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Research Article

Selective Enhancement of Chemotherapeutic Agent-Induced Tumor Cell Killing by Acetoacetate and 3-Hydroxybutyrate

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Abstract

Aims: We have studied the effects of ketone bodies on cancer cells in culture alone and in combination with rapamycin, a chemotherapeutic agent. The goal was to determine if, as previously shown, ketone bodies inhibit proliferation and further the enhance tumor cell killing effect of rapamycin, without affecting normal cells, thereby lowering their effective dose with a consequent reduction in toxicity and side effects.

Methods: SW480, a human colon cancer cell line was treated with ketone bodies (sodium acetoacetate or sodium 3-hydroxy butyrate [commonly called beta-hydroxybutyrate]) in the presence or absence of rapamycin. Cells were incubated for 96 hours in DMEM with 10 mM glucose medium. HSF2617, a human fibroblast line served as control, and cells were subjected to similar treatment as the SW480 cells. Cell proliferation was determined using the crystal violet assay.

Results: Both acetoacetate and 3-hydroxybutyrate inhibited proliferation of SW480 cells in culture in a dose-dependent manner over a wide dose range (0 - 60 mM) while they had no effect on the growth of the normal HSF2617 fibroblasts, suggesting these agents are selective for cancer cells. Rapamycin also blocked proliferation and induced cell death in a dose-dependent manner. The IC_{50} for rapamycin in inducing cell death was significantly reduced (up to a factor of 37) by the presence each ketone body.

Conclusion: The results suggest that the normal control cells can switch to ketogenic metabolism while the cancer cells, which proliferate poorly, cannot. The results bear on the recent reports of a mouse model showing the synergy of rapamycin and a ketogenic diet. The ketone bodies enhance the effect of the chemotherapeutic agent by reducing cancer, but not normal, cell proliferation lowering its IC_{50} on the cancer cells. These reductions make it possible to treat cancers at significantly lower concentrations of chemotherapeutic agents thereby reducing the incidence of detrimental or toxic side-effects.

Keywords: *Ketone bodies; Rapamycin Cancer Cells; IC_{50}*

Abbreviations

KB: Ketone Bodies; AcAc: Acetoacetate; 3-HB: 3-Hydroxybutyrate

Introduction

In a number of prior *in vivo* studies, low carbohydrate and ketogenic diets have been found to induce diminution of *in vivo* cell proliferation of tumors [1-7]. In corresponding *in vitro* studies on cells in culture, ketone bodies, acetoacetate and 3-hydroxybutyrate have been found to block the growth of a variety of cancer cells while not affecting the growth of normal or untransformed cells [8]. This selective

inhibitory effect on cancer cells is such that cells remain viable but replicate at very diminished rates. Associated with this effect, ATP production in cancer cells is significantly diminished, but ATP production is not blocked in the counterpart untransformed cells [8].

These results are compatible with the Warburg effect [9] wherein cancer cells have been observed to produce most of the their ATP via glycolysis and not via the Krebs (tricarboxylic acid) cycle even in the presence of oxygen. Ketone bodies are known to be involved in the so-called Randall Cycle [8] in which these end products of lipid metabolism block glycolysis. Since cancer cells require glycolysis for ATP generation and ketone bodies block glycolysis, cancer cell growth is inhibited providing an explanation for the observed *in vivo* effects of ketogenic diets.

A number of *in vivo* studies show that ketogenic diets increase the efficacy of chemotherapeutic agents [1-7] and, in a recent study, we have found that mice expressing a spontaneous breast tumor with lung metastases responded to a ketogenic diet: tumor size was reduced, and longevity increased. Importantly, the combination of the ketogenic diet and the chemotherapeutic agent, rapamycin, extended lifespan to a greater extent than either treatment alone [6]. The results suggest that these agents can interact cooperatively with anti-cancer agents, possibly reducing the required doses of these agents thereby diminishing potential side effects of these agents.

Purpose of the Study

The aim of this study was to determine the effect of ketone bodies, sodium acetoacetate (NaAcAc) and 3-hydroxybutyrate (3-HB) on a human colon cancer cell line, SW480, in culture and to determine if these agents enhance the anti-tumor effect of the chemotherapeutic agent, rapamycin. Normal human HSF2617 fibroblasts served as controls.

Materials and Methods

Cell lines and cultures: SW480 and HSF2617 fibroblasts were obtained from the ATCC (Manassas, VA, USA). All cell lines were cultured in DMEM at 37^0C and 5% CO_2 pressure. Medium was supplemented with 10% fetal bovine serum, 4 mM glutamine, 1 mM pyruvate, 10 and 25 mM glucose, and 1% (v/v) penicillin and streptomycin. Cell culture reagents were from Corning through ThermoFisher (Fairlawn, NJ, USA).

