Cordycepin/Hydroxyurea Synergy Allows Low Dosage Efficacy of Cordycepin in MOLT-4 Leukemia Cells

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Abstract. *Background: Cordycepin requires the relatively toxic co-drug, deoxycoformycin, for full efficacy as an anti-cancer agent. We sought to improve cordycepin efficacy using other, less toxic co-drugs. Materials and Methods: We evaluated the ability of hydroxyurea (HU) to enhance the effects of cordycepin against MOLT-4 leukemia cells with the MTT cell viability assay. We determined the relationship of the combination drug treatment with CalcuSyn statistical analysis program according to the Chou-Talalay method. Results: HU (50 μg/ml) was found to reduce the IC_{50} of cordycepin from 100 μM to 0.3 μM, a reduction similar to that observed for deoxycoformycin. CalcuSyn analysis of the cordycepin/HU combination revealed the dose effect as synergistic. Further statistical analysis demonstrated a clear synergy between the two drugs at a range of dosages. Conclusion: HU was identified as a promising potential alternative for anti-cancer therapy with cordycepin, thus eliminating the need for the toxic deoxycoformycin.*

Cordycepin is a drug with several applications and several proposed modes of action. It is an analog of adenosine lacking the 3'-OH and an effective chain terminator against RNA synthesis. The effects of cordycepin *in vivo* have also been suggested to be the result of the depletion of cellular ATP pools and its ability to substitute in ATP-dependent reactions (1). Cordycepin has anti-inflammatory action (2), inhibits platelet aggregation (3) and has shown effect against fungal infection (4) and trypanosomes (5). However, cordycepin has been studied most extensively as an anti-cancer agent (6-8). For full efficacy, cordycepin is best used along with the relatively toxic co-drug, deoxycoformycin, which protects it from degradation by cellular adenosine deaminase (9).

We used a dual approach to improve the efficacy of cordycepin. We have previously collaborated in the development of cordycepin analogs that are resistant to adenosine deaminase (10). In this study, we sought a less toxic, synergistic co-drug for cordycepin that could work *via* mechanisms other than adenosine deaminase resistance. Specifically, we explored drugs, such as hydroxyurea (HU), which are known to produce a nucleotide pool imbalance and thus trigger apoptosis (11). HU abrogates the synthesis of DNA by inhibiting ribonucleotide reductase, the enzyme responsible for the conversion of ribonucleotide diphosphates to the deoxyribonucleotide form (12). The results of our study demonstrate that HU can be used to increase the efficacy of cordycepin in MOLT-4 leukemia cells as an alternative to the much more toxic deoxycoformycin.

Materials and Methods

Drugs. Cordycepin and HU were purchased from Sigma (St. Louis, MO, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at –20˚C. The working solutions were diluted in culture medium to the desired concentration before use; the highest concentration of DMSO was 0.1% (v/v).

Cell culture. MOLT-4 leukemia cell line was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained in RPMI-1640 supplemented with penicillin-streptomycin, fungizone® and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), and maintained at 37˚C humidified 5% CO_2 atmosphere.

Cell viability assay. Cell viability assays were assessed using a modified MTT assay (13). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT) was purchased from Sigma. For each drug treatment, triplicate wells were seeded with cells ($5.0x10^3$/well) and conditioned overnight. Drugs were prepared by

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serial dilution with RPMI media and added to the corresponding wells. After 96 hours, the MTT assay was performed. Viability was measured as a percent of control untreated cells. Experiments were run in triplicate unless otherwise mentioned. Data are expressed as the mean±95% confidence limits from at least 3 separate experiments. The difference between groups was analyzed using a double-sided Student's *t*-test. Statistical significance was considered as $p<0.05$.

CalcuSyn analysis. The commercial software CalcuSyn (BIOSOFT®, Cambridge, UK) was used to analyze dose effect relationship by the Chou-Talalay method (14). The combination index (CI) method was used as a non-constant ratio combination design of MOLT-4 cells treated with cordycepin (12.5 μM, 25 μM, and 50 μM) and HU (1 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml) as single drugs or combination exposure. For each drug treatment, triplicate wells were seeded with cells (5.0×10^3/well) and conditioned overnight. Drugs were prepared by serial dilution with RPMI media and added to the corresponding wells. After 96 hours, the MTT assay was performed and percent survival was calculated as described previously. The effect of drug treatment was calculated by subtracting the percent at treatment level divided by the percent survival of the control (100%) from one. The effect was entered as the input for CalcuSyn analysis as a non-constant ratio combination and the CI was recorded as the output value. The drug treatment was characterized based on the table for describing synergism or antagonism with the CI method.

