Combination of Nicotinamide and Hyperthermia to Eliminate Radioresistant Chronically and Acutely Hypoxic Tumor Cells

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ABSTRACT

The interaction among nicotinamide, radiation, and heat was studied in vivo using a C3H mouse mammary carcinoma grown in the feet of CDF, mice. Response following local tumor treatment was assessed by tumor control and regrowth delay. Nicotinamide (1000 mg/kg i.p.) produced maximal radiosensitization when injected 30 min to 2 h before irradiation [enhancement ratios (ERs), 1.2–1.5]. Radiation damage was also increased by heating tumors (43.5°C for 60 min) 4 h after irradiation (ERs = 1.6–2.6). This combined radiation and heat treatment was enhanced by nicotinamide but the effect depended on the assay procedure, such that although a significant increase was observed with the tumor control assay, only a slight increase was seen using regrowth delay as the end point. The development of moist desquamation in normal feet was used to estimate skin damage after irradiation. Nicotinamide and heat both resulted in a small yet significant increase in skin damage (ERs <1.2 and 1.1, respectively). A combined treatment resulted in a greater ER of 1.7, but when compared to the tumor response it still gave a therapeutic gain. A histological fluorescent staining technique was used to assess functional tumor vasculature at two periods in time separated by 20 min. Under normal conditions 7.7% of the vessels in this tumor were functional at one time but not the other. This value was reduced to 2.8% after nicotinamide administration. Since these fluctuations in blood flow can result in acute hypoxia we conclude that while heat eliminates chronically hypoxic tumor cells, nicotinamide probably removes the presence of acute hypoxia.

INTRODUCTION

It has been clearly shown that solid tumors contain hypoxic cells (1, 2) and there is evidence to strongly suggest that these same cells can influence clinical response to treatment (3–5). Overcoming hypoxia therefore continues to be a major interest in radiation biology and oncology (6–9). Numerous efforts to eliminate hypoxia have been tried. These include decreasing the level of hypoxia by increasing the availability of oxygen, as can occur with hyperbaric oxygen (10, 11), perfluorochemical emulsions (12, 13), hemoglobin oxygen modifiers (14, 15), and calcium antagonists (16, 17). Alternatively, methods have been studied in which hypoxic cells are actually sensitized to radiation or preferentially killed by agents like bioreductive drugs (18, 19) and hyperthermia (20, 21), although in the latter example the increased cytotoxicity is not due to hypoxia per se but rather a consequence of hypoxia-induced metabolic changes (21–23).

While these approaches can yield improvements in tumor response to treatment, the results have been far from satisfactory. One important reason for this is that in general these treatments specifically overcome long term “chronic hypoxia” which was first described by Thomlinson and Gray (24) in 1955 and typically arises as a result of a diffusion limitation of oxygen. There is now evidence to suggest that hypoxia can result from transient fluctuations in microregional blood flow (25–28). This type of hypoxia is generally considered to be acute and would be unresponsive to most of the treatments designed to overcome chronic hypoxia.

Nicotinamide is a compound which has been gaining interest over the past few years as a potential tumor radiosensitizer. It is relatively nontoxic both in mice (29, 30) and humans (31) and in general appears to enhance radiation damage in several tumor models in preference to normal tissues (30, 32). Rather than directly radiosensitizing hypoxic cells, its major mode of action appears to involve an increase in tumor oxygenation status at the time of irradiation, apparently as a result of an increase in tumor blood perfusion (33, 34). More recent data suggest that rather than simply reducing the level of chronic hypoxia in tumors, nicotinamide may also be capable of decreasing the presence of radioresistant acutely hypoxic cells (35, 36). If nicotinamide can eliminate acute hypoxia then this compound may have a role to play in combination with agents which are used to specifically overcome radioresistant chronic hypoxia.

One of the most efficient agents for killing chronically hypoxic cells is hyperthermia (20, 37), and numerous studies have shown that as a result of this, heat can substantially improve the response of murine tumors to radiation (for review, see Ref. 38). The following study was therefore undertaken to determine whether or not nicotinamide could enhance the combined effect of radiation and heat in vivo, using a C3H mouse mammary carcinoma.

