Punicic Acid and Ferroptotic Cell

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Plant-derived conjugated linolenic acids (CLnA) have been widely studied for their preventive and therapeutic properties against diverse diseases such as cancer. In particular, punicic acid (PunA), a conjugated linolenic acid isomer (C18:3 c9t11c13) present at up to 83% in pomegranate seed oil, has been shown to exert anti-cancer effects, although the mechanism behind its cytotoxicity remains unclear. Ferroptosis, a cell death triggered by an overwhelming accumulation of lipid peroxides, has recently arisen as a potential mechanism underlying CLnA cytotoxicity. In the present study, we show that PunA is highly cytotoxic to HCT-116 colorectal and FaDu hypopharyngeal carcinoma cells grown either in monolayers or as three-dimensional spheroids. Moreover, our data indicate that PunA triggers ferroptosis in carcinoma cells. It induces significant lipid peroxidation and its effects are prevented by the addition of ferroptosis inhibitors. A combination with docosahexaenoic acid (DHA), a known polyunsaturated fatty acid with anticancer properties, synergistically increases PunA cytotoxicity. Our findings highlight the potential of using PunA as a ferroptosis-sensitizing phytochemical for the prevention and treatment of cancer.

Keywords: conjugated linolenic ; acids ; punicic acid ; carcinoma cells ; ferroptosis ; docosahexaenoic acid ; spheroids ; lipid peroxidation

1. Introduction

For centuries, plant-derived lipids have been used for their therapeutic properties against various diseases [1][2]. Fatty acids are the building blocks of natural lipids and represent a large class of compounds that are diverse in composition. They act as a source of energy as well as structural and functional components of cells [1]. Saturated fatty acids have no double bond while unsaturated fatty acids present at least one double bond in their carbon chain [3]. Polyunsaturated fatty acids (PUFAs) are classified according to the number and position of double bonds in the carbon chain. Most PUFAs present double bonds separated by a methylene (-CH_2-) group. In contrast, some PUFAs display double bonds that are not interrupted by a methylene group and are known as conjugated fatty acids [1][4]. Conjugated linoleic acids (CLAs) have two conjugated double bonds while conjugated linolenic acids (CLnAs) have three double bonds of which at least two are conjugated [5]. The three double bonds of plant-derived CLnAs are in a conjugated configuration. In contrast to CLAs that are particularly found in dairy products, CLnAs are mainly contained in diverse plant seed oils, such as pomegranate seed oil rich in punicic acid (PunA, C18:3 c9t11c13) and tung, bitter gourd or ricinodendron seed oil rich in α-eleostearic acid (α-ESA, C18:3 c9t11t13) [6][7][8].

Among the conjugated fatty acids, CLAs have been the most extensively studied for their beneficial effects on human health. These include anti-obesity, anti-atherogenic, anti-diabetic, anti-carcinogenic and immunomodulatory properties [7][9][10][11][12][13]. However, the interest towards CLnAs has significantly increased over the last two decades, partially due to their high content in some seed oils, suggesting that CLnAs may be more available for preventive or even therapeutic purposes than previously expected [2]. In fact, CLnAs have been shown to possess anti-inflammatory [14][15][16], anti-obesity [14][17][18], anti-diabetic [19][20] and anti-cancer activities [1][2][21][22][23]. Specifically, PunA has been reported to exert a strong anti-cancer activity, both in vitro [7][24][25][26] and in vivo [4][27][28]. PunA anti-cancer activity is thought to be associated with lipid peroxidation [7]. In fact, CLnAs are more susceptible to autoxidation than their non-conjugated counterpart, namely α-linolenic acid (C18:3 c9c12c15), due to the ease of free radical formation by the quick electron delocalization at the level of the conjugated double bonds [29]. However, the exact mechanisms behind PunA cytotoxicity towards cancer cells remain poorly understood.

Ferroptosis is a form of iron-catalyzed regulated cell death that is morphologically, biochemically and genetically distinct from other regulated cell deaths, such as apoptosis and necroptosis [30][31]. Ferroptotic cell death is characterized by the overwhelming accumulation of lipid hydroperoxides, a form of reactive oxygen species (ROS) generated through oxidation of PUFAs [32][33][34][35]. This mode of cell death is executed through PUFA-containing phospholipid peroxidation as well as the presence of redox-active iron and a defective lipid peroxide repair (i.e., deficiency in glutathione and impairment of

glutathione peroxidase 4) [36]. More recently, other key ferroptosis suppressors have been identified, such as the ferroptosis suppressor protein 1, which regenerates the radical trapping-reduced form of ubiquinone and the Ca^{2+}-independent phospholipase $A_2\beta$, which hydrolyses lipid hydroperoxides from cell membranes [37][38]. Mechanistically, the formation of lipid hydroperoxides requires di-oxygenation of lipid double bonds, which occurs either spontaneously by autoxidation or through enzyme-catalyzed processes controlled by lipoxygenases [39][40][41] and oxidases [42]. Accumulation of lipid hydroperoxides eventually leads to the disruption of cell membranes, production of reactive aldehydes and finally cell death.

In the last decade, multiple drugs impacting the activity of various enzymes or transporters have been identified as ferroptosis inducers in cancer cells [30][43][44][45]. Another strategy, as yet largely unexplored for the induction of ferroptosis, may lie in the promotion of lipid hydroperoxide production by preferentially introducing high amounts of peroxidable PUFAs in cancer cells. In fact, several lines of research suggest that a wide range of PUFAs might sensitize cancer cells to ferroptosis by causing a dramatic accumulation of phospholipid-derived peroxides [37][46][47][48]. Recently, ferroptosis has appeared as a potential cell death pathway underlying CLnA cytotoxicity [49]. However, more work is warranted to further dissect the potential pro-ferroptotic effects of PunA. This is even more necessary as PunA, unlike other CLnAs, is a readily available phytochemical. It is indeed present in large amounts in pomegranate seed oil [6], the only CLnA-rich oil widely recognized as edible on the market [14].

