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Selective toxicity of caffeic acid in hepatocellular carcinoma cells

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ABSTRACT

Caffeic acid is a natural phytochemical structurally similar to other cinnamic acids. In this study we found caffeic acid (CA) but not ferulic, sinapic or cinnamic acids inhibited proliferation of hepatocellular carcinoma cells (HCC) and reduced cell numbers by inducing apoptosis. Only transient exposure to CA was required for these lethal effects that are associated with disruption of mitochondrial membrane potential and induction of reactive oxygen species. By comparison, primary hepatocytes resisted CA toxicity for nearly 48 h, consistent with selective sensitivity of HCC to CA. These results support use of CA as an anti-tumor agent to inhibit HCC, especially if delivered by locoregional catheterization in an embolization procedure.

1. Introduction

Patients with hepatocellular carcinoma (HCC) have limited choices for therapies. One option is trans arterial embolization (TAE), a procedure performed via the femoral artery to advance a catheter through the mesenteric system to the arterial vessel immediately supplying the tumor [1]. The catheter is positioned to deliver embolic particles that occlude the artery and arrest blood flow to deprive the tumor of oxygen and nutrients. However, clinical results have been less than satisfactory because a majority of tumors survive TAE and regrow, with no apparent benefit in terms of overall survival [2–7]. We have been seeking agents that could be added to TAE protocols to enhance therapeutic efficacy.

One approach is to block lactate export from HCC tumor cells that are dependent on anaerobic metabolism. Build up of excess intracellular lactate exerts inhibitory effects on glycolysis and cell growth through inhibition of phosphofructokinase, the rate-limiting enzyme for glycolysis [8–10] Reports in the literature suggested that the natural phytochemicals caffeic acid (CA) and ferulic acid (FA) would inhibit lactate transport via monocarboxylate transporter proteins [11]. These compounds are structurally similar; CA and FA only differ by a single methyl group (Fig. 1). We previously have shown that CA and FA were equally effective at inhibiting lactate export by rat N1S1 HCC cells, using a Seahorse metabolic analyzer to measure Extra Cellular Acidification Rate (ECAR) [12]. We observed significant regression of tumors when these agents were tested *in vivo* in combination with TAE with orthotopic syngeneic N1S1 tumors in rats compared to TAE alone. The response to CA + TAE was somewhat greater than the response to FA + TAE, raising a question about whether CA had other effects on HCC, in addition to inhibition of lactate transport. That question prompted the studies reported here.

The preferred animal model for HCC is woodchucks, because tumors arise *de novo* secondary to hepatitis virus infection and need not be implanted [13,14]. Moreover, woodchucks have arteries large enough to allow for catherization, so TAE procedures with woodchucks closely resemble those in clinical practice [15]. Therefore, future *in vivo* testing will be done in woodchucks, so here we used the woodchuck HCC cell line (WHC-17). We discovered profound differences between the effects of CA and related compounds on HCC cells in terms of proliferation, mitochondrial integrity and apoptosis. The results support choice of CA for future locoregional therapy of HCC using a TAE protocol.

Abbreviations: CA, caffeic acid; FA, ferulic acid; HCC, hepatocellular carcinoma; ECAR, extra cellular acidification rate; TAE, transarterial embolization; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; TMRE, tetramethylrhodamine, ethyl ester; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; DCFDA, 2′,7′-dichlorohydrofluorescein diacetate; ROS, reactive oxygen species; 5-LO, 5-lipoxygenase.

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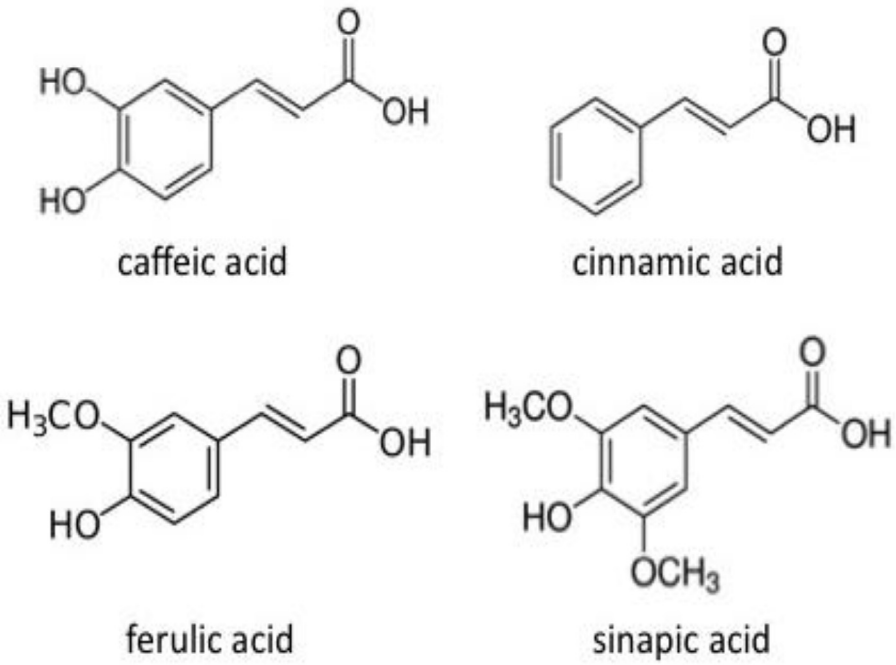

Fig. 1. Chemical structures of caffeic acid, ferulic acid, cinnamic acid (3-phenylacrylic acid), and sinapic acid, 4-hydroxy, 3,5-dimethoxy substituted cinnamic acid.

