG (2000 mg/kg) group tumors to reach 600 mm^3 was significantly longer than that for the paclitaxel and paclitaxel + 2-DG (500 mg/kg) groups ($P < 0.05$). The Paclitaxel + 2-DG (1000 mg/kg) and paclitaxel + 2-DG (2000 mg/kg) groups were not significantly different from each other. A statistical analysis was not done on the rate of growth of the NSCLC tumors due to the large reduction in tumor growth rate between 10 and 20 days (Fig. 2). Combination chemotherapy with 2-DG and either ADR or paclitaxel improves therapy over chemotherapy alone in both osteosarcoma and NSCLC xenograft models of cancer in nude mice. It should be noted that an effective dose of 2-DG was achieved orally in these animals, which offers an important advantage over i.v. delivery of conventional chemotherapeutic agents, if similar results can be obtained in human patients.

Discussion

The *in vivo* results reported here demonstrate that combining 2-DG with either ADR or paclitaxel clearly increases the efficacy of each of these chemotherapeutic agents in retarding tumor growth and prolonging survival. This outcome can be explained in part by our previous *in vitro* results, which illustrated that when oxidative phosphorylation is inhibited by either chemical (mitochondrial inhibitors), genetic (Rho 0 cells), or environmental (hypoxia) means, cells must rely on glycolysis for survival and thus become hypersensitive to inhibitors of glycolysis (9–11). However, because we could not observe an advantage in arresting tumor growth when animals were treated with 2-DG alone (although an effect on slowly proliferating hypoxic cells might have been masked by the rapidly dividing cells in the tumor), additional factors must account for the enhanced effect we find with combination treatment.

One of these factors is that chemotherapeutic agents including paclitaxel and ADR display antiangiogenic and or anti-HIF activity (12–14). Thus, tumors treated with these agents may become more hypoxic, leading to enhanced efficacy of 2-DG. Moreover, if anti-HIF agents reduce the overexpression of glycolytic enzymes, then theoretically, this should reduce the amount of glycolytic inhibitor necessary to shut down glycolysis. Therefore, the multiple effects that anti-HIF agents such as 2-methoxyestradiol (15) have on tumor metabolism in blocking both angiogenesis and reducing the expression of glycolytic enzymes would theoretically increase the effectiveness of 2-DG or other inhibitors of glycolysis.

Another factor that may be contributing to the results presented

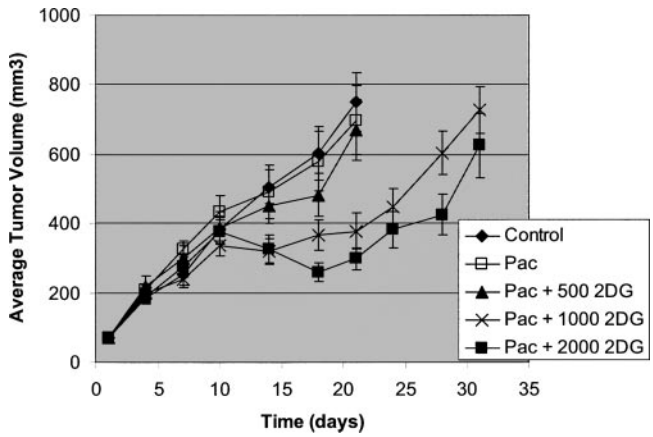

Fig. 2. Effect of 2-DG and paclitaxel, alone and in combination, on average tumor volume of human NSCLC xenografts in nude mice: Numbers in the *inset* symbol definitions represent dose in mg/kg. Treatment was started when tumors were 70 mm^3. Starting on day 1, animals in the 2-DG treatment groups were dosed orally, twice daily, with an aqueous solution of 2-DG at 500 or 1000 mg/kg for the duration of the experiment or with 2000 mg/kg for 10 days. Paclitaxel was given alone or in combination with 2-DG, by i.p. injection at 16 mg/kg, once a day for 5 days, beginning 5 days after the first day of 2-DG treatment. Note the increased tumor growth inhibition in the combined paclitaxel + 2-DG group as compared with the paclitaxel alone or vehicle-treated control groups.

here is based on reports that the combination of 2-DG and cisplatin is more effective than either agent alone when applied to various cell lines that are rapidly proliferating *in vitro* (16). One of the explanations for this effect is that cells treated with drugs like cisplatin, which are known to cause DNA damage, cannot repair lesions as readily when their ATP levels are reduced as a result of glycolytic inhibition (16). Similar *in vitro* synergism has been observed with the combination of 2-DG and ADR in MCF-7 cells (17), however, other studies have reported antagonistic effects (18). Thus, further experimentation will be required to determine whether the increased effectiveness of the combination therapy presented here is due to 2-DG preventing cells from repairing damage caused by ADR or paclitaxel.

An alternative explanation for how 2-DG increases the activity of chemotherapeutic agents is based on the fact that the p-glycoprotein effluxing pumps require ATP for their activity (19). If ATP concentrations are reduced, as has been reported to occur when cells are treated *in vitro* with 2-DG (16), the pumps will cease to function, and drug accumulation should increase intracellularly, thereby killing the cell. Thus, treating such resistant tumors with a combination of glycolytic inhibitor and any of the numerous anticancer agents known to be recognized by this mechanism (19, 20) should provide clinical benefit. Although we reported previously that the osteosarcoma cell line used here expressed low levels of multidrug resistance-related protein (MRP), and the MDR1 protein was undetectable, it remains possible that other effluxing pumps may be present in this cell line as well as in the NSCLC cell line used in these experiments.

In summary, regardless of the underlying mechanisms, it is demonstrated here that 2-DG does indeed increase the efficacy of standard chemotherapeutic drugs when applied to human tumors *in vivo*. We believe that this strategy of inhibiting glycolysis will have broad clinical benefit as an adjunct to cancer therapies. Our approach should be particularly applicable to antiangiogenic treatment and to the new agents tested against HIF, both of which should make the tumor more hypoxic and therefore more sensitive to inhibitors of glycolysis. In conclusion, we anticipate that inhibitors of glycolysis could have widespread application for a variety of tumor types not only in combination with existing anticancer agents but also with emerging new treatments that target the metabolic state of a tumor.

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