Results

Cordycepin efficacy is enhanced by HU. We examined hydroxyurea as a co-drug to increase the efficacy of cordycepin, which is usually administered with the relatively toxic co-drug, deoxycoformycin. In preliminary cytotoxicity studies in MOLT-4 leukemia cells, broad concentration ranges of 1-50 μM cordycepin and 25-100 μg/ml HU were examined (Figure 1). The single-drug treatment IC_{50} values were 100 μM for cordycepin and 82 μg/ml for HU. In combination treatment, the cordycepin IC_{50} value dropped to 0.3 μM in the presence of 50 μg/ml HU. This indicated that HU could strongly improve efficacy of cordycepin.

These results led us to design an experiment to statistically determine if synergy was occurring. The lower to mid-range of concentrations of both drugs were selected. Again, lower levels of cordycepin provided greater cytotoxicity when administered in combination with HU (Figure 2). In the absence of hydroxyurea, cordycepin concentrations up to 50 μM showed little to no toxicity. In contrast, cell survival fell below 20% for all cordycepin concentrations in the presence of 25 μg/ml HU. Thus, HU improved the efficacy of cordycepin in a concentration-dependent manner.

Hydroxyurea mediates enhanced cordycepin efficacy synergistically. To verify the apparent synergy, the data of Figure 2 were analyzed by the Chou-Talalay method (14), using the CalcuSyn statistical analysis program. In this analysis, the general range of CI values of less than 0.90 indicate various levels of synergy, while values of 0.90-1.10 indicate addition, and >1.10 indicates antagonism. The HU/cordycepin values generated CI values between 0.3-0.7 and 0.7-0.85 indicating, specifically, "synergy" and "moderate synergy", respectively. The analysis demonstrated clear synergy with all cordycepin/HU combinations except those at the highest HU concentration of 50 μg/ml (Figure 3). In this set of assays, HU was unexpectedly more potent (IC_{50}=17 μg/ml for the HU alone curve). This may explain why only moderate synergy was observed at 50 μg/ml HU. Thus, HU is a co-drug alternative for cordycepin efficacy *via* a synergistic mechanism.

Discussion

We began our search for a synergistic cordycepin co-drug by evaluating compounds that affect the cellular nucleotide balance, in order to complement, rather than duplicate, cordycepin action. We soon discovered HU as a potential synergistic co-drug in the MOLT-4 leukemia cells. In this study, we have demonstrated that cordycepin IC_{50} values can be improved to below 1 μM in combination with HU, in contrast to 100 μM without the co-drug. This compares favorably with deoxycoformycin usage in the work of Kodama *et al.* (6), in which the IC_{50} value for cordycepin in MOLT-4 cells was 0.92 μM with deoxycoformycin and >100 μM without it.

HU inhibits ribonucleotide reductase, the enzyme which converts ribonucleotide diphosphates (NTPs) to the deoxyribonucleotide (dNTPs). It has low toxicity and is currently seeing extensive use as a long-term treatment for sickle-celled anemia (15). It also has a long history of clinical trials as an anticancer agent. However, its efficacy against cancer has proven to be only modest; therefore, its success as a single agent for chemotherapy for any solid tumors is limited (12). Hence, HU is utilized in many combination treatments and as an enhancer or modulator of other agents employed in cancer treatment. For example, HU has shown synergy with 5-fluorouracil in a human colon cancer cell line (16) and with ganciclovir against herpes simplex virus in cell culture (17). Here, we report that HU can also function *in vitro* synergistically with cordycepin against MOLT-4 leukemia cells.

The mechanism of the anti-cancer action of cordycepin is the subject of much study. Cordycepin may act by its known ability to inhibit poly(A) polymerase (18) or DNA primase (19), or to collapse intermediate filaments (20). Moreover, cordycepin action has been specifically attributed to its targeting the adenosine A3 receptor (21) or terminal deoxynucleotidyl transferase (6). HU is expected to increase NTP cellular pools, reducing