Chemicals and drugs: Sodium DL-3-hydroxybutyrate and cell culture grade dimethyl sulfoxide (DMSO) were purchased from Millipore-Sigma (Burlington, Mass, USA). Crystal violet and rapamycin were purchased from Alfa Aesar Chemicals of ThermoFisher. A stock of 1 mg/ml rapamycin was prepared by solution in sterile cell culture grade DMSO and maintained at -80^0C. The final DMSO concentration was maintained at 0.5% (v/v) either in the control or the treatment samples in all experiments. Sodium acetoacetate was prepared by reaction of equimolar ethyl acetoacetate and NaOH for 12 hours at 4^0C [10]). Acetoacetate concentration was determined by a colorimetric method [11].

Cell treatment, cell proliferation assay: Cells were grown to 80% confluence and detached with trypsin-EDTA 1X solution. Experimental cells were treated with chemicals and drugs at the same time in 10 mM glucose DMEM/low glucose in 96 wells tissue culture plates along with controls without any additives. The crystal violet proliferation assay [12] was used to determine viable cells in all treatment conditions. Assays were measured on a Perkin-Elmer (Waltham, Mass, USA) Victor3 multilabel plate reader.

Results and Discussion

Ketone bodies inhibit growth in SW-480 cancer cells but do not affect normal cells

We chose to study the effects of the two ketone bodies and the chemotherapeutic agent, rapamycin, on the human colon cancer cell line, SW480, since these cells proliferate rapidly (doubling time approximately 38h [SW480 (RRID:CVCL_0546]) and have a high metastatic potential. Recently, the agent, rapamycin, has been found to be an effective cytotoxic agent against human colon cancers [13] blocking cell division at the mTOR level, and we therefore used this compound as the chemotherapeutic agent. Proliferation at 96 hours incubation time was determined by the crystal violet assay, as shown in figure 1. Proliferation of cells was inhibited in a dose-dependent manner

by AcAc and, to a lesser extent, by 3-HB. Figure 1 also shows that, in contrast, there was no inhibitory effect on the growth of normal human fibroblast controls. These differences are evident in the micrographs of SW480 cells (top panels of figure 2) and control HSF2617 fibroblasts (bottom panels). The effect of the ketone bodies, shown in figure 2, is to reduce the cell density of the SW480 cells but not the control cells. This conclusion is supported by the results of trypan blue exclusion assays on the SW480 cells incubated with 40 mM AcAc. Over the 96h incubation period, there was an absence of dead cells. We conclude that, as in our previous study [8], the ketone bodies reduce the cell count but do not cause cell death.

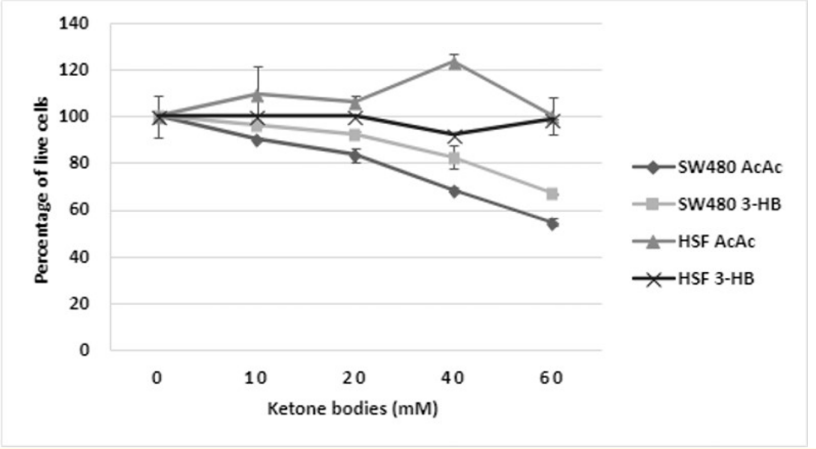

Figure 1: *Effects of acetoacetate (filled diamonds) and 3-hydroxybutyrate (gray-filled squares) on SW480 human colon cancer cells and the effects of acetoacetate (gray-filled triangles) and 3-hyrdoxybutyrate (curve with X characters) on untransformed HSF human fibroblasts.*

