Alpha-Lipoic Acid in the Treatment of Hepatocellular Carcinoma

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**Hypothesis**

Alpha-lipoic acid’s capability to generate reactive oxygen species in cancer cells leads to apoptosis of the cancer cells and a decrease in its metastatic potential.

**Article Word Count**

6032
Abstract

Alpha-lipoic acid is a potent generator of reactive oxygen species and works through several mechanisms to reduce inflammation and modify gene expression. Along with these benefits, alpha-lipoic acid is proposed to potentially cause apoptosis of cancer cells and decrease its metastatic potential.

A literature review of ten studies was performed to confirm the hypothesis. The analyzed studies were primary research studies with the aim of establishing a theorized mechanism of action in a study population of human liver and breast cancer cell lines, rat and mouse liver cancer cell lines, and rhesus monkey livers.

Results showed that alpha-lipoic acid was an effective generator of reactive oxygen species in cancer cells as well as a potent regenerator of other antioxidants, such as glutathione. Alpha-lipoic acid was also shown to effectively inhibit pro-inflammatory cytokines and increase anti-inflammatory cytokines. Additionally, alpha-lipoic acid was effective in controlling cancer’s metastatic ability in vitro by inhibiting matrix metalloproteinase gene transcription and translation. The major drawback to alpha-lipoic acid was its potential lethality in mega-doses when given to rhesus monkeys. Mega-doses of alpha-lipoic acid however, have not been used with humans.

The results of alpha-lipoic acid’s effects on cancer provide statistically significant data showing that it is useful in treating hepatocellular carcinoma. As alpha-lipoic acid has been shown to be beneficial in animal and human clinical studies, human clinical trials of alpha-lipoic acid should be performed in
combination with current conventional hepatocellular carcinoma treatment guidelines and as a stand-alone treatment for comparative analysis.

Word Count – 242

Keywords – Alpha-lipoic acid, reactive oxygen species, inflammation, metastasis, cell migration, apoptosis, mitochondria, matrix metalloproteinases, cytokines

Ultramini Abstract

Alpha-lipoic acid is a powerful antioxidant that has potential to be used in the treatment of hepatocellular carcinoma. It treats cancer by targeting it at multiple stages in progression, from growth to metastasis. Alpha-lipoic acid also reduces the inflammation associated with cancer and increases other powerful antioxidants, such as glutathione.

Introduction

Cancer is a cellular metabolism defect characterized by poor oxygen consumption and excessive lactate production leading to a decreased intracellular pH level. Under normal aerobic conditions, healthy cells undergoing glycolysis convert glucose into CO₂ and ATP, whereas cancer cells inadvertently ferment this glucose into lactate regardless of whether or not oxygen is present (Warburg, 1956). This process of cancer cell metabolism is termed the Warburg effect, named after Dr. Otto Warburg, who discovered this process in 1924 and subsequently won the Nobel Prize.

Cancer cells, as dictated by the Warburg effect live in an environment with elevated reactive oxygen species (ROS). ROS are signaling molecules vital to cancer proliferation and the suppression of apoptosis. As such, cancer is also characterized
by its irregular growth pattern and ability to metastasize. This irregularity in growth may lead to increased levels of inflammation due to the fact that parts of the tumor not receiving enough blood supply become necrotic.

ALA is an endogenous molecule and cofactor for a variety of enzyme complexes that control metabolism with the most important being pyruvate dehydrogenase complex (PDH), which is found in glycolysis. PDH, under aerobic conditions, converts pyruvate into acetyl-CoA for use in the tricarboxylic acid (TCA) cycle in the mitochondrion (Berkson et al., 2009).

Apart from glycolysis, ALA is also a potent antioxidant that decreases oxidative stress by acting as a free radical scavenger. It does so by participating in the Fenton reaction (Abdan, 2012). ALA is also a potent stimulator of glutathione peroxidase activity and other powerful antioxidants such as ubiquinone, vitamin E, and ascorbic acid (vitamin C) (Shi et al., 2010, Kim et al., 2012, Abdan, 2012). The antioxidant effects of ALA are particularly beneficial in diseases such as heavy metal poisoning, diabetes, cancer, and liver disease (Mark et al., 2003). In particular, ALA has been demonstrated to revert liver enzymes to near normal levels in hepatocellular carcinoma (HCC) bearing mice (Abdan, 2012).

**Methods**

The databases used for this literature review project were **MEDLINE, PLOS, Academic Search Premier** and **PubMed**. When searching PubMed, **MeSH** terms were used. All journal articles used for this project were peer-reviewed. When searching these databases for relevant articles, the search was limited to articles within the last ten years, except for one article dating back to 1956 and another
article from 2003, both of which were used in the introduction only. The article from 1956 was used for historical relevance. The key terms used in the search were “Alpha Lipoic Acid”, “Hepatocellular Cancer”, “Apoptosis”, and “Antioxidant”. About one quarter of the articles related to ALA's use in the treatment of hepatocellular cancer. Most of the articles were very narrow in their research, for example, just focusing on one particular pathway of the entire intrinsic apoptotic pathway.