MATERIALS AND METHODS

Animal and Tumor Model. All experiments were performed on 10–14-week-old female CDF, mice. The tumor model used was the C3H/Tif mouse mammary carcinoma. Its derivation and maintenance have been described previously (39). Experimental tumors were produced following sterile dissection of large flank tumors. Macroscopically viable tumor tissue was minced with a pair of scissors, and 5–10 μl of this material were injected into the foot of the right hind limb of the experimental animals. This location ensured easy access to the tumor for treatment without involvement of critical normal tissue in the treatment field. In addition, anesthetization of the animals during treatment could be avoided. Treatments were carried out when tumors had reached a tumor volume of about 200 mm3, which generally occurred within 3 weeks after challenge. Tumor size was determined by the formula

$$D_1 \times D_2 \times D_3 \times \pi/6$$

(where the $D$ values represent three orthogonal diameters).

Drug Preparation. Nicotinamide (Sigma Chemical Co., St. Louis, MO) was dissolved at a concentration of 50 mg/ml in a sterile saline solution (0.9% NaCl solution) immediately before each experiment. It was injected i.p. into mice at a constant injection volume of 0.02 ml/g body weight.

Radiation Treatment. Irradiations were given with a conventional therapeutic X-ray machine (250 kV; 15 mA; 2-mm Al filter; 1.1 mm
Clamping the tumor-bearing leg 5 min before and during the period of irradiation, the tumors only were immersed in a water bath with about 5 cm of water between the leg and the water surface. Previous measurements of intratumoral temperature showed stabilization within 2 min to ±0.2°C of the adjusted temperature. The water bath was covered with a Lucite plate with holes allowing immersion of the foot approximately 1 cm below the water surface. Preparations for hyperthermia were achieved by constriction of the blood flow using a rubber tube tightened around the leg.

**Hyperthermia Treatment.** Nonanesthetized animals were placed in Lucite jigs and their tumor-bearing feet were fixed as described previously. Hyperthermia was delivered by immersing the leg into a circulating water bath (type TE 623; Heto, Birkerød, Denmark) stabilized to ±0.2°C of the adjusted temperature. The water bath was covered with a Lucite plate with holes allowing immersion of the foot approximately 1 cm below the water surface. Previous measurements of intratumoral temperature showed stabilization within 2 min to ±0.2°C below the water bath temperature (40). The temperature of the water bath was therefore adjusted to 0.2°C above the desired tumor temperature. All temperature measurements were calibrated against a certified precision mercury thermometer.

**Tumor Response.** The response of tumors to treatment was assessed using both a regrowth delay assay and local tumor control. The regrowth delay assay procedure involved measuring tumor volume, as described earlier, on a daily basis following treatment. The time taken to reach 3 times the treatment volume was determined and from this value the mean ± SE was calculated from all the mice in each treatment group. For the local tumor control assay, tumor-bearing mice were followed at weekly intervals up to 90 days posttreatment. Tumor response was calculated as the percentage of animals in each treatment group showing local tumor control at 90 days.

**Estimation of Tumor Hypoxic Fraction and Cell Survival.** The procedure and rationale for these calculations have been described in detail previously (41, 42). Basically the hypoxic fraction (HF) was calculated from local tumor control data as

\[
HF = \exp \left( \frac{TCD_{50}\text{ (clamp)} - TCD_{50}\text{ (air)}} {D_0\text{ (hypoxic)}} \right)
\]

where \(TCD_{50}\) (air) and \(TCD_{50}\) (clamp) represent the \(TCD_{50}\) doses in normal and clamped animals, respectively, and \(D_0\) (hypoxic) represents a \(D_0\) of 3.2 Gy for hypoxic cells (43). The total number of tumor cells \(N\) (total) were determined as

\[
N \text{ (total)} = \exp \left( \frac{TCD_{50}\text{ (clamp)}} {D_0\text{ (hypoxic)}} \right) \cdot \ln (2) \cdot n
\]

where \(N\) is the number of tumor cells and \(n\) is the extrapolation number of 3 (43). The total number of hypoxic tumor cells \(N\) (hypoxic) is estimated by

\[
N \text{ (hypoxic)} = \exp \left( \frac{TCD_{50}\text{ (air)}} {D_0\text{ (hypoxic)}} \right) \cdot \ln (2) \cdot n
\]

and finally the relative number of surviving aerobic tumor cells [SF (air)] by

\[
SF \text{ (air)} = \frac{N \text{ (air, radiation + treatment)}} {N \text{ (air, radiation)}}
\]