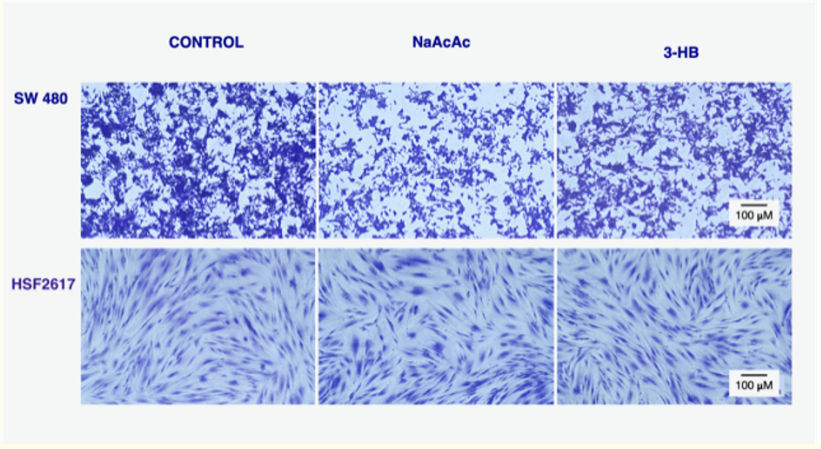

Figure 2: *Morphology of SW480 and fibroblast controls after treatment with ketone bodies. SW480 (upper panel) or SF2617 (lower panel) cells were cultured as described in the Materials and Methods section. Both cell lines were treated with 40mM acetoacetate or 3-hydroxybutyrate for 96 hours. The left-most panels show the morphology of the two cell lines when they were incubated for 96 hours with no ketone bodies. The two middle panels show the cells after 96 hour treatment with sodium acetoacetate, and the two right-most panels show the cells after treatment with 3-hydroxybutyrate.*

Ketone bodies enhance SW 480 cancer cell killing by rapamycin

We tested the effects of the ketone bodies on the efficacy of the anti-tumor agent, rapamycin (range 0 - 6 nM), on SW480 colon cancer cells during a 96h incubation period. Figure 3A shows that increasing concentrations of AcAc (0 - 60 mM) enhance cell killing by rapamycin. Similar results were found for 3-HB (Figure 3B) over the same concentration range.

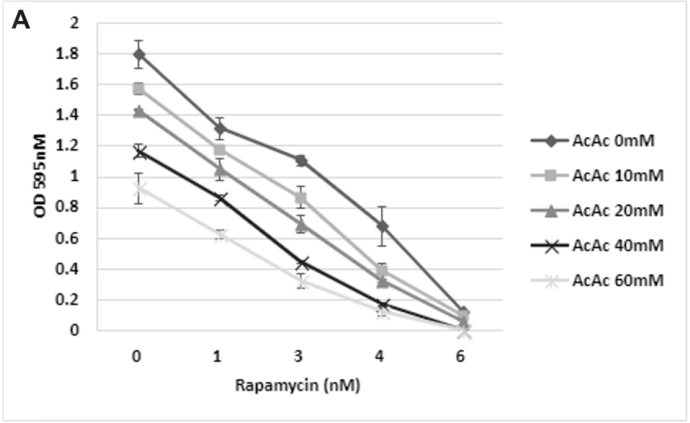

Figure 3A: *Dose response curves for the treatment of SW480 human colon cancer cells with rapamycin at five different concentrations of acetoacetate (0, 10, 20, 40 60 mM) as labeled in the figure.*

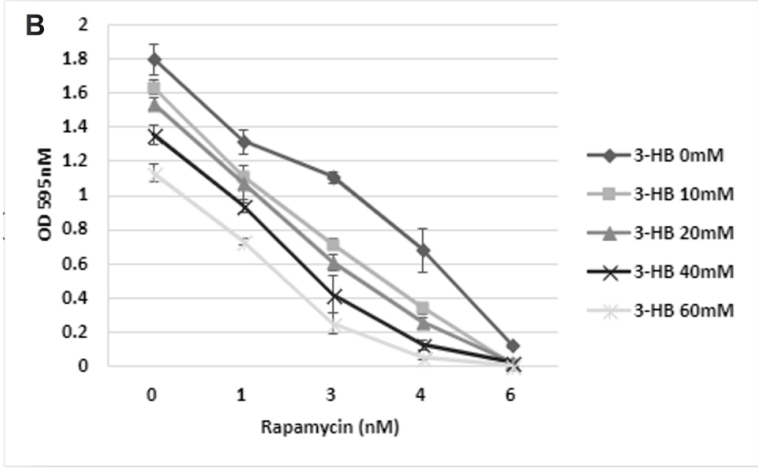

Figure 3B: *Dose response curves for the treatment of SW480 human colon cancer cells with rapamycin at five different concentrations of 3-hydroxybutyrate (0, 10, 20, 40 60 mM) as labeled in the figure.*

Table 1 shows that increasing the concentration of AcAc successively reduces the IC_{50} values for rapamycin. At 60 mM AcAc, there is a thirty seven-fold reduction of the IC_{50}. Similar results were obtained for 3-HB although the reductions are lower than that obtained with AcAc; the IC_{50} for rapamycin cell killing at 60 mM 3-HB were reduced by a factor of 6, consistent with the observation that inhibition is lower with 3-HB than with AcAc. This diminished effect may be due, in part, to the necessity of converting 3-HB to AcAc by the enzyme,

3-hydroxybutyrate dehydrogenase which may be rate-determining. In addition 3-HB has other effects in the cell notably as a histone deacetylase inhibitor [1,2].

