



Evaluation of cytotoxic and anticytotoxic properties of apocynin

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Abstract

Toxic substances present in food, environment and in several drugs can cause DNA damage, chromosomal aberrations and alteration of genetic material leading to cytotoxicity and cancer. Natural compounds can be employed to support innate detoxification systems and protect cellular components. Oxidative stress is a prominent adverse effect of cytotoxic substances. Apocynin, a plant compound, is an inhibitor of NADPH oxidase complex, and thus, a strong antioxidant. In the current study, the cytotoxic/anticytotoxic activity of Apocynin was evaluated in a normal cell line CHOk1 and Cancer cell line HepG2, using the chemotherapy drug cisplatin as the cytotoxic agent. Apocynin did not exhibit cytotoxicity towards either of the cell lines, nor has a synergistic effect with cisplatin. On the other hand, Apocynin shows significant anticytotoxicity as evidenced by the lowering of cisplatin-induced cell inhibition in both the cell lines.

Keywords: acetovanillone, cell proliferation, cell inhibition, antioxidants, cisplatin, CHOk1, HepG2

Introduction

Several chemicals present in the food, environment and those used in medical treatment can alter the genetic material leading to different forms of cancer [1]. Toxicity of substances is manifested in various forms like DNA damage, chromosomal aberrations, formation of micronuclei and cell death. Innate detoxification systems are present in living organisms to counteract the effects of toxic chemicals. Several plant compounds can support the innate detoxification systems and protect the cellular components from the adverse effects of DNA damaging chemicals [2]. The contribution of plant compounds in prevention and treatment of human diseases is vast and has been in practice since centuries, across different systems of traditional medicines [3]. Plants used in traditional medicines have been valuable leads for therapeutic drug development in modern medicine [4].

Research and development of new anticancer agents which exhibit efficient and selective toxicity to tumour cells is enticing increased attention. Chemotherapeutic drugs exert toxicity to normal cells also, which causes unpleasant side effects to the patients. One of the mechanisms by which the toxicity is caused is oxidative stress. When excessive amounts of Reactive oxygen species (ROS) are produced, a disturbance in the pro-oxidant/antioxidant balance in favor of the prooxidant state may occur, which may lead to cell damage [5]. ROS affect cell function by directly acting on cell components, including lipids, protein, and DNA, and destroying their structure [6]. Use of Natural antioxidants can help in counteracting these adverse effects. Natural compounds have also been shown to possess anti-proliferative activities in various cancer cells without exhibiting

considerable damage to normal cells [7-9].

Apocynin (IUPAC name: 1-(4-Hydroxy-3-methoxyphenyl) ethanone-4-hydroxy-3-methoxyacetophenone or acetovanillone) a pharmacologically active plant phenol was identified as the biologically active substance in the roots of *Picrorhiza kurroa* Royle ex Benth, a perennial plant growing in the alpine Himalayas. Extracts from the roots are used in the Ayurvedic medical tradition of India and Sri Lanka, for treatment of ailments of liver, heart, joints, and lungs [10]. Since its characterization as a NADPH Oxidase assembly inhibitor in 1994 [11], apocynin has been widely studied in experimental models of disease in which the involvement of ROS is well-established; for instance, in vascular, inflammatory and neurodegenerative pathologies. The clinical applicability of apocynin as an anti-inflammatory drug has been studied by several groups, which showed that it efficiently decreased symptoms of rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, neurodegenerative pathologies like Parkinson's disease and asthma [12-14].

The current study was taken up to study the cytotoxic / anticytotoxic ability of apocynin, and its potential use as adjuvant therapy to counteract the cytotoxic action of chemotherapeutic agents on normal cells. Our earlier studies had shown apocynin to be a potential antigenotoxic agent in reducing micronuclei formation in mice bone marrow cells and chromosomal aberrations in onion root tip meristems [15]. The cytotoxic/anticytotoxic properties of apocynin against cisplatin were studied using the MTT tetrazolium assay technology that has been widely adopted for assaying cell viability [16].

Materials and Methods

Materials Required: MTT Powder (Sigma chemicals -5 mg/10 ml of MTT in 1X PBS - the solution is filtered through a 0.2 μ m filter and stored at 2–8 °C for frequent use or frozen for extended periods), DMSO (Sigma chemicals), DMEM (Dulbecco’s Modified Eagle’s Medium), Apocynin (purchased from Natural Remedies Pvt. Ltd., Bangalore.-purity >95% by HPLC), Cisplatin (Uniplatin, United Biotech (P) Ltd), CO₂ incubator, Tecan Plate reader, Centrifuge.