The research criteria of the literature review included primary research, preclinical trials and clinical studies, with most of the information coming from the primary research. Test subjects for the primary research included cultured human hepatic, breast and lung cell lines, rat and mouse models, and rhesus monkeys. Human patients were the source of the clinical studies. There was no restriction with the number or gender of mice used. However, some mice were fed a restricted diet or had certain experimental procedures performed, such as an injection of Ehrlich ascites carcinoma cells or thioacetamide into the peritoneum. For the clinical studies, a particular subset of patients was used. The patients had at least stage III primary or metastatic pancreatic or hepatocellular carcinoma.

The main criteria used to assess the quality of the research were the level of conflicts of interest (if any) and the clinical relevance of the research. Research that held little or no clinical relevance was not used.

**Important Terms/Abbreviations:**

- ALPHA-LIPOIC ACID (ALA) – a potent antioxidant endogenous to the human body essential for aerobic metabolism
• REACTIVE OXYGEN SPECIES (ROS) – highly reactive oxygen-based molecules containing an unpaired electron

• APOPTOSIS – process by which the cell undergoes controlled death without producing inflammation

• INFLAMMATION – the process mediated by cytokines that attracts leukocytes to fight infection, disease, or foreign invaders

• AEROBIC GLYCOLYSIS – the process of energy production from glucose performed in either an aerobic or anaerobic environment

• WARBURG EFFECT – the process by which cancer cells undergoing glycolysis produce lactate as the main product regardless of oxygen content

Results

The first set of research pertains to ALA’s anti-metastatic properties using cultured human hepatoma and breast cell lines. The most common method employed was based upon time-dependent observations using various lab techniques, as described, to measure the data. To observe the metastatic potential, assorted cytokine levels and invasion properties of the cells were measured. Controls were used in both cell lines. The results were all statistically significant with the exception of some differing concentrations of ALA.

Cell migration and matrix metalloproteinase (MMP) activity are increased in metastatic cancers such as hepatocellular and breast carcinomas, and as such are potential targets of ALA. MDA-MB-231 breast cancer migration is decreased in a dose-dependent manner when treated with ALA as demonstrated using a Boyden chamber assay (Lee et al., 2010). The results demonstrated that concentrations of
ALA greater than 250 μmol/L (51 mg/L) for 12 hours decrease the migration of MDA-MD-231 breast cancer cells, while concentrations greater than 1000 μmol/L (206 mg/L) for 12 hours have a 50% reduction in migration. A control was measured where 0 μmol/L (0 mg/L) of ALA was used (Lee et al., 2010). Concentrations of ALA at 250 μmol/L to 1000 μmol/L (206 mg/L) were statistically significant in reducing motility with p < 0.05.

Increased MMP activity allows for tumors to metastasize by degrading the extracellular matrix. Using zymography and real-time polymerase chain reaction (RT-PCR), ALA’s ability to decrease MDA-MB-231 cell mRNA expression of MMP-2 and MMP-9 was recorded in a dose-dependent manner. (Lee et al., 2010). With respect to MMP-2, the concentration of ALA at 500 μmol/L is statistically significant with p < 0.05, while any concentration above or below is statistically insignificant compared to 500 μmol/L. Targeting MMP-9, statistical significance was demonstrated at concentrations of 250 μmol/L and 500 μmol/L recorded by zymography, while RT-PCR demonstrated statistical significance only at the concentration of 250 μmol/L, both having p < 0.05.

Invasion through the basement membrane is another vital step in metastasis. ALA was able to inhibit invasion of MDA-MB-231 cells through a Matrigel invasion chamber with statistical significance measured in all concentrations at p < 0.05. With increasing concentrations of ALA a further decrease in invasion is noted, however. A control was used with a 0% decrease in invasion. At the dose of 250 μmol/L, a near 60% reduction in invasion is seen, while concentrations of 500
μmol/L and 1000 μmol/L decreased invasion by nearly 70% and 80%, respectively (Lee et al., 2010).

IL-8 is a cytokine that promotes tumor invasiveness. ALA was demonstrated to be a statistically significant inhibitor of IL-8 in human hepatoma (Huh7) and hepatocellular carcinoma cell lines if it was used for more than 72 hours. The p value was measure at p < 0.05. When compared against other antioxidants such as caffeic acid and 2-S-lipoyl-caffeic acid, ALA was superior in the hepatocellular carcinoma cell lines with a maximum efficacious concentration of 0.5 mM (103 mg/L). Concentrations of 0.05 mM to 1 mM were used ranging in 50% increases from the previous dose as shown in Figures 1 and 2. In the hepatocellular carcinoma cell lines ALA was a more potent antioxidant compared to caffeic acid and 2-S-lipoyl-caffeic acid at concentrations of 0.05 mM to 0.5 mM, however at 1.0 mM, ALA and caffeic acid were of near equal efficacy (Guerriero et al., 2011). In Huh7 cell lines, ALA was statistically significant in reducing the IL-8 concentration at p < 0.05, but compared to caffeic acid and 2-S-lipoyl-caffeic acid, it was of near equal efficacy.

