Heptelidic acid displays cytotoxicity against rhabdoid tumor of the kidney *in vitro*

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Background

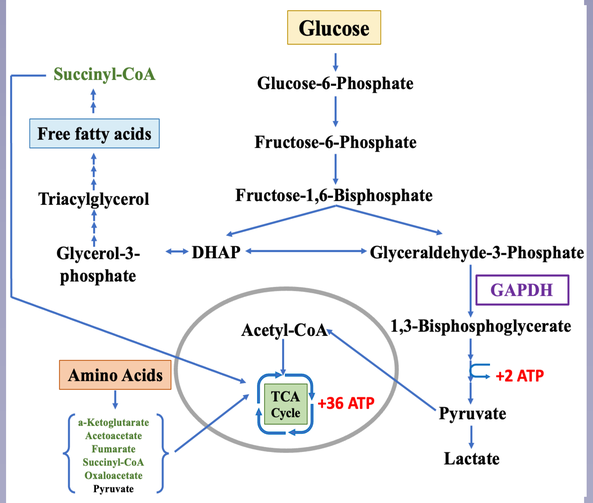
Rhabdoid tumor of the kidney (RTK) is a rare but aggressive cancer that occurs in infancy and early childhood.[1] Current treatment protocols for RTK included a combination of surgery, radiation therapy, and chemotherapy, however the prognosis remains poor with a 5-year survival between 20-25%.[1] **Therefore, the need to develop therapeutic strategies to treat RTK is still greatly unmet.**

High dependence on glycolysis by cancer cells, known as the Warburg effect [2], demonstrates a weakness that can be harnessed to target cancer cells and provides rationale for exploring antiglycolytic approaches for targeting cancer cells. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) is an essential enzyme within the glycolysis pathway (Figure 1) making GAPDH a potential therapeutic target for inhibiting tumor growth and progression [3].

Aspergillus oryzae is a probiotic fungus that is used in the production of Japanese fermented food, including soybean paste and soy sauce. Heptelidic acid (HA) is a sesquiterpene lactone derived from *A. oryzae* and has recently been shown to exert antitumor effects against multiple cancers including pancreatic and extraintestinal melanomas. [4,5] HA is a specific and irreversible inhibitor of GAPDH.[6] Its GAPDH inhibition activity is shown to contribute to its antitumor effects. [4,5].

The purpose of this study is to assess the therapeutic potential of HA on RTK. This study gives the first evidence that HA shows cytotoxicity in RTK and provides rationale to ultimately develop a therapy using HA for RTK.

Figure 1: Pathways of Human Metabolism



Objective

To determine the therapeutic effect of HA on rhabdoid tumor of the kidney (RTK).

Methods

1. Sulforhodamine B assay:

RTK cells lines G-401 and JMU-RTK-2 cells were seeded onto 96-well microplates at 7.5x10^4 cells/well and cultured for 24 hours. After 48 hours of HA treatment (n=3) at different concentrations (0, 100, 200, 400, 800 ng/mL) the cells were fixed in a 10% trichloroacetic acid for 1 hr at 4C and washed five times in distilled water. The microplates were then dehydrated at room temperature, stained with 0.057% (wt/vol) Sulforhodamine B (SRB) in 1% (vol/vol) acetic acid at 100uL per well, washed five times with 0.1% acetic acid, and re-dehydrated at room temperature. The stained cells were lysed in 10mM unbuffered Tris base solution, and optical density was measured at 510 nm. [7]

2. GAPDH activity assay:

The activity of GAPDH was determined using a GAPDH Activity Assay Kit (Abcam). G-401 and JMU-RTK-2 cells were seeded onto 12-well microplates at 1x10^5 cells/well and cultured for 24 hours. After 24 h of HA treatment at 400ng/mL, the cells were lysed using GAPDH assay buffer and then its activity was determined (mU/mL) using the GAPDH Activity Assay Kit.

3. ATP assay:

The inhibition of ATP production following HA treatment was determined using an ATP Assay kit. G-401 and JMU-RTK-2 cells were seeded onto 12-well microplates at 1x10^5 cells/well and cultured for 24 hours. After 24 h of HA treated at 400ng/mL, the cells were incubated with ATP Assay Kit according to the manufacturer's recommended protocols. The group without the treatment was set as control.