**Skin Treatment and Response.** Irradiations and heat treatments were performed locally on normal foot skin as described for the tumor experiments. However, due to the absence of a tumor, it was not possible to loosely attach the leg to the restraining jig with tape. Fixing of the leg in the correct position for treatment was therefore achieved by the use of histocryl glue. Essentially a small drop of glue was placed on the jig in the region of the uppermost part of the leg. The leg was then firmly compressed against the jig with tape for a 5-min period, after which time the tape was loosened and the mouse was left for 10 min before treatment was started. By this procedure blood flow to the foot at the time of treatment was not impaired and the use of anaesthetics was avoided. The leg was easily detached from the jig after treatment. Mice were then observed on a daily basis between 11 and 30 days following treatment and the percentage of animals in each treatment group showing moist desquamation of the treated foot was recorded.

**Histological Evaluation of Microregional Perfusion.** The use of sequential injections of Hoechst 33342 and DiOC7(3), to quantify changes in the functional tumor vasculature, has been described in detail previously (44, 45). Briefly, the basis of the technique stems from the properties of the two fluorescent dyes: i.e., they have different fluorescence spectra permitting selective visualization of the stains; they have short plasma distribution half-lives after i.v. administration; and their diffusion properties are such that they provide selective staining of the cells bordering the functional vasculature. For the present series of experiments, Hoechst 33342 was administered i.v. at 10 μg (in 0.05 ml phosphate-buffered saline) either 20 min prior to or at the same time as an i.v. injection of DiOC7(3) at 1 μg/g (in 0.05 ml of 75% dimethyl sulfoxide). Five min after DiOC7(3) the animals were sacrificed, the tumors were then excised, embedded, frozen, and sectioned on a refrigerated microtome. Fluorescence microscopy was performed using a Zeiss microscope with epifluorescence condenser, 100-W mercury light source, and Neofluor objectives. For each tumor a minimum of 1000 blood vessels were counted and the percentage of vessels marked with either Hoechst 33342 or DiOC7(3) but not both dyes, was determined. This value was expressed as the “staining mismatch.”

**Data Analysis.** For the local tumor control and skin moist desquamation results, all the lines through the data were determined by logit analysis. With the regrowth delay data, lines were either fitted by eye or estimated following regression analysis using all the results from individual animals. Statistical analysis was performed using the Student t test after testing for variance homogeneity by an F test. \(P = 0.05\) was used as the level of significance.

**RESULTS**

The ability of nicotinamide to enhance radiation damage in this C3H mouse mammary carcinoma under normal conditions is illustrated in Fig. 1. Maximum sensitization was observed when the drug was administered between 30 min and 2 h prior to irradiation. At longer time intervals this radiosensitization decreased, disappearing completely when the two treatments were separated by a 6-h time period. Little or no enhancement was also observed if nicotinamide was injected either immediately prior to or 30 min after the radiation. As a result of the data shown in Fig. 1, for all other experiments involving nicotinamide, the drug was routinely administered 30 min before irradiation.

In Fig. 2 are shown the changes in the radiation dose-response curve when nicotinamide (1000 mg/kg) was injected 30 min prior to local tumor irradiation. These results were obtained using both a local tumor control assay and regrowth delay.

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The abbreviations used are: TCD\(_{50}\), radiation dose producing tumor control in 50% of treated mice; DD\(_{50}\), radiation dose producing moist desquamation in 50% of treated mice; ER, enhancement ratio; TGF, therapeutic gain factor.
NICTAMIDE, RADIATION, AND HEAT IN VIVO

From the tumor control experiment the TCD50 values (radiation doses producing tumor control in 50% of treated animals) were calculated to be 53.80 Gy for radiation alone and 44.27 Gy for nicotinamide plus radiation (Table 1). The ratio of these doses gave an ER of 1.22. The characteristics of the regrowth delay curves are shown in Table 2. A significant increase in the slope of the radiation dose-response curve, from 0.68 to 0.97, was obtained when nicotinamide was injected before irradiating, equivalent to an ER of 1.43.