Figure 1. IL-8 levels in HepG2 (human hepatocellular carcinoma) cell line after 72 hours of treatment with concentrations of ALA, caffeic acid and 2-S-lipoyl-
caffeic acid between 0.05 mM and 1 mM. At concentrations between 0.05 mM and 0.5 mM, ALA is a more potent antioxidant compared to caffeic acid and 2-S-lipoyl-caffeic acid, however at 1.0 mM, ALA and caffeic acid are of near equal efficacy (Guerriero et al., 2011).

Figure 2. IL-8 levels in Huh7 (human hepatoma) cell lines after 72 hours of treatment with concentrations of ALA, caffeic acid and 2-S-lipoyl-caffeic acid between 0.05 mM and 1 mM. ALA is statistically significant in reducing the IL-8 concentration, but compared to caffeic acid and 2-S-lipoyl-caffeic acid it is of near equal efficacy (Guerriero et al., 2011).

The next study demonstrates ALA’s ability to reestablish near normal values of certain liver enzymes and to regenerate other powerful antioxidants such as glutathione. The study used Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice in which the EAC cells were injected directly into the peritoneum of the mice. In Figure 3, daily oral administration of 50 mg/kg/day for 30 days of ALA is capable of reestablishing near normal levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT). In EAC bearing mice it is shown that levels of ALP, ALT, AST and GGT are elevated, but with the addition of ALA the liver markers are returned to near baseline levels. When ALA is administered in healthy mice however, the liver markers have minimal associated change from baseline. The results of ALA in EAC-
bearing mice are all statistically significant with \( p < 0.05 \) (Abdan, 2012). Healthy mice were used as the control to measure baseline levels.

<table>
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<th>EAC + LA</th>
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<td>ALP K.A.U/100 mL</td>
<td>130.6 ± 14</td>
<td>125 ± 15</td>
<td>162.4 ± 11*</td>
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<td>ALT U/l</td>
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<td>71.8 ± 4.42</td>
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<td>80.5 ± 4.1*</td>
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<td>AST U/l</td>
<td>54.5 ± 3.5</td>
<td>50.1 ± 3.2</td>
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<td>56.2 ± 4.2*</td>
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<td>GGT U/l</td>
<td>6.0 ± 0.09</td>
<td>6.1 ± 0.01</td>
<td>11.9 ± 0.9*</td>
<td>7.9 ± 0.08*</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SE

*Significant at \( p < 0.05 \) as compared with control group

**Significant at \( p < 0.05 \) as compared with EAC group.

**Figure 3.** Activities of ALP, ALT, AST and GGT in EAC-bearing mice and healthy mice (control) (LA, lipoic acid). In EAC-bearing mice it is shown that levels of ALP, ALT, AST and GGT are elevated, but with the addition of ALA the liver markers are returned to near baseline levels. When ALA is administered in healthy mice, however, the liver markers have minimal associated change from baseline (Abdan, 2012).

ALA is also able to replenish redox liver enzymes glutathione (GSH), total thiols (T-SH), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) in EAC-bearing mice as seen in Figure 4. In EAC-bearing mice SOD is inhibited, while the levels of CAT, GST, GSH, and T-SH are greatly decreased from baseline. When ALA is administered to EAC-bearing mice SOD is much less inhibited, while the levels of CAT, GST, and T-SH are returned to near baseline levels with the exception GSH, which is still diminished. When ALA was administered to healthy mice, minimal change in the liver redox enzymes was noted. The results of ALA in EAC-bearing mice on the effect of liver redox enzymes was also statistically significant with \( p < 0.05 \) (Abdan, 2012). Healthy mice were used as the control to measure baseline levels.
Figure 4. Activities of the liver redox enzymes SOD, CAT, GST, GSH, and T-SH in EAC-bearing mice and healthy mice (control) (LA, lipoic acid). In EAC-bearing mice, liver redox enzymes were all decreased as a result of SOD becoming inhibited. When ALA was administered to EAC-bearing mice the levels of the liver redox enzymes were able to return to near baseline levels. When ALA was administered to healthy mice minimal change from baseline was noted (Abdan, 2012).

The next set of studies relates to ALA’s ability to mediate intrinsic apoptosis in cancer through three alternative pathways, each converging upon and increasing the activity of caspase-3 and caspase-9. The pathways include cellular membrane protein signaling, endoplasmic reticulum signaling, and the release of cytochrome c from the mitochondrion. The first pathway, consisting of the up-regulation of phosphatidyl inositol-3,4,5-triphosphate phosphatase (PTEN) and down-regulation of Protein Kinase B (Akt) are proteins used in cellular membrane protein signaling termed the PTEN/Akt pathway (Shi et al., 2008). In human hepatoma cells 5 mM (1031 mg/L) of ALA is demonstrated to activate phosphorylated PTEN and inhibit phosphorylated Akt by western blot analysis. The activity of phosphorylated PTEN continued to increase from the start of ALA treatment to 72 hours of treatment. The inhibition of Akt however, was statistically significant only up to 48 hours of treatment with p < 0.05 (Shi et al., 2008).
The next subset of studies pertaining to apoptosis are the ER stress-related proteins and the release of cytochrome c from the mitochondrion. These two pathways are direct activators of caspase-3 and caspase-9.

