Original Article

The Triad Indole-3-Acetic Acid Ethyl Ester/Esterase/Horseradish Peroxidase as a New Cytotoxic Prodrug/Enzyme Combination

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Abstract

The antibody-directed enzyme prodrug therapy (ADEPT) is a means of restricting the action of toxic drugs to the tumor site. The enzyme/prodrug pair horseradish peroxidase (HRP)/indole-3-acetic acid (IAA) has been studied as a combination with potential application in ADEPT strategies. In this combination, the non-toxic plant hormone IAA is activated to cytotoxic species by the catalytic action of HRP. **Objective:** We studied the use of the ethyl ester of IAA as a new prodrug that could be activated by two enzymes, HRP and esterase. **Methods:** The oxidation of IAA and its ethyl ester, catalyzed by HRP, was monitored by the consumption of dioxygen and liquid chromatography. The cytotoxicity of IAA and its ethyl ester in combination with HRP and esterase was assessed using the lineage McCoy cells through the trypan blue and neutral red assays. **Results**: We found that HRP was not able to catalyze the oxidation of IAA-ethyl ester in the absence of an additional esterase. Hence, the potential cytotoxicity of the IAA-ethyl ester could be controlled by sequential treatment with esterase, to liberate the carboxyl group, and HRP, for oxidation and generation of cytotoxic species. We present evidence for the potential application of the combination IAA-ethyl ester/esterase/horseradish peroxidase as a new ADEPT, GDEPT or related strategy. **Conclusions**: We suggest that this technique could provide more selectivity in the generation of cytotoxic drugs at tumor sites.

Keywords: Indole-3-acetic acid synthase; Indole-3-acetic acid ethyl ester; horseradish peroxidase, esterases

Introduction

Anticancer drugs are characterized by a narrow therapeutic window that results mainly from their high systemic toxicity combined with an evident lack of tumor selectivity.[1] Hence, the strategy known as antibody–directed enzyme prodrug therapy (ADEPT) is adopted as a means of restricting the action of toxic drugs to the tumor site. In ADEPT, an enzyme is linked to an antibody that targets the tumor cells. After administration of this conjugate and clearance of the unbound circulating antibody, a nontoxic prodrug, a substrate for the enzyme, is then given. By cleaving an inactivating component from the prodrug, the enzyme generates a potent cytotoxic agent on the surface of the tumor cells.[2-6] The ideal drugs for ADEPT are small molecules that can diffuse into the tumor tissues and cause the cytotoxic effect. Additionally, the enzyme system should not have a human homologue, to avoid prodrug activation away from the tumor site.[7] Some recent developments in this area include (enzyme/prodrug): β–glucosidase/glycoside of toxoflavin;[8] β–galactosidade/β–galactoside of suberoylanilide hydroxamic acid;[9] carboxypeptidase/diethylstilbestrol-glutamate;[10] β–D–glucuronidase/glucuronide paclitaxel;[11] β–galactosidase /carbohydrate-geldanamycin conjugates.[12]

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In 1998, Folkes et al.[13] proposed the pair horseradish peroxidase (HRP)/ indole-3-acetic acid (IAA) as an enzyme/prodrug combination for potential application in ADEPT.[13] This combination meets the criteria for the ADEPT strategy, since HRP is a plant enzyme and IAA, a plant hormone, is nontoxic for humans. In fact, there are many mammalian endogenous peroxidases; however, HRP is the only one able to catalyze the oxidation of IAA in the absence of hydrogen peroxide, which is crucial for its potential use in ADEPT. Under the enzymatic action of HRP, IAA is oxidized to produce cytotoxic radical and non-radical species. Following this seminal work, many papers have presented and discussed the mechanism of cytotoxicity of HRP/IAA in several tumor cell lines. For instance, 3-methylene-2-oxindole has been identified as a cytotoxic species generated during the oxidation of IAA, which reacts with endogenous nucleophiles such as glutathione, cysteine, and a cysteinyl peptide.[14] The successful use of antioxidants, to block the apoptosis of human melanoma cells, induced by IAA/HRP, is clear evidence that free radicals play an important role in the mechanism of cytotoxicity.[15] The finding that catalase prevents IAA/HRP-induced apoptosis of G361 human melanoma cells indicates that hydrogen peroxide may also be involved in the mechanism of action.[16] IAA has also been used in experimental photodynamic therapy for liver cancer cell in combination with intense pulsed light;[17] the combination of radiotherapy with the plasmid phTERTp-HRP (human telomerase reverse transcriptase promoter), in which expression of enzyme HRP is controlled by hTERTp, resulted in increased apoptosis and necrosis of tumor cells after administration of IAA and may overcome radioresistance in laryngeal squamous carcinomas cells;[18] IAA also presents protective effect on diethylnitrosamine-induced hepatocarcinogenesis in mice.[19]

Considering the high specificity of HRP towards indole-3-acetic acid, we hypothesized that its ethyl ester (IAA-ethyl ester) might not be recognized by the enzyme, so that, we could link the generation of cytotoxic species to two enzymes. Here, we present evidence of the potential combined use of indole-3-acetic acid ethyl ester, esterase and horseradish peroxidase in a new strategy for ADEPT or related techniques.