Cell Lines: Hepatocarcinoma cell line HepG2 and normal cell line CHOk1 procured from ATCC were maintained and cultured for the experimental procedures.

Procedure: The cell lines HepG2 and CHOk1 are collected when they reach about 70-80% confluence. They are checked for the viability and centrifuged. About 10,000 cells per well were seeded in a 96 well plate and incubated for 24 hrs. at 37°C, 5 % CO₂ incubator. For determination of IC₅₀ value, the samples to be tested, apocynin and cisplatin, individually are added from 0 – 120 μM/ml concentration in DMEM without FBS and are incubated for 24 hrs. After incubation with test samples, 100 μl/well (50 μg /well) of the MTT solution was added to the wells and incubated for 3 to 4 hours. After incubation, the MTT reagent was discarded by removing the media with a pipette without disturbing cells and 100 μl of DMSO was added to each well to rapidly solubilize the formazan. The absorbance, at 590 nm was measured using a Tecan plate reader. For studying the cytotoxic/anticytotoxic effect of apocynin, the assay was performed by treating the cells with different combinations of apocynin and cisplatin as mentioned in Table No.2. All treatments were done in

triplicates.

$$\% \text{ Inhibition} = 100 - (\text{OD of sample} / \text{OD of control}) \times 100$$

Results

Apocynin showed very little cytotoxicity on both the cell lines CHOk1 and HepG2 at all the tested concentrations. Cisplatin showed significant toxicity on the cancer cells HepG2 as well as CHOk1. The IC₅₀ value of cisplatin was calculated using a dose response curve (Table No.1). The concentration of 30μM cisplatin was chosen for further studies. The effect of presence of apocynin on cisplatin induced cell growth inhibition was studied by treating the cells with three different proportions of apocynin and cisplatin simultaneously followed by MTT (Table No.2). All the combination treatments showed a significant inhibition of cell growth as compared to the control which was considered as zero inhibition. The results show that apocynin lowered the inhibition caused by cisplatin very significantly in both CHOk1 and HepG2 cells. In both cases, the presence of apocynin, even at a concentration lower than cisplatin also quite significantly lowers the inhibition of cell growth as compared to cisplatin alone, although it is less than the protection by higher concentrations of apocynin. A combination of Apo: Cis in 1:1 proportion showed the highest protection of both the cultures. In case of CHOk1, the difference in protection offered by different proportions of apocynin against the same concentration of cisplatin is significant, where as in case of HepG2, apocynin did not show a dose dependent relation with protection against cisplatin induced cytotoxicity.

Table 1: Cell proliferation studies - Apocynin on CHOk1 and HepG2 cells and Cisplatin on CHOk1 and HepG2 cells (10,000cells/well)

| | Conc. μM of Apocynin | OD at 590 nm | % Inhibition | Conc. μM of cisplatin | OD at 590 nm | % Inhibition | IC50 μM of cisplatin |
|-------|----------------------|--------------|--------------|-----------------------|--------------|--------------|----------------------|
| CHOk1 | Control | 0.487 | 0.00 | Control | 0.473 | 0.00 | 15.23 |
| | 1.87 | 0.480 | 1.44 | 1.87 | 0.434 | 8.25 | |
| | 3.75 | 0.453 | 6.98 | 3.75 | 0.400 | 15.43 | |
| | 7.5 | 0.445 | 8.62 | 7.5 | 0.356 | 24.74 | |
| | 15 | 0.421 | 13.55 | 15 | 0.321 | 32.14 | |
| | 30 | 0.408 | 16.22 | 30 | 0.265 | 43.97 | |
| | 60 | 0.402 | 17.45 | 60 | 0.213 | 54.97 | |
| | 120 | 0.399 | 18.07 | 120 | 0.198 | 58.14 | |
| HepG2 | Control | 0.499 | 0.00 | Control | 0.488 | 0.00 | 33.65 |
| | 1.87 | 0.488 | 2.20 | 1.87 | 0.456 | 6.56 | |
| | 3.75 | 0.465 | 6.81 | 3.75 | 0.423 | 13.32 | |
| | 7.5 | 0.455 | 8.82 | 7.5 | 0.399 | 18.24 | |
| | 15 | 0.432 | 13.43 | 15 | 0.367 | 24.80 | |
| | 30 | 0.417 | 16.43 | 30 | 0.289 | 40.78 | |
| | 60 | 0.401 | 19.64 | 60 | 0.211 | 56.76 | |
| | 120 | 0.389 | 22.04 | 120 | 0.178 | 63.52 | |