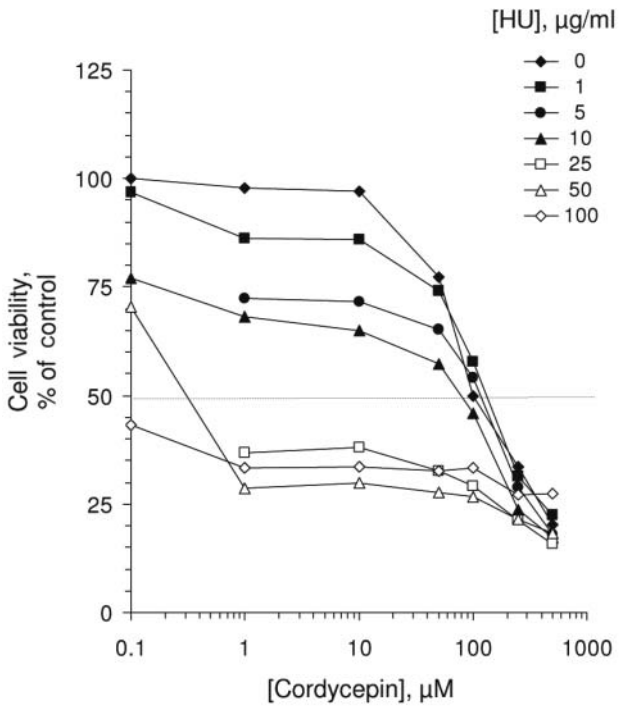

Figure 1. *Hydroxyurea improves cordycepin cytotoxicity. MOLT-4 cells (5.0x10^3) were seeded in 96-well plates and conditioned overnight followed by addition of hydroxyurea and or cordycepin at the indicated concentrations for 96 hours. The effect of drugs as single agents or in combination was assessed using the MTT cell viability assay and expressed as a percentage of the control. The graphical results represent the mean of at least four experiments each performed in triplicate.*

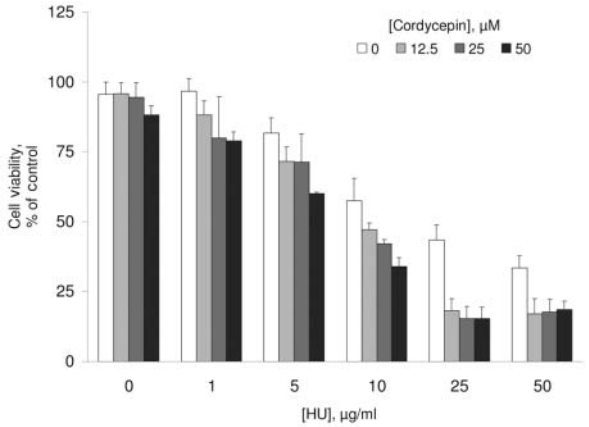

Figure 2. *Low dose cordycepin efficacy is enhanced by hydroxyurea. MOLT-4 cells (5.0x10^3) were seeded in 96-well plates and conditioned overnight followed by addition of hydroxyurea and or cordycepin at the indicated concentrations for 96 hours. The drug effects were assessed using the MTT cell viability assay and expressed as a percentage of the control. The graphical results represent the mean ±95% confidence level of at least four experiments each performed in triplicate.*

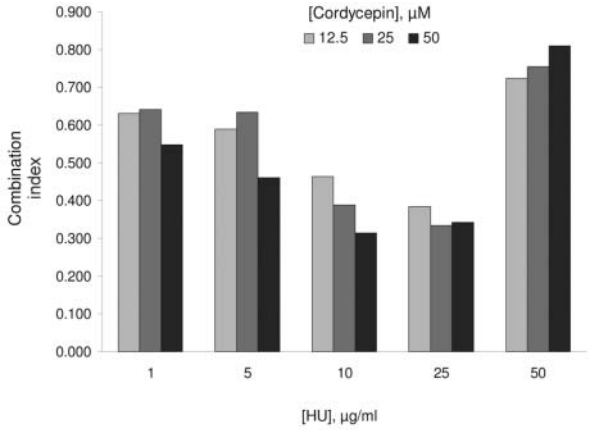

Figure 3. *Combination index (CI) values of cordycepin and hydroxyurea co-drug treatment indicates synergy. To confirm the apparent synergy, the data of Figure 2 were analyzed by CalcuSyn according to the method of Chou and Talalay (10). The general range of CI values of less than 0.90 indicate synergy, 0.90-1.10 indicate addition, and >1.10 indicates antagonism. The results were quantitated specifically with CI values between 0.3-0.7 and 0.7-0.85 indicating "synergy" and "moderate synergy," respectively.*

cordycepin concentration relative to ATP, which would reduce, not enhance, its effect on these targets. Thus, the synergy between cordycepin and HU might be due to the interaction of two independent, rather than supplementary, modes of action. Future studies are needed to to clarify the mechanism of synergy between cordycepin and HU.

The clinical impact of these results is significant and warrant further studying. Cordycepin/HU efficacy should be explored in other cancer cell lines and against fungal and trypanosomal cells. Importantly, it can now be determined if the synergy seen with HU can be extended to adenosine deaminase-resistant cordycepin analogs (10), and whether this leads to a synergistic or supplementary effect. Other small molecules affecting the cellular nucleotide balance might be profitably explored for those which increase the efficacy of cordycepin. The combination of cordycepin analogs and various other nucleotide balance altering compounds may provide synergistic leads for a variety of clinical applications.

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