First, the expression ER stress-related proteins, such as glucose-regulated protein 78 (GRP78) and C/EBP-homologous protein (CHOP) were up-regulated in the presence of ALA. Apoptosis was only detected after 12 hours or more of treatment of human lung cancer (A549) cell lines with 1 mM (206 mg) of ALA as detected using western blot analysis. Once apoptosis occurred at the 12-hour mark it continued to increase in a time-dependent manner. Subsequently, the level of inactive poly ADP-ribose polymerase (PARP) decreased while the activities of both X-linked inhibitor of apoptosis protein (XIAP) and caspase-3 increased (Kim et al., 2012). Heat shock cognate protein 70 (Hsc 70) was used as the control. Statistical significance was not mentioned.

After it was determined that ALA through the ER pathway induced apoptosis after 12 hours another lab was performed. The time of observation was 18 hours with concentrations of ALA ranging from 0.1 mM to 1 mM (0.02 mg/L to 206 mg/L). As the concentration of ALA increased so to did the expression of ER stress-related proteins such as the amino acid sequence KDEL (lysine, aspartate, glutamate, leucine), CHOP, and myeloid cell leukemia (Mcl-1). The activity of caspase-3 increased slightly with its substrate, PARP, showing a dramatic decrease in activity (Kim et al., 2012). The results were visualized using western blot analysis. There was no mention of statistical significance.
The next study within this subject involves the release of cytochrome c from the mitochondrion. Visualization by western blot analysis of rat hepatoma (FaO) cells and human hepatocellular carcinoma (HepG2) cells with 500 μM (103 mg/L) of ALA induces a gradual release of cytochrome c from the mitochondria into the cytosol. Despite this observation of the mitochondrial protein marker, COX-4, it was not detected in the cytosol of either cell line (Simbula et al., 2006). The measurements of cytochrome c and COX-4 were recorded at 24 hour intervals ranging from time 0 hours to 72 hours. Statistical significance was measured in HepG2 and FaO cell lines as time dependent variations with the p value ranging from p < 0.05 to p < 0.001 at 24 hour to 72 hour intervals, respectively. No control was used.

The last set of studies pertaining to apoptosis deals with the regulators of the intrinsic apoptotic pathway. These include caspase-3, caspase-9, bcl-XL and Bax. The direct effect of caspase activity in SMMC-7221 human hepatoma cells was detected following 3 days of treatment with ALA at 5mM (103 mg/L). ALA induced a 420% increase in caspase-3 expression and a 630% increase in caspase-9 expression. The effect on caspase-8, an extrinsic apoptotic mediator was not statistically significant (Shi et al., 2008). The statistical significance of both caspase-3 and caspase-9 increase was statistically significant with p < 0.05.

Next, the proteins Bax (pro-apoptotic) and bcl-XL (anti-apoptotic) were increased and decreased, respectively, with ALA treatment. With regards to Bax activation, it is suspected that the phosphorylation of p53 induces the activation of Bax. This was tested using 500 μM (103 mg/L) of ALA at 24-hour intervals using
HepG2 and FaO cells (Simbula et al., 2006). Statistical significance was measured in HepG2 and FaO cell lines as time dependent variations with the p-value ranging from $p < 0.05$ to $p < 0.001$ at 24 hour to 72 hour intervals, respectively. No control was used.

With respect to bcl-XL, human colorectal carcinoma (HT-29) cells were exposed to 1 mM (206 mg/L) of ALA or dihyrdro-lipoic acid (DHLA). What makes bcl-XL interesting is that an increase in mitochondrial superoxide production preceded the down-regulation of bcl-XL in HT-29 cells (Wenzel et al., 2005). Superoxide production was visualized using proxyfluorescamine dye and MitoTracker. It was observed that ALA had an increased effect on superoxide production compared to DHLA with a 28% and 21% increase, respectively. The bcl-XL down-regulation was visualized by Western blot. Actin was used as the control for both studies. No statistical significance was noted for bcl-XL down-regulation specifically, however, statistical significance was mentioned for the amount of cells displaying apoptotic features to be $p < 0.01$ and $p < 0.001$ for ALA and DHLA treatment, respectively.

The next topic of research pertains to ALA’s ability to dampen the inflammatory response on all levels. Some of the key modulators of the immune system include pro-inflammatory cytokines, white blood cells, and cellular membrane signaling via a decrease in cyclic adenosine monophosphate (cAMP) production. Arguably, one of the most important mediators of inflammation are the pro-inflammatory cytokines. It was shown in Figures 5 and 6 that ALA has a negative effect on IL-1β, IL-8, TNF-α and a positive effect on IL-10. IL-8 was previously described as also being a promoter of tumor invasion. The production of
these cytokines was evaluated in HepG2 and Huh7 cells by Bio-Plex assay. The time of measurement was taken at 72 hours because at 48 hours no significant change in production was noted. The dose of ALA and other antioxidants including, caffeic acid and 2-S-lipoyl-caffeic acid ranged from 0.05 mM to 1 mM (103 mg/L to 206 mg/L).