Materials and Methods

Chemicals and Cell Culture

Indole-3-acetic acid, indole-3-acetic acid ethyl ester, melatonin, ascorbic acid, neutral red, esterase from porcine liver (ammonium sulfate suspension, 160unit/mL) and HRP (Type VI) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trypan blue was purchased from Vetec (RJ, Brazil). McCoy mouse fibroblast cells (ATCC CRL 1696) were obtained from the Cell Culture Section of the Instituto Adolfo Lutz (SP, Brazil). This continuous cell line was used to assess the *in vitro* basal toxicity. A 5% trypan blue solution was prepared in 0.9% NaCl. The neutral red stock solution was 4 mg/mL in PBS and a working solution was prepared by diluting this stock solution to 50 μg/mL in Eagle's medium.

Trypan Blue Assay

McCoy cells were assessed for viability by the trypan blue exclusion assay, in which living cells with intact cell membranes do not take up the dye, while nonviable cells are unable to exclude the dye and stain dark blue. The cells (1×10^6/mL) were incubated in Dulbecco's phosphate-buffered saline (PBS-D) in various combinations of the test compounds, HRP (1 μM) and esterase (5 units/mL) for 1 hour, at 37°C. These cell suspensions were then mixed (1:1) with trypan blue solution and incubated for five minutes. Cells were observed under a microscope and stained and unstained cells were counted in a hemocytometer and the percentage of dead cells calculated.[20]

Neutral Red Assay

The McCoy cells were maintained in Eagle's medium (Instituto Adolfo Lutz, SP, Brazil) with 7.5% fetal bovine serum. After trypsinization, 0.2 mL aliquots of medium containing approximately 10^4 cells/mL were seeded into 96-well tissue-culture plates and incubated at 37 °C. After 24 h, the Eagle's medium was removed and the cells were covered with unmodified medium (control) or a modified medium with various concentrations of test compounds and enzymes (1 μM HRP and 5 units/mL esterase). After incubating for another 24 h, the medium was removed and the plates were prepared for microculture neutral red assay.[21] After a brief agitation, the plates were transferred to a microplate reader (Spectra and Rainbow (Shell) Readers − Tecan, Austria) and the optical density of each well was measured, using a 540 nm filter and 620 nm reference wavelength. All experiments were performed at least four times.

Oxygen Uptake Study

The reaction mixtures consisting of 1 mM IAA, 1 mM IAA-ethyl ester, 0.5 µM HRP, and various concentration of esterase in 0.05 M phosphate buffer pH 5.5 were monitored with a Clark-type oxygen electrode (Yellow Spring Instruments 5300A) coupled to an X-Y recorder (EG & G, Princeton Applied Research). The reactions were performed at 37°C in a final volume of 3 mL and triggered by adding the enzymes.

High Performance Liquid Chromatographic (HPLC) Study

The reaction mixtures consisting of 1 mM IAA or 1 mM IAA-ethyl ester, 0.5 µM HRP and/or 3.0 units/mL esterase in 0.05 M phosphate buffer (pH 5.5) were incubated at 37°C, for 30 min. Next, an aliquot of 20 µL was injected into the HPLC system (Waters 2690 Separation Module in line with a Waters 996 UV-Vis Detector set at 254 nm). The separation was carried out isocratically on a Luna C18 reversed-phase column (250 x 4.6 mm, 5 µm), with a mobile phase of 75:25 water/ acetonitrile flowing at 1.0 mL/min).

Results

The oxidations of IAA and its ethyl ester, catalyzed by HRP, were monitored by the consumption of dioxygen dissolved in the reaction medium. It is well established that the mechanism for HRP-catalyzed oxidation of IAA, in the absence of H_2O_2, involves an oxidative decarboxylation, leading to a skatolyl radical[22-23]. Here, the oxidation studies were conducted in a slightly acid medium (pH 5.5), which is ideal for HRP-catalyzed oxidation of IAA.[22-24] The radical coupling between the skatolyl and dioxygen explains the fast oxygen uptake when IAA was mixed with HRP (Figure 1A).[13] The oxidations were also studied at the physiological pH 7.4. Although the reaction rate for oxidation was decreased, compared to that at pH 5.5, similar results were obtained at pH 7.4 (Fig. 1B).