Table 2: % Inhibition observed in CHOk1 and HepG2 cells on treatment with different combinations of apocynin and cisplatin

| | Treatment | OD value at 590nm | % inhibition | | Treatment | OD value at 590nm | % inhibition |
|-------|--------------------|-------------------|--------------|--|--------------------|-------------------|--------------|
| CHOk1 | Control | 0.453 | 0 | | control | 0.467 | 0 |
| | | 0.443 | 0 | | | 0.489 | 0 |
| | | 0.463 | 0 | | | 0.476 | 0 |
| | Cisplatin alone | 0.243 | 46.35 | | Cisplatin alone | 0.257 | 44.97 |
| | | 0.221 | 50.11 | | | 0.224 | 54.19 |
| | | 0.236 | 49.03 | | | 0.261 | 45.16 |
| | Apo:Cis 1:1 (30μM) | 0.367 | 18.98 | | Apo:Cis 1:1 (30μM) | 0.356 | 23.77 |
| | | 0.389 | 12.18 | | | 0.377 | 22.90 |

| | | | | | | | | |
|-------------------|--|-------|-------|-------------------|-------------------|-------|-------|-------|
| | | 0.377 | 18.57 | | | 0.356 | 25.21 | |
| Apo:Cis 1:2(30μM) | | 0.267 | 41.06 | HepG2 | Apo:Cis 1:2(30μM) | | 0.308 | 34.05 |
| | | 0.278 | 37.25 | | | | 0.322 | 34.15 |
| | | 0.298 | 35.64 | | | | 0.311 | 34.66 |
| | | 0.311 | 31.35 | | | | 0.367 | 21.41 |
| Apo:Cis 2:1(30μM) | | 0.322 | 27.31 | Apo:Cis 2:1(30μM) | | 0.378 | 22.70 | |
| | | 0.333 | 28.07 | | | 0.368 | 22.70 | |
| | | | | | | | | |

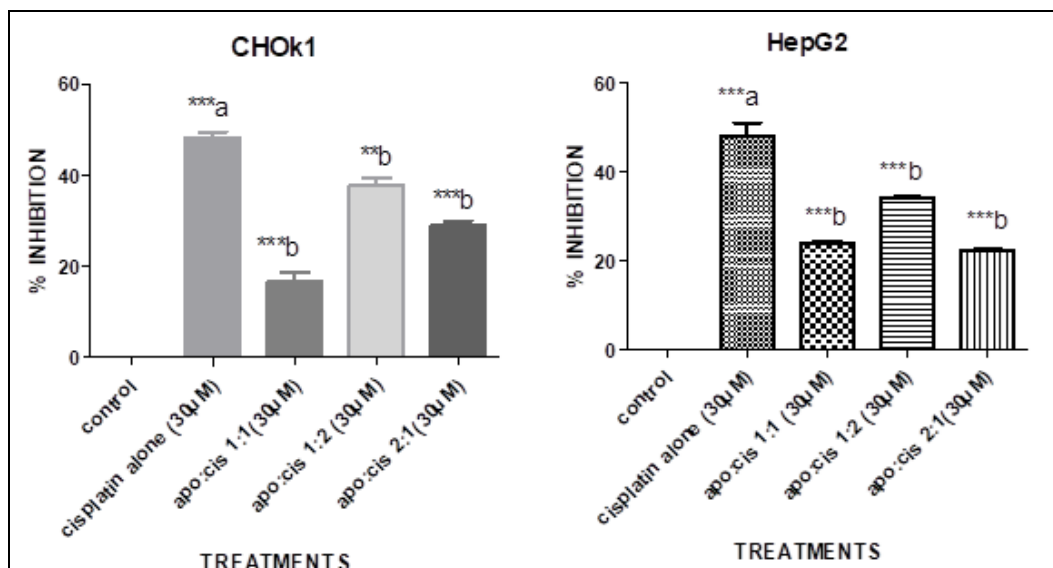


Fig 1: Treatment vary significant at $P < 0.05$ a- in comparison with control; b- in comparison with cisplatin alone