Fig. 3 shows the effect that heat and nicotinamide plus heat had on the radiation dose-response curve. Heating tumors alone at 43.5°C for 60 min resulted in a TCD50 which was equivalent to an ER of 2.32 (Table 1). However, nicotinamide did not significantly change the slope of the radiation plus heat tumor growth time curve (Table 2), although there was a parallel upward shift of the curve (Fig. 3), an effect that appeared to be entirely a consequence of nicotinamide enhancing the heat response alone in this tumor.

The response of normal foot skin to radiation is shown in Fig. 4 and the DD50 values (the radiation dose required to produce moist desquamation in 50% of treated animals) are

Table 1 Interaction among nicotinamide, radiation, and heat in a C3H mouse mammary carcinoma and normal foot skin∗

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TCD50 (Gy)</th>
<th>ER</th>
<th>DD50 (Gy)</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD alone</td>
<td>53.80</td>
<td>32.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIC + RAD</td>
<td>44.27</td>
<td>2.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAD + heat</td>
<td>32.34</td>
<td>1.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIC + RAD + heat</td>
<td>23.22</td>
<td>1.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

∗ Values calculated from results shown in Figs. 2, 3, and 4.

Table 2 Characteristics of the growth delay dose-response curves for the interaction between nicotinamide, radiation, and heat∗

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intercept (days)</th>
<th>Slope (Gy)</th>
<th>Slope ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD alone</td>
<td>3.43 ± 2.80</td>
<td>0.68 ± 0.04</td>
<td>1.43 ± 0.08</td>
</tr>
<tr>
<td>NIC + RAD</td>
<td>3.38 ± 3.27</td>
<td>0.97 ± 0.07</td>
<td>1.66 ± 0.14</td>
</tr>
<tr>
<td>RAD + heat</td>
<td>13.11 ± 3.48</td>
<td>1.71 ± 0.14</td>
<td>2.51 ± 0.15</td>
</tr>
<tr>
<td>NIC + RAD + heat</td>
<td>16.15 ± 4.02</td>
<td>1.54 ± 0.26</td>
<td>2.26 ± 0.26</td>
</tr>
</tbody>
</table>

∗ Values calculated from data shown in Figs. 2 and 3.
hypoxic cells, and the hypoxic fraction. Table 3 and Fig. 6 summarize the values calculated from our results. Nicotinamide did not reduce the total number of tumor cells but it significantly decreased the number of hypoxic cells, such that the hypoxic fraction (±SE) for this tumor, which was calculated to be 6.62% (3.82–9.41), was reduced to 0.34% (0.14–0.54) following drug treatment. A heat treatment at 43.5°C for 60 min reduced both the aerobic and hypoxic cell populations, an effect that was even greater when nicotinamide and heat were combined.

The data of Fig. 7 show the results from an experiment to investigate the effect of nicotinamide on microregional blood flow using the fluorescent stains Hoechst 33342 and DiOC$_7$(3). A simultaneous injection of the two stains gave a percentage mismatch (±SE) of 3.42% (3.03–3.82). This was significantly increased to 7.73% (6.65–8.82) when the stains were separated by a 20-min time interval. Injecting nicotinamide 40 min prior to the sequential administration of the two stains reduced the percentage mismatch to 2.80% (2.42–3.18), a value that was not significantly different from that obtained when the two stains were given simultaneously.

**DISCUSSION**

Using a regrowth delay assay and local tumor control we were able to demonstrate that a large single dose of nicotinamide (1000 mg/kg) could enhance the radiation response of a C3H mouse mammary carcinoma. Radiosensitization was maximal when nicotinamide was injected between 30 min and 2 h prior to local irradiation of the tumor and resulted in ERs of 1.2 to 1.5. These findings are consistent with the radiosensitization reported for nicotinamide and related compounds in a range of murine tumors in vivo by both us and other workers (29, 30, 35, 46–48). Of more interest was the observation that the enhanced response to radiation seen when tumors were subsequently heated could also be substantially increased by a prior injection of nicotinamide.