4. Statistical analysis:

The assay data were analyzed using Student's unpaired t-test. A p-value of <0.05 was considered statistically significant.

Results

1) HA demonstrates cytotoxicity in G-401 and JMU-RTK2 cell lines. The growth of both G-401 and JMU-RTK-2 cells were significantly suppressed by HA in a concentration dependent manner. **(Figure 2A,B)**

Figure 2A: SRB Assay with HA Treated G-401 Cells

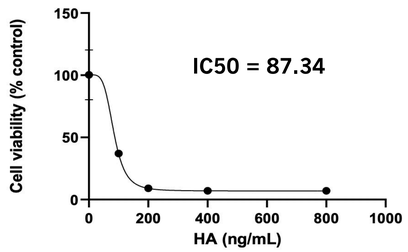


Figure 2B: SRB Assay with HA Treated JMU-RTK-2 Cells

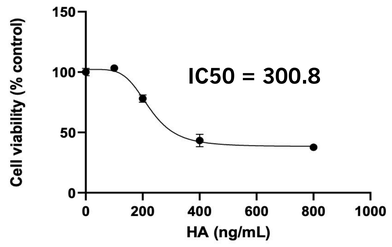


Figure 2 A) HA exhibits cytotoxicity in G-401 cells in a concentration dependent manner. Absolute IC50 was calculated using Prism. P-value (0nM vs 100nM) = 0.005. p-value (0nM vs 200nM) = 0.001. p-value (0nM vs 400nM) = 0.001. p-value (0nM vs 800nM) = 0.001 **B)** HA exhibits cytotoxicity in JMU-RTK-2 cell line in a concentration dependent manner. Absolute IC50 was calculated using Prism. P-value (0nM v 400nM) = 0.00007. P-value (0nM v 800nM) = 0.000006.

Results

2) HA inhibits GAPDH activity in G-401 and JMU-RTK2 tumor cells

HA has been shown to exert its anti-tumor effects via inhibition of GAPDH. To determine whether HA suppresses GAPDH in RTK cells, GAPDH activity was assessed in both G-401 and JMU-RTK-2 cell lines before and after treatment with HA. GAPDH activity was significantly reduced in both cell lines following treatment with HA. **(Figure 3A, B).**

Figure 3A: GAPDH Assay in HA Treated G-401 Cells

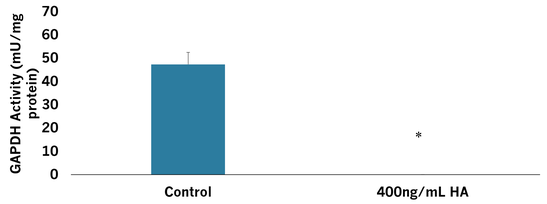


Figure 3B: GAPDH activity in HA Treated JMU-RTK2 Cells

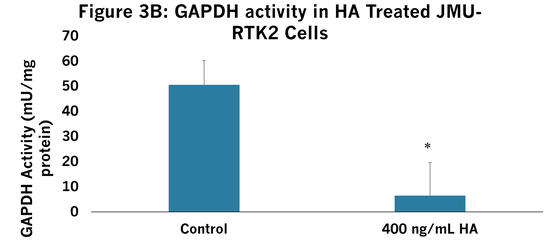


Figure 3: A) GAPDH activity in G-401 cells is significantly decreased compared with that in the negative control group following treatment with 400ng/mL of HA. Concentration of HA was determined from previous SRB assay experiments. Error bars represent standard deviation (SD). **B)** GAPDH activity in JMU-RTK-2 cells is significantly decreased compared with that in the negative control group following treatment with 400ng/mL of HA. Error bars represent standard deviation (SD). P-value = 0.008. *p<0.05

3) HA decreases content of ATP in G-401 and JMU-RTK2 cells lines.