Our data indicate that PunA induces ferroptosis in carcinoma cells by triggering an intense lipid peroxidation, a phenomenon that is prevented by ferroptosis inhibitors. In addition, a combination of PunA with docosahexaenoic acid (DHA, C22:6 c4c7c10c13c16c19), another PUFA recently shown to induce ferroptosis under acidosis [50], increases its effect in a synergistic manner. These findings suggest that PunA, possibly in combination with DHA, could be used as an anti-cancer agent.

2. Punicic acid as a ferroptosis-sensitizing agent

In the present work, we show that PunA is cytotoxic for hypopharyngeal (FaDu) and colorectal (HCT-116) carcinoma cells in vitro, either grown in monolayers or as three-dimensional spheroids. At micromolar doses, only PunA was cytotoxic to carcinoma cells, with a dramatic loss of viability in both HCT-116 and FaDu cells, whereas DHA, a known omega-3 PUFA, has no cytotoxic impact and even increased HCT-116 cell viability. We found that the viability of HCT-116 and FaDu carcinoma cells exposed to both DHA at 100 µM and PunA at a sub-lethal dose of 7 µM for 72 h was more greatly reduced than upon treatment with PunA alone. The combination of PunA with DHA also significantly decreased spheroid growth (vs. single fatty acid treatments), suggesting that PunA and DHA act in a supraadditive way to impact on carcinoma cell viability. Next, we evaluated the impact of two ferroptosis inhibitors, namely ferro- statin-1 (fer-1) and α-tocopherol (α-T), on the cytotoxicity of PunA on HCT-116 and FaDu carcinoma cells. PunA cyto- toxicity on HCT-116 and FaDu carcinoma cells was inhibited by the addition of fer-1 and α-T, as well as by the iron chelator deferoxamine mesylate (DFOM). On the contrary, neither the apoptosis inhibitor ZVAD-fmk nor the necroptosis inhibitor necrostatin-1 prevented PunA cytotoxicity on HCT-116 and FaDu carcinoma cells, further supporting ferroptosis as the cell death pathway triggered upon PunA exposure. Both α-T and fer-1 also strongly inhibited the cytotoxicity of PunA on HCT-116 and FaDu spheroids. As ferroptosis execution is characterized by an abundant accumulation of lipid peroxide species, we investigated whether PunA treatment triggers an increase in lipid peroxidation in carcinoma cells. We used the C11-BODIPY assay, which measures the ability of cells to peroxidize the double bonds of this BODIPY probe, as well as the MDA assay, which measures the secondary products resulting from intracellular lipid peroxidation. We showed that PunA triggers lipid peroxidation in carcinoma cells in a manner and a time course that are consistent with the induction of ferroptosis.

3. Development and Findings

Despite great advances in treatment, cancer remains the second cause of mortality worldwide, with the majority of cancer deaths caused by carcinoma [51]. Many anti-cancer drugs aim at triggering apoptosis as a strategy to eliminate cancer cells. However, the effectiveness of these drugs is limited by the tendency of cancer cells to acquire resistance to apoptosis [52]. In this regard, exploiting other types of cell death mechanisms such as ferroptosis opens up new therapeutic avenues. PunA, a CLnA isomer, is cytotoxic to different carcinoma cell lines by triggering intracellular lipid peroxidation and these effects are completely prevented in the presence of ferroptosis inhibitors. Neither apoptosis nor necroptosis inhibitors blocked PunA cytotoxicity, further supporting the ability of PunA to specifically trigger ferroptosis in cancer cells. Ferroptotic effects of PunA were observed from low concentrations, both on carcinoma cells grown as monolayers and on cells organized as three-dimensional spheroids.

Our results indicate that DHA and PunA may work synergistically to induce cell death in carcinoma cells. To our knowledge, this is the first study suggesting synergies between PUFA and CLnA cytotoxicities on cancer cells and spheroids. Even though in vivo evidence of their synergistic mechanism should be provided, such a combination of PUFAs and CLnAs may be an interesting therapeutic option to increase their respective effects in cancer patients.

As indicated by our results as well as by other in vitro and in vivo studies [1][49], CLnAs are phytochemicals with promising anti-cancer effects that could be exploited as preventive or even therapeutic agents. CLnAs are mainly found in seed oils of specific plants and the CLnA content can reach up to 80% of the total lipids [5], making CLnAs accessible nutritional phytochemicals. However, CLnA-rich seed oils remain too little known and available on the market to be used on a large scale as anti-cancer agents. Only pomegranate seed oil is currently widely recognized as an edible oil on the market [14], making PunA the most relevant CLnAs to be further investigated as a potential anti-cancer agent. A strategy to make these phytochemicals more available could be to include CLnAs in frequently consumed food items. A different approach would be the production of enriched food supplements that could be taken up by cancer patients. However, as an oral intake of PunA would mainly lead to its incorporation into triglycerides as part of complex lipids (i.e., chylomicrons and lipoproteins), whether cancer cells are able to take up PunA in the form of triglycerides should be carefully investigated. Such enriched food products and supplements open up new opportunities to promote the use of CLnAs as health beneficial phytochemicals in the context of cancer prevention and treatment. Further studies are, however, needed to assess whether PunA-induced lipid peroxidation may cause adverse effects on the long term.

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