In HepG2 cell lines, ALA compared to the other antioxidants was not as effective in decreasing the concentrations of IL-1β and TNF-α. ALA, however was more efficacious in decreasing the concentration of IL-8. With respect to increasing the concentration of IL-10, ALA was equally efficacious with respect to the other tested antioxidants, except at a concentration of ALA at 1 mM where ALA was the most efficacious. The control was untreated HepG2 or Huh7 cells. The results were all statistically significant at 72 hours with p < 0.05 (Guerriero et al., 2011). In Huh7 cell lines, ALA was equally efficacious with the other antioxidants in decreasing IL-1β, IL-8 and TNF-α. ALA, however, was the least efficacious in increasing IL-10 in Huh7 cell lines compared to caffeic acid and 2-S-lipoyl-caffeic acid.
Figure 5. Bio-Plex assay demonstrating the cytokine levels in HepG2 (human hepatocellular carcinoma) cell line after 72 hours of treatment with concentrations of ALA, caffeic acid and 2-S-lipoyl-caffeic acid between 0.05 mM and 1 mM. ALA compared to the other antioxidants was not as effective in decreasing the concentrations of IL-1β and TNF-α. ALA, however, was more efficacious in decreasing the concentration of IL-8. With respect to increasing the concentration of IL-10, ALA was equally efficacious with the other tested antioxidants, except at a concentration of ALA at 1 mM where ALA was the most efficacious (Guerriero et al., 2011).

Figure 6. Bio-Plex assay demonstrating the cytokine levels in Huh7 (human hepatoma) cell line after 72 hours of treatment with concentrations of ALA, caffeic acid and 2-S-lipoyl-caffeic acid between 0.05 mM and 1 mM. ALA was equally efficacious with the other antioxidants in decreasing IL-1β, IL-8 and TNF-α. ALA, however, was the least efficacious in increasing IL-10 compared to caffeic acid and 2-S-lipoyl-caffeic acid (Guerriero et al., 2011).

IL-6 is another important inflammatory cytokine that also reduces the negative acute-phase reactants, retinol-binding protein (RBP) and transthyretin (TTR) by nearly 50% as measured in HepG2 cells (El-Saadany et al., 2007). To determine ALA efficacy many antioxidants including ALA were used in HepG2 cells to evaluate the positive effects on TTR and RBP levels after they were inhibited by IL-6. The HepG2 cells were cultured for 32 hours and treated with IL-6 for another 8
hours after which the concentrations of TTR and RBP were measured by ELISA. Changes in TTR and RBP levels were then measured with ELISA again after being exposed to the different antioxidants. The results showed that all antioxidants increase TTR and RBP levels compared to the non-antioxidant treated control with the strongest effects achieved with the highest concentration of each antioxidant (Figure 7). ALA at 0.8 mM was the most efficacious while ascorbic acid at 1 mM was the least efficacious (El-Saadany et al., 2007). The antioxidant effects on RBP were greater compared to TTR and are measured as a percent increased after a pre-treatment with IL-6. Statistical significance was not mentioned for the effectiveness of the antioxidants, however, with respect to IL-6, the p-value of IL-6’s inhibitory effects on TTR and RBP was measured as p < 0.001.

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<tr>
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<th>RBP</th>
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<tr>
<td>Lipoic acid</td>
<td>93.5 ± 13.4</td>
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<tr>
<td>EGCG</td>
<td>84.8 ± 14.2</td>
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<td>ECG</td>
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<td>Alpha-tocopherol</td>
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<td>EGC</td>
<td>72.2 ± 4.6</td>
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</tr>
<tr>
<td>EC</td>
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<td>107.9 ± 2.7</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>60.9 ± 3.5</td>
<td>104.2 ± 3.3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>52.5 ± 14.3</td>
<td>104.1 ± 15.7</td>
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Data represent the mean of three replicates ± SD vs IL-6 non-treated control.

Figure 7. The effect of the highest concentration of each antioxidant on IL-6 inhibited transthyretin (TTR) and retinol binding protein (RBP) production as measured in percent increased. ALA was the most efficacious in increasing TTR and RBP concentrations, while ascorbic acid was the least efficacious. The highest concentrations of each antioxidant are as follows: ALA = 0.8 mM (165 mg/L), EGCG = 25 μM (1.15 mg/L), ECG = 25 μM (1.11 mg/L), α-tocopherol = 50 μM (2.15 mg/L), EGC = 50 μM (1.53 mg/L), EC = 50 μM (1.45 mg/L), N-acetylcysteine = 1 mM (163 mg/L), Ascorbic acid = 1 mM (176 mg/L),
EGCG (epigallocatechin gallate), ECG (epicatechin gallate), EGC (epigallocatechin), EC (epicatechin) (El-Saadany et al., 2007).