2. Materials and methods

Cell Proliferation – Woodchuck WCH-17 cells were purchased from American Type Culture Collection (ATCC) that provided authentication. Primary hepatocytes were harvested from a woodchuck that had been used for catherization and embolization procedure (REF). The woodchuck was procured from Northeastern Wildlife (Harrison, Idaho) and housed and cared for in AALAC-approved facilities at University of Virginia under a protocol approved by the institutional animal care and use committee.

Cells were seeded at 10,000 cells/well and allowed to attach in an incubator overnight at 37 °C in humified 5% CO_2. Fresh DMEM medium (with 10% fetal bovine serum (FBS), without Phenol Red) was supplemented with 1 mM Caffeic Acid (CA), 1 mM Ferulic Acid (FA), or 1% DMSO as vehicle control. After 12, 24, 36, and 48 h media was removed and plates frozen at −80 °C. CyQuant assay (Thermo Fisher Scientific) was done by adding 200 μl CyQuant GR dye diluted 80 times in cell-lysis buffer. After 5 min incubation fluorescence was measured (480/520 nm). Mean ± SD was calculated for n = 3 independent replicates, each done in triplicate. Transient exposures involved incubation with CA, FA or DMSO for 2, 4, 6 or 12 h, followed by rinsing and exchange into fresh medium without additions for 24 or 48 h, followed by Alamar Blue fluorescence assay for 2 h (560/590 nm).

Caspase assay - Cells were seeded 10^6 cells/100 mm dish and allowed to attach overnight. Medium was replaced with fresh DMEM containing 10% FBS plus 1 mM CA, 1 mM FA or 1% DMSO, as a control. After 0, 12, 24, 36, 48 h cells were trypsinized collected by centrifugation (500x g, 5 min), and washed with ice cold PBS, pelleted and dissolved in 100 μl of lysis buffer. Samples were frozen and thawed twice, centrifuged at 13,000 g for 10 min and the supernatants frozen at −80 °C. Bradford assay was performed to determine protein concentration and Caspase 3/7 Assay (Promega) was done in 96 well plate using 50 μg of total protein, following manufacturer's directions. Absorbance was read at 405 nm after 4 h of incubation at 37 °C. Caspase specific activity calculated as mean value for samples done in triplicate.

Mitochondrial membrane potential. Cells were seeded 10,000 cells/well in 96 well plate and treated as described for proliferation assay. Cells were incubated with 1 μM TMRE (Abcam) for 30 min at 37 °C. After this, samples were washed 2x with PBS and 200 μl of PBS was added to measure fluorescence (549/575 nm). As an additional control FCCP (20 μM) was added to samples in parallel. Mean ± SD was calculated for n = 3 independent replicates, each done in triplicate.

Radical Oxygen Species (ROS) measurement. WCH-17 cells were seeded 20,000 cells/well in 96 well plate and allowed to attach overnight. After adding 100 μl of 25 μM H2DCFDA (2′,7′−dichlorohydrofluorescin diacetate) (Thermo Fisher Scientific) for 45 min cells were washed with PBS. Medium with 1 mM CA, 1 mM FA, or 1%DMSO as a control was added and samples incubated for 0, 2, 4, 6 h, then fluorescence intensity measured (495/529 nm). After diffusion into cells H2DCFDA is deacetylated by cellular esterases to H2DCF, which is oxidized by ROS into highly fluorescent 2′, 7′−dichlorofluorescein (DCF).

3. Results

3.1. Effects of caffeic acid on HCC cell proliferation and survival

We cultured woodchuck WHC-17 cells in 96 well plates and assayed for proliferation by quantifying DNA content at 12 h intervals using the CyQuant protocol. As shown in Fig. 2 addition of caffeic acid (CA) to the medium resulted in a complete block of proliferation, and in fact a net decrease in the number of viable cells, noticeable within the first 12 h. The response to CA was similar to the response to the chemotherapeutic agent doxorubicin, which caused a reduction in the number of viable cells (Fig. 2A). In contrast, there was no difference in the rate of proliferation of WHC-17 cells grown in ferulic acid (FA) compared to controls that had 1% DMSO added. We independently replicated these experiments, and included samples with added cinnamic acid and sinapic

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