Cancer cells have a high dependence on glycolysis to produce ATP [3]. By blocking glycolysis through inhibition of GAPDH, we wanted to determine if it would decrease ATP content as well. Treatment of HA in both G-401 and JMU-RTK-2 cell lines resulted in significantly decreased ATP content **(Figure 4).**

Figure 4: ATP Assay G-401 and JMU-RTK2 Cell Lines Following HA Treatment

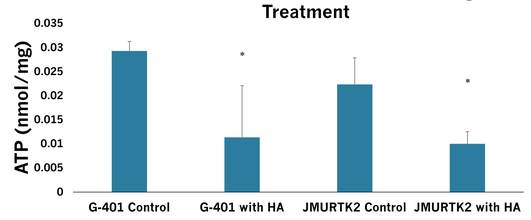


Figure 4: ATP content was decreased in both G-401 and JMU-RTK-2 lines following treatment with 400ng/mL of HA compared to negative controls. Error bars represent standard deviation (SD). P-value (G-401 Control v treatment) = 0.046. P-value (JMU-RTK-1 Control v Treatment) = 0.02. * p<0.05.

Summary

1. HA shows cytotoxicity in both RTK cell lines in a concentration dependent manner.

2. HA suppresses GAPDH activity and ATP content in both RTK cell lines.

Limitations of Study

- SRB assays demonstrated differences in HA efficacy between the two cell lines.
- G-401 is more sensitive to HA suggesting glucose as its primary source of energy whereas JMU-RTK2 may also use another energy source such as fat or protein which are not dependent on GAPDH.
- Other possibilities include differences in growth rate. In the SRB assay JMU-RTK-2 control group had significantly fewer viable cells compared to G-401 control group and showed a less drastic drop in cell viability following HA treatment. Slower growth rate would make JMU-RTK-2 cells less susceptible to blocking glycolysis due to reduced energy requirement.
- Further studies will be needed to understand the differences between these two cell lines.

Future Directions

- Determine the mechanism of cell death by which HA kills RTK cells via apoptosis assays.
- Determine cytotoxicity of HA against RTK in *in vivo* mouse models.
- Determine the effect of HA on GAPDH activity in RTK tumors *in vivo.*
- Determine safety profile and toxicity of HA *in vivo.*

References

1. Geller JI, Roth JJ, Biegel JA. Biology and Treatment of Rhabdoid Tumor. Crit Rev Oncog. 2015;20(3-4):199-216. doi: 10.1615/critrevoncog.2015013566. PMID: 26349416; PMCID: PMC6087667.
2. Warburg O. On respiratory impairment in cancer cells. Science. 1956;124:269–70.
3. Ganapathy-Kanniappan S. Evolution of GAPDH as a druggable target of tumor glycolysis? Expert Opin Ther Targets. 2018;22:295–8. https://doi.org/10.1080/14728222.2018.1449834.
4. Konishi H, Isozaki S, Kashima S, Moriichi K, Ichikawa S, Yamamoto K, Yamamura C, Ando K, Ueno N, Akutsu H, Ogawa N, Fujiya M. Probiotic Aspergillus oryzae produces anti-tumor mediator and exerts anti-tumor effects in pancreatic cancer through the p38 MAPK signaling pathway. Sci Rep. 2021 May 26;11(1):11070. doi: 10.1038/s41598-021-90707-4. PMID: 34040123; PMCID: PMC8154913.
5. Isozaki S, Konishi H, Tanaka H, Yamamura C, Moriichi K, Ogawa N, Fujiya M. Probiotic-derived heptelidic acid exerts antitumor effects on extraintestinal melanoma through glyceraldehyde-3-phosphate dehydrogenase activity control. BMC Microbiol. 2022 Apr 22;22(1):110. doi: 10.1186/s12866-022-02530-0. PMID: 35459092; PMCID: PMC9026996.
6. Endo A, Hasumi K, Sakai K, Kanbe T. Specific inhibition of glyceraldehyde-3-phosphate dehydrogenase by koningic acid (heptelidic acid). J Antibiot (Tokyo). 1985;38:920–5. https://doi.org/10.7164/antibiotics.38.920.
7. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006;1:1112–6. https://doi.org/10.1038/nprot.2006.179.