Hyperthermia is a modality which has been shown in vitro to be preferentially cytotoxic to cells under hypoxic conditions (20, 22). This increased toxicity is not a consequence of hypoxia per se, because under well defined nutrient conditions acute hypoxia alone does not have any significant influence on cellular response to heat (20, 23). Sensitivity to heat is, however, increased when cells are exposed to prolonged oxygen deprivation (20, 21). This chronic hypoxia generally leads to metabolic depletion which in turn alters several other parameters, especially cellular acidity, and it is these changes which are believed to be responsible for the increase in sensitivity (21–23). The hyperthermic response of tumors in vivo can also be enhanced if tumors are made totally hypoxic by clamping during the heating period (37, 49) or if mice are treated with drugs, such as hydralazine, which are known to decrease tumor oxygenation status by decreasing tumor blood flow (40). Our own results (Table 3) not only demonstrated that heat could substantially decrease the number of hypoxic cells in this C3H mouse mammary carcinoma but also showed that it was capable of killing some aerobic cells, although these latter cells were probably those which were not fully radiobiologically hypoxic yet existed under conditions of some nutrient deprivation and relatively high acidity. Since heat can reduce the number of radioresistant cells in a tumor, it does become an attractive modality to use in combination with radiation. In our current study heat was given 4 h after radiation. This time interval was selected because previous studies had demonstrated that any
interaction between the two modalities could be avoided and thus any enhancement was simply a consequence of heat killing cells which survived the radiation treatment (38). However, our results in Fig. 3 suggested that even with a 4-h interval some heat sensitization might be occurring. Nevertheless, heating tumors 4 h after irradiating resulted in an ER of 1.6–2.6, an effect which is consistent with that reported by others (38).

Nicotinamide has been reported to be capable of decreasing tumor hypoxia and appears to do so as a result of an increase in tumor perfusion (33, 34). A reduction in hypoxia was also observed in this C3H mouse mammary carcinoma in which the normal hypoxic fraction of 6.62% was found to be significantly decreased to 0.34% after nicotinamide administration. The data of Table 3 also showed a reduction in the total number of hypoxic cells, but more importantly this reduction occurred without any change in the total number of tumor cells, suggesting that nicotinamide was not cytotoxic to the cells and that its mechanism of action simply involves a conversion of hypoxic cells into aerobic ones. The absence of any nicotinamide radiosensitization in clamped tumors (Fig. 5) also supports the suggestions previously made by us with the EMT6 tumor (34), that the effect of nicotinamide does not involve any direct tumor radiosensitization, nor does it involve an inhibition of potentially lethal damage repair as reported to occur in vitro (50–52). More recent data using the SCCVII tumor suggested that nicotinamide actually decreased the presence of both chronic and acute hypoxia (35). In one series of experiments we investigated the effect of nicotinamide on the opening and closing of vessels in this C3H mouse mammary carcinoma. Functional vasculature was visualized at 2 periods in time, separated by a 20-min interval, using 2 fluorescent markers. The results shown in Fig. 7 indicated that the number of “mismatched” vessels (i.e., functional at one time but not the other) was reduced from 7.73% to 2.80% by prior administration of nicotinamide, an effect which is consistent with that reported for both nicotinamide and its structurally related analogue pyrazinamide in the SCCVII tumor (36, 48). Since transient fluctuations in blood flow are known to result in the development of acute hypoxia (27, 28) the data for nicotinamide strongly support our suggestion that this drug can eliminate acute hypoxia.

Nitroaromatic compounds such as misonidazole and nimorazole have also been reported to be capable of enhancing the response of this C3H mouse mammary carcinoma to both radiation and radiation plus heat (53, 54). If we look in closer detail at the results for misonidazole from the earlier study by Overgaard (53), we see that at a dose of 1000 mg/kg, misonidazole enhanced radiation response by a factor of 2.2 but increased the effect of radiation plus heat by a factor of only 1.5. Misonidazole is capable of sensitizing any hypoxic cell to radiation whether it be acutely or chronically hypoxic. However, in combination with radiation and heat, the hyperthermia would have accounted for the chronically hypoxic cells, leaving only those which were acutely hypoxic for misonidazole to sensitize, hence the smaller increase by misonidazole when all 3 agents were combined. Nicotinamide in our study reduced the TCD_{50} for radiation plus heat from 32.34 to 23.22 Gy, equivalent to an ER of 1.4, similar to the value reported with misonidazole. However, on its own nicotinamide produced only a 1.2-fold increase in radiation response, much lower than the ER of 2.2 seen with misonidazole plus radiation. This may therefore indicate that unlike its effect in the SCCVII tumor, in this C3H mouse mammary carcinoma nicotinamide primarily overcomes
acute hypoxia and probably has little or no effect on diffusion-limited chronic hypoxia.