Discussion

Plant compounds are being evaluated for their antiproliferative effects, alone as well as in combination with chemotherapeutic agents. Natural substances play important roles in multiple mechanisms, which may be responsible for their anticarcinogenic effects. Antioxidant activity, iron chelating activities as well as inhibition of bio activating enzymes and induction of detoxifying enzymes may provide protection against cancer initiation [17-20]. Natural compounds have also shown protective effects against cytotoxicity induced by toxic substances [21, 22]. Extracts of *Ficus deltooides* inhibited growth of human ovarian cancer cells *in vitro* [23]. *E. guineensis* extract was shown to have potent cytotoxic activity against MCF7 cells [24]. Research and development of new classes of anticancer agents which exhibit efficient and selective toxicity in tumor cells is enticing increased attention. Mangiferin in combination with low non cytotoxic concentrations of cisplatin and 5-fluorouracil increased the cytotoxicity of these chemotherapeutic agents in mouse colon carcinoma cell lines without significant increase of cell death in CHO-K1 cells under the same experimental conditions [25]. *Solanum nigrum* methanolic extract showed a greater inhibitory activity on proliferation of *HeLa* cell line and little activity on *Vero* cell line [26]. Marine yeast may be a potential chemotherapeutic or a chemo preventive agent based on its ability to induce apoptosis in cancer cells with relatively low toxicity to normal cells using a vero cell line and two tumor cell lines of human hepatocarcinoma HepG2 and human breast carcinoma cell line MCF-7 [9].

There is increasing support to consider using apocynin as a therapeutic agent for treatment of inflammatory diseases. The

property of apocynin as an inhibitor of NADPH-oxidase is a key aspect in exploring its wider applications [12]. It has been shown to have a protective effect against antimycinA induced cell damage by its antioxidant effects and attenuation of mitochondrial dysfunction in osteoblastic cells [27]. It ameliorated diabetes-related erectile dysfunction by reducing ROS production in experimental models of diabetic rats [28] and also prevented microglial activation induced by oligomeric amyloid- β [29]. Diapocynin exhibits profound neuroprotective effects in a pre-clinical animal model of Parkinson's disease by attenuating oxidative damage and neuroinflammatory responses [14]. Apocynin is used as an inhibitor of the multienzymatic NADPH oxidase complex and concomitant reactive oxygen species production in experimental models involving phagocytic cells [11, 30, 31]. In this context it is essential to study and understand the cytotoxic and anticytotoxic properties of apocynin to know its potential as an agent for adjuvant therapy. An agent which shows selective inhibition of cancer cells or which shows selective protection of normal cells from the adverse effects of a chemotherapeutic drug without lowering the toxicity of the drug towards the cancer cells is a suitable candidate for use as an adjuvant in chemotherapy procedures.

Cisplatin [Cis-diamminedichloroplatinum (II)] is a chemotherapeutic drug used in the treatment of solid organ tumors of the head, neck, ovary and breast [32]. Cisplatin induced cell death is mediated by mechanisms involving mitochondrial dysfunction, oxidative stress and activation of inflammatory pathways [33]. Treatment with cisplatin and its analogs is often hindered by side effects, including nephrotoxicity, peripheral neurotoxicity, and ototoxicity [34].

Renal side effects include reduction of antioxidant plasma levels and generation of free radicals in normal cells [35]. Cisplatin's side effects are due to various mechanisms like DNA damage [36] and increase the intracellular production of ROS via NADPH oxidase [37, 38]. Several studies have shown that the administration of antioxidants can reduce the side effects associated with cisplatin [39-41]. Root extracts of *B.diffusa* could reduce the nephrotoxicity caused by cisplatin by attenuating ROS production [42]. Bixin, a carotenoid, was shown to function as a selective protective agent, reducing cisplatin-induced DNA damage in PC12 cells [43]. Wang *et al.* reported that acetovanillone (apocynin) attenuated cisplatin induced markers of kidney damage such as oxidative stress, cell death, inflammatory cytokine production and nephrotoxicity but also enhanced cancer cell killing efficacy of cisplatin [44].

Our current study reveals that apocynin does not show an inherent cytotoxicity towards either of the tested cell lines CHOK1 and HepG2. It also did not exhibit a synergistic effect with the cytotoxicity of cisplatin. Apocynin counteracted the cytotoxicity of cisplatin, which can be attributed to its strong ability to inhibit NADPH oxidase, thereby indicating its potential as an anticytotoxic agent. Our results also indicate that apocynin can ameliorate the cytotoxic damage caused by cisplatin in general but is not selective towards protection of non-tumor cells as it decreased the cytotoxicity of cisplatin towards the tumor cells HepG2 also. Further studies with different cytotoxic agents and cell lines could give us insights into the possible use of apocynin as an adjuvant in chemotherapeutic strategies.

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