In a hepatocarcinogenesis rat model, it was shown that COX-2 and HO-1 (heme oxygenase-1) macrophages were present in the liver along with CD3+ T-cells, as shown in Figure 8. 36 rats received an intraperitoneal injection of N-diethylnitrosamine (DEN) at 200 mg/kg body weight. 12 of these rats were fed a basal diet and were used as the control. 12 other rats were treated with thioacetamide (TAA) drinking water (0.02% TAA in drinking water) to induce a hepatocellular carcinoma state. The last 12 rats were treated with TAA and DEN plus 0.02% ALA in their diet (Fujii et al., 2013).

It was found that with TAA the number of COX-2 and HO-1 hepatic macrophages and CD3+ T-cells were greatly increased compared to DEN alone. Next, ALA was added to the DEN and TAA treated liver. This demonstrated that ALA returned the number of COX-2 and HO-1 hepatic macrophages as well as the number of the CD3+ T-cells to near baseline (control) levels (Fujii et al., 2013). The results were obtained after a 6-week hepatocellular carcinoma promotion period and were all statistically significant with COX-2 macrophages and CD3+ T-cells being $p < 0.01$ and HO-1 macrophages as $p < 0.05$. 
Figure 8. Immunohistochemical staining of a) COX-2 hepatic macrophages, b) HO-1 hepatic macrophages and c) CD3+ T-lymphocytes. DEN with TAA greatly increases COX-2 and HO-1 hepatic macrophages and CD3+ T-cells, while treatment with ALA returned the cells to near baseline levels. (Fujii et al., 2013)

The last study pertaining to ALA deals with its potentially dangerous side effect of lipid peroxidation of the mitochondria at high concentrations intravenously (IV). It was shown that doses of 90 mg/kg of body weight to 100 mg/kg of IV ALA were lethal to half of the rhesus monkeys experimented on. It should be noted, however, in patients the typical maximum dose administered is usually no more than 10 mg/kg. The mitochondria exposed to mega-doses ALA had obvious swelling and damage to the cristae. This is thought to be caused by the peroxidation of lipids in mitochondrial membrane (Vigil et al., 2014). No statistical evidence was gathered and no control group was used. Post-mortem dissection of the rhesus monkey liver exposed to mega-doses of ALA also displayed evidence of acute hepatic necrosis.
with multiple infarcts. Under electron microscopy the mitochondria was noted to have visible swelling and damage to the cristae (Vigil et al., 2014).

**Discussion**

After a thorough analysis of the research it is evident that ALA is beneficial in treating hepatocellular carcinoma. The first set of research suggested ALA is a powerful anti-metastatic antioxidant against human breast cancer and human hepatoma cell lines in vitro. Breast cancer has an important relationship to hepatocellular carcinoma being that the liver is one of the first organs infiltrated by metastatic breast cancer. One of the most important factors in the initiation of metastasis is invasion through the basement membrane mediated by metalloproteinases (MMPs), also known as extracellular matrix degradation proteinases, while IL-8 is important in maintaining cancer's ability to metastasize. It was demonstrated that ALA decreases the mRNA expression MMP-2 and MMP-9, the key proteinases involved in type IV collagen degradation (Lee et al., 2010). Also, by decreasing the MMP expression the migration of the breast cancer cells was also effectively reduced. This then supports the data that MMPs, especially MMP-2 and MMP-9 are essential for the migration of cancer. ALA had a dose-dependent relationship occurring after 24 hours against both MMP expression and migration. Concentrations of ALA ranging between 250 μmol/L and 1000 μmol/L (50 mg/L and 100 mg/L, respectively) were beneficial in decreasing the expression of MMP-2 and MMP-9, therefor reducing breast cancer's ability to metastasize (Lee et al., 2010).
IL-8 is a pro-inflammatory cytokine that also has pro-angiogenic activity further increasing the invasiveness and metastatic potential of late stage HCC (Guerriero et al., 2011). It was found that ALA had a dose dependent relationship on IL-8 concentration with concentrations ranging between 0.05 mM and 1 mM (103 mg/L and 206 mg/L, respectively). The most efficacious concentration was 0.5 mM (Guerriero et al., 2011).

Overall, with respect to metastasis, ALA was found to be efficacious in decreasing the initiation of tumor invasion and migration by decreasing MMP mRNA expression and IL-8 concentration. This has potential influence in how doctors may use ALA and when. Since invasion begins early by the actions of MMPs it would be beneficial to start ALA treatment early. However, in both studies, the research was performed in vitro with no supporting in vivo studies.

In HCC, the hepatic enzymes are usually markedly elevated and potent antioxidants are diminished. ALA was demonstrated to normalize ALP, ALT, AST and GGT to near normal values and replenish GSH, T-SH, GST, SOD and CAT in Swiss albino mice. ALA also participates in the Fenton reaction scavenging superoxide molecules, hydrogen peroxide and hydroxyl radicals and chelating ferrous ions (Abdan, 2012). However, the experimental dose of 50 mg/kg/day is not used in medical practice because it is approaching hepatotoxic levels. Although, no human trials have been studied to establish the toxic doses of ALA (Orsucci et al., 2009).