We had previously shown that nicotinamide could enhance the interaction observed when radiation and heat were given at the same time and not separated by a 4-h interval (55). There have been 2 other recent reports into the effect of nicotinamide on a simultaneous radiation and heat treatment, one using local tumor control in a mammary adenocarcinoma (56) and the other with growth delay assessment in a C3H mouse mammary carcinoma (57). Both studies reported a radiosensitizing effect by hyperthermia and nicotinamide. However, although Kim et al. found an additional effect on tumor control when all 3 agents were combined, Kjellen et al. failed to obtain any additional enhancement in the growth delay assay. Our own experience with regrowth delay (Fig. 3) showed only a small influence of nicotinamide on radiation plus heat and this was simply a consequence of an interaction between heat and the drug, similar to that reported to occur in vitro with structurally related compounds (58). Kjellen et al. did not find this interaction between nicotinamide and heat, which would account for the absence of any effect when all 3 agents were combined and may be explained by the fact that the nicotinamide dose they used was only 200 mg/kg. In our hands the tumor growth time of 13 days obtained with heat alone was increased to 16 days by nicotinamide. It is unlikely that this small yet significant increase in growth time was responsible for the substantial decrease in the radiation plus heat TCD50 value, obtained when drug, radiation, and heat were given together. Why we failed to see a large effect with the regrowth delay procedure is not clear, but it may reflect an inadequacy of this technique to detect these changes after such a severe treatment. Nevertheless, when all 3 agents were combined we did find an increase in local tumor control, an assay which is the most clinically relevant.

In previous studies we had investigated the ability of nicotinamide to enhance radiation damage in various normal tissues (30, 32). Although sensitization was observed in intestine, skin, and testis, the ERs were never greater than 1.2 and always lower than that observed in tumors. Using moist desquamation as the end point of skin damage (Fig. 4; Table 1) we have again found a small but significant enhancement of skin damage (ER < 1.2). Comparing the ERs from the tumor data (ERs of 1.22 from the tumor control assay and 1.43 from regrowth delay) with that obtained in skin (ER = 1.15) results in a small TGF of 1.1–1.2. When heat was given 4 h after radiation, the relatively large effect in tumors (ERs of 1.66 and 2.51 from the tumor control and regrowth delay assays, respectively) compared to the small enhancement in skin (ER = 1.08) gives rise to a much larger TGF of 1.5–2.3, an effect which is consistent with other reports (for review, see Ref. 38). Although the administration of nicotinamide, radiation, and heat resulted in a rather large enhancement of skin damage (ER = 1.72), this value was still lower than the ERs of 2.32 and 2.26 calculated from the two tumor assays; hence a TGF of 1.3–1.4 was obtained.

There is now evidence to suggest that hypoxia does influence the clinical response of tumors to radiation (3–5), although it is not known at this time whether this is simply due to chronic or acute hypoxia or a combination of both types. Our present study does, however, clearly demonstrate that the radio-resistance produced by both chronic and acute hypoxia can be overcome in vivo by combining nicotinamide and heat with radiation and thus substantially improve the tumor response to treatment. There is now abundant evidence from clinical Phase I and II studies of combined heat and radiation showing that hyperthermia can enhance the radiation effect to a significant degree (for review, see Ref. 59). Nicotinamide has also been well established in the clinic for the treatment of several disorders including psoriasis (60), pellagra (61), and schizophrenia (62–64). Thus the potential for administering nicotinamide, radiation, and heat together in the clinic clearly exists. However, additional testing in other tumor and normal tissue systems in vivo with nicotinamide or related compounds, and perhaps with other established clinically relevant methods of overcoming chronic hypoxia, is clearly required.

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