Agent	Concentration (mM)	IC_{50} for Rapamycin	Agent	Concentration (mM)	IC_{50} for Rapamycin
AcAc	0.0	3.48	3-HB	0.0	3.48
	10	2.78		10	2.07
	20	1.84		20	2.74
	40	0.89		40	1.16
	60	0.09		60	0.58

Table 1: *Effects of acetoacetate and 3-hydroxybutyrate on the IC_{50} values for rapamycin that induces cancer cell death.*

Figure 3 also indicates a significant shift in dose efficacy at the lower KB concentrations. For example, in Figure 3A, 1 nM rapamycin, with no ketone body present, results in a decrease in absorbance due to cell killing from 1.8 to 1.3. In the presence of 20 mM acetoacetate, at which concentration there is no effect on normal cells, there is a further decrease from 1.3 to 1.0. The total decrease in absorbance is therefore 1.8-1.0 or 0.8. To obtain this absorbance change in the absence of ketogenic agent, the required dose of rapamycin would be greater than 3 nM. Thus, cell killing by 1 nM rapamycin is enhanced by acetoacetate threefold and is equivalent to 3nM rapamycin alone.

In the absence of ketone bodies (Figure 3), rapamycin kills all of the cancer cells at a concentration of 6nM. This concentration is significantly lower than reported in the literature where the effective concentration range for this agent against breast cancer cells is in the micromolar range [14]. This difference is due at least in part to the incubation periods. Previously reported concentrations were for incubation periods of 48h. We performed a time course study of the effect of 4 nM rapamycin on SW480 cells and found that at 48h, only about 10 percent of the cells were killed whereas at 96h, 75 percent of the cells were killed suggesting that increased incubation (and possibly treatment) times result in more effective cancer cell killing.

It may be noted that the concentrations of the ketone bodies used in this study were non-physiological in the sense that such high doses could not be practicably administered *in vivo*. On the other hand, even the highest concentration used, 60 mM, had no effect on the viability or growth properties of the normal fibroblast cells (Figure 1) suggesting that the ketone bodies are non-toxic to normal cells and would not damage normal cells even at high concentrations - ketone bodies are, in fact, substrates. In addition, concentrations of anti-tumor agents used in cell culture studies have been found to be effective *in vivo* at much lower concentrations than used in cell culture studies. For example, rapamycin has an IC_{50} on retinoblastoma cells of 0.122 uM whereas a normal effective serum level for treatment of human cancer is about 11 nM. Thus, the ratio of an effective dose in cell culture is on the order of 11 times that for effective treatment *in vivo*. The anti-cancer drug 5-fluorouracil, for example was found to have an IC_{50} of 185 uM against HCT116 human colon cancer cells but has a maximum therapeutic serum level of about 23 uM or 1/8 that of the *in vitro* IC_{50} value. Based on these considerations, it may be expected that therapeutic ranges for the ketogenic agents would be reduced around ten-fold, concentrations that would not be toxic to normal cells.

Conclusion

We have shown here that ketone bodies induce significant enhancements of the efficacy of rapamycin by inhibiting cancer cell proliferation without causing cell death, thereby lowering the number of cancer cells that encounter the chemotherapeutic agent that itself induced cell death. Ketone bodies do not, however, affect the proliferation of normal cells even at high concentrations. This finding is consistent with the hypothesis that ketone bodies, block cancer cell growth because of their requirement for glycolysis as the source of ATP. Normal cells, in distinction, can utilize ketone bodies which are converted to acetyl Coenzyme A (acetyl-CoA), the substrate for the

tricarboxylic acid cycle and can therefore generate ATP and proliferate. In cancer cells, acetyl-CoA is an inhibitor of glycolysis.

Our findings suggest that either administration of ketone bodies such as AcAc or ketogenic diets or possibly some derivative of acetyl-Co enzyme A itself can significantly enhance the effects of chemotherapeutic agents which can thereby be administered in lower doses minimizing off-target effects.

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