In medical practice 10 mg/kg every other day is usually the maximum dose given to patients. In rhesus monkeys however, ALA in concentrations exceeding 90 mg/kg/day was given resulting in severe hepatotoxicity and in some cases lethality.
The cause of hepatotoxicity was theorized to be destruction of the mitochondrial membrane by lipid peroxidation cascading the healthy cell into apoptosis or worse, necrosis (Vigil et al., 2014).

As shown in the results, ALA is able to induce apoptosis in cancer cells by three different pathways; membrane protein signaling, ER stress-related proteins, and cytochrome c release from the mitochondrion induced by the generation of free radical species. All of these pathways increase the activity of caspase-3 and caspase-9, activators of intrinsic apoptosis. ALA also increased Bax, a pro-apoptotic protein and decreased bcl-X$_L$, an anti-apoptotic protein (Simbula et al., 2006, Wenzel et al., 2005). Bax and bcl-X$_L$ are considered the regulators of intrinsic apoptosis. If Bax is in greater proportion than bcl-X$_L$, apoptosis will occur, whereas if bcl-X$_L$ is in greater proportion to Bax, apoptosis will not occur.

The first pathway in relation to apoptosis pertains to cellular membrane protein signaling via the PTEN/Akt pathway in human hepatoma cells. Akt is anti-apoptotic promoting the growth and survival of the cell. Akt is positively regulated by PI3K and negatively regulated by PTEN. When phosphorylated PTEN increased it was shown to cause a decrease in phosphorylated Akt, leading to apoptosis. It is therefore indicated that apoptosis induced by ALA is facilitated by the PTEN/Akt pathway (Shi et al., 2008).

ER stress-related proteins as it relates to apoptosis were found to be up-regulated in the presence of ALA. The described proteins are GRP78, CHOP, PARP, KDEL and Mcl-1. GRP78 is an ER stress-responsive protein while KDEL and Mcl-1 are ER stress-related proteins. CHOP increases the expression of DR5, which then
mediates ER-mediated apoptosis. PARP cleavage was increased only after it was induced by the increased expression of caspase-3. The only protein that was down-regulated was XIAP, an inhibitor of apoptosis in the ER pathway (Kim et al., 2012).

Activation of ER stress-related apoptosis is due to improper protein folding. Apoptosis by this pathway is then therefore mediated by a failed unfolded protein response (UPR). UPR activates transcription factor 6 (inositol-requiring enzyme 1) to prevent further protein misfolding, but if any of these steps fail to occur apoptosis ensues (Kim et al., 2012).

The next pathway involving intrinsic apoptosis is induced by cytochrome c release from the mitochondrion. ALA induces cytochrome c release by increasing reactive oxygen species (ROS) generation (Wenzel et al., 2005), leading to DNA damage, causing p53 to become phosphorylated (active). Activated p53 then up-regulates p21 and p27 (Simbula et al., 2006), which leads to cell cycle arrest at the G₁ to S phase and the induction of Bax protein. Overexpressed Bax and inactivated Bcl-2 and bcl-xL proteins (Kisurina-Evgenieva et al., 2010) are followed by the release of cytochrome c from the mitochondrion into the cytosol (Simbula et al., 2006). Cytosolic cytochrome c then binds to Apaf-1 leading to assembly of the apoptosome and stimulating it to activate caspases 3 and 9. Once caspase-3 and caspase-9 are finally activated, apoptosis will soon follow (Borges et al., 2008).

A concentration of only 500 μM (103 mg/L) over a 24-hour period was needed to induce cytochrome c release from rat hepatoma and human hepatocellular carcinoma cell lines. This concentration is usually only about one-third to one-sixth the average dose given intravenously in medical practice,
however, so the full benefit of ALA if used in humans at this experimental may not reach its full efficacious potential.

The final aspects of apoptosis involve caspases, pro-apoptotic proteins Bax, Bim, Bmf, Bak and jBid, and anti-apoptotic proteins bcl-2 and bcl-Xl, with Bax and bcl-Xl being the most important (Kisurina-Evgenieva et al., 2010). Caspase-9 is involved in the intrinsic apoptotic pathway, whereas caspase-8 is involved in the extrinsic apoptotic pathway. The two pathways converge into caspase-3 and induce apoptosis. ALA promotes caspase-3 and caspase-9 activity, but does not induce caspase-8, however (Shi et al., 2008). Caspases 3 and 9 are up-regulated whenever there is irreversible cell damage. Caspase-3 is the converging protein for the three separate pathways involved in intrinsic apoptosis.

Bax and bcl-Xl are regulatory proteins that must be in balance with each other to promote cell survival. When irreversible cellular injury occurs such as stress induced by an increase in the amount of reactive of species (ROS), Bax becomes up-regulated while bcl-Xl becomes down-regulated. This shift in balance then promotes the cell to undergo apoptosis. While Bax is directly up-regulated by ROS and phosphorylated p53 (Simbula et al., 2006), bcl-Xl down-regulation is immediately preceded by an increase in mitochondrial superoxide production (Wenzel et al., 2005). ALA at clinically useful concentrations induces ROS formation and increases mitochondrial superoxide production, therefore promoting an up-regulation and down-regulation of Bax and bcl-Xl, respectively in cancer cells (Simbula et al., 2006, Wenzel et al., 2005).
The last anti-cancer effect of ALA discussed was based on its anti-inflammatory properties. It was shown with Huh7 and HepG2 cell lines that concentrations of ALA between 0.05 mM (103 mg/L) to 1 mM (206 mg/L) were more efficacious than caffeic acid and 2-S-lipoyl-caffeic acid in reducing the pro-inflammatory cytokines IL-1β, TNF-α, while also promoting an increase in IL-10, an anti-inflammatory cytokine (Guerriero et al., 2011). Increasing the levels of IL-10 should be one of the goals in treating any form of cancer as it blocks the activation of NF-κB leading to apoptosis of tumor cells (Guerriero et al., 2011).

TNF-α is one of the more potent inflammatory cytokines. Decreasing its level is very important in treating cancer. The decreased effects of TNF-α in cancer is two-fold; 1) it decreases cachexia and 2) it may promote better tolerance to chemotherapeutic drugs (xenobiotics) (Salinthone et al., 2010).

IL-6, another pro-inflammatory cytokine was also inhibited by ALA. IL-6 is an acute phase reactant that decreases the transcription of negative acute-phase reactants, such as TTR and RBP, as was shown in HepG2 cells (El-Saadany et al., 2007). ALA, compared to other antioxidants was shown to have an inhibitory effect on IL-6 thus promoting the transcription and secretion of TTR and RBP (El-Saadany et al., 2007).

In TAA induced hepatocarcinogenesis in rat models, enzymatic activity of COX-2 in hepatic macrophages was reduced while the enzymatic activity of HO-1 was increased by a poorly understood mechanism. In TAA-induced hepatocarcinogenesis, transcription levels of Ptgs2 coding COX-2 was significantly increased with ALA effectively reducing both. HO-1 is theorized to increase in the
presence of ALA because it too has anti-inflammatory properties as it helps to protect against oxidative cellular stress (Fujii et al., 2013).

TAA-induced hepatocarcinogenesis also promoted an increase in CD3+ T-cells, while ALA effectively reduced the amount of CD3+ T-cells present. This reduction is thought to occur due to a decrease in TNF-α (Fujii et al., 2013), while TNF-α itself is decreased by the activation of the cAMP-dependent pathway. This pathway provides strong stimulatory signals for the production of Th2 helper T-cells while also providing an effective inhibitory signal for Th1 helper T-cell generation (Salinthone et al., 2010).

Finally, Berkson et al. have done several case studies of patients with stage 4 metastatic pancreatic and hepatic cancers. They have shown that IV therapy of 300 mg/L to 600 mg/L of ALA two to three times a week is very efficacious in treating these cancers when all other conventional treatments have failed (Berkson et al., 2009, Berkson, 2006). Dr. Berkson reported that one of his patients with metastatic pancreatic carcinoma treated with the ALA protocol was free of symptoms after 51 months and even returned to work at time of reporting. This was after the patient had been treated with conventional cancer therapy without benefit (Berkson, 2006).

While all of the previously mentioned research showed promising results, a minority must be evaluated with caution. Human clinical trials of ALA comparing it to standard pharmaceutical therapy are very limited (Orsucci et al., 2009). Therefore, no conclusive evidence favoring ALA over its pharmaceutical counterpart has been established. With respect to the research pertaining to metastasis, all of the research was performed in vitro rather than in vivo. As a result, the data may look more

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promising than what actually occurs in the human body or even a test animal’s body. Most of the research used concentrations of ALA comparable to what is used in clinical practice. The only study that used decreased concentrations of ALA pertained to cytochrome c. Even though a greatly diminished concentration of ALA was used it should be noted that the effect on cytochrome c was still very desirable. With respect to toxicity, no human trials have been performed to establish baseline toxic dose concentrations of ALA (so all the data on human toxicity has been established through case studies, case reports and clinical experience (Orsucci et al., 2009). This can be worrisome especially for a doctor who has never used ALA before in clinical practice. Overall, even though there are a few misfortunes with ALA the vast majority of the research is very positive. However, it would be of great importance and benefit to the medical community to have a comparative analysis of ALA versus standard chemotherapy in the treatment of hepatocellular carcinoma.

The optimal goal of cancer therapy is to cure and promote health in the patient with as little side effects as possible. Therefore, ALA should be considered early on as a cancer therapy option as it can be used with conventional cancer approaches safely. By doing so, the therapeutic and negative side effects of conventional cancer therapy may be enhanced and decreased, respectively.

In conclusion, ALA is a very potent antioxidant capable of treating hepatocellular cancer at any stage. It is shown to be very efficacious in regenerating GSH and other potent antioxidants. It can cause apoptosis in hepatic tumor cells by several well-outlined mechanisms and reduce inflammation associated with cancer.
Negative side effects of ALA are few with the most important being an overdose leading to apoptosis of healthy liver tissue.
References


inflammatory cell responses in a two-stage hepatocarcinogenesis model in rats. *Chemico-Biological Interactions, 205(2),* 108-118.