The HRP was completely inactive when IAA was replaced by its ethyl ester. However, when an esterase was added to the reaction mixture, leading to hydrolysis of IAA-ethyl ester to the precursor IAA, the oxidation took its natural course. The assumption that esterase was catalyzing the hydrolysis of the IAA-ethyl ester to IAA was confirmed by HPLC. The chromatograms depicted
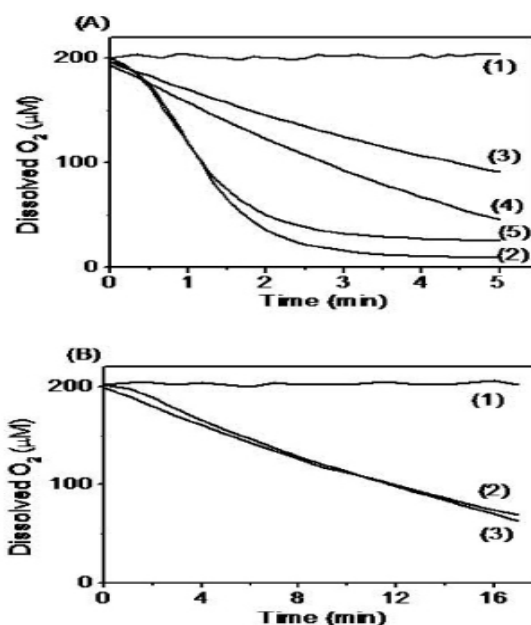

Figure 1- Dioxygen consumption provoked by the oxidation of IAA or IAA-ethyl ester catalyzed by HRP. Reaction conditions: when present, 1 mM IAA, 1 mM IAA-ethyl ester, 0.5 µM HRP and various concentrations of esterase in 0.05 M phosphate buffer at 37°C. The final volume was 3 mL. (A) Reactions at pH 5.5. Trace 1: IAA-ethyl ester and HRP. Trace 2: IAA and HRP. Trace 3: IAA-ethyl ester, 0.5 units/mL esterase and HRP. Trace 4: IAA-ethyl ester, 1.5 units/mL esterase and HRP. Trace 5: IAA-ethyl ester, 3.0 units/mL esterase and HRP. (B) Reactions at pH 7.4. Trace 1: IAA-ethyl ester and HRP. Trace 2: IAA and HRP. Trace 3: IAA-ethyl ester, HRP and 3.0 unit/mL esterase.

in Fig. 2 show the peak for IAA (trace A), the peak for IAA-ethyl ester (trace B), the peak for IAA-ethyl ester after hydrolysis (trace C) and the peaks obtained when the reaction mixture containing IAA-ethyl ester, esterase and HRP was injected (trace D).

The potential cytotoxicity of IAA and its ethyl ester in combination with HRP was assessed using the lineage McCoy cells. Using the Trypan Blue exclusion assay, which is based on the rupture of the cell membrane of dead cells, it was found that the combination HRP/ IAA is highly cytotoxic in this experimental model (Figure 3). The inset in Fig. 3 also shows that neither HRP nor IAA were cytotoxic on their own.

The mechanism of cytotoxicity of IAA/HRP is linked to the generation of oxidant species such as the peroxyl radical and hydrogen peroxide.[19,25] In corroboration of this, Figure 4 shows that antioxidant substances, such as melatonin and ascorbic acid, partially
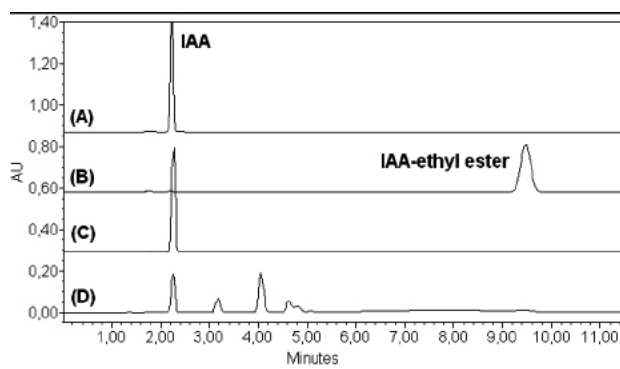
Figure 2– Chromatographic profile of IAA and IAA-ethyl ester. Reaction conditions: 1 mM IAA or 1 mM IAA-ethyl ester, 0.5 μM HRP and 3.0 units/mL esterase in 0.05 M phosphate buffer pH 5.5 at 37°C. The reaction mixture (20 μL) was analyzed 1 h from the start of the reaction. Trace A: Peak for IAA. Trace B: peak for IAA-ethyl ester. Trace C: peak for IAA-ethyl ester after hydrolysis catalyzed by esterase. Trace D: peaks obtained after oxidation of the hydrolyzed IAA-ethyl ester by HRP.

impeded the death of cells. When the reaction mixture was made anaerobic, by bubbling nitrogen during the course of the reaction, the cytotoxicity of IAA/HRP was significantly decreased (Figure 4). This is further evidence of the generation of peroxyl radicals. As expected, considering the lack of reactivity between HRP and the IAA-ethyl ester, this enzyme/prodrug combination was significantly less cytotoxic in the absence of the esterase (Figure 5).

Similar results were obtained using the neutral red assay, which is based on the uptake of neutral red in the lysosomes of living cells.[21] In this case, the cells were incubated for 24 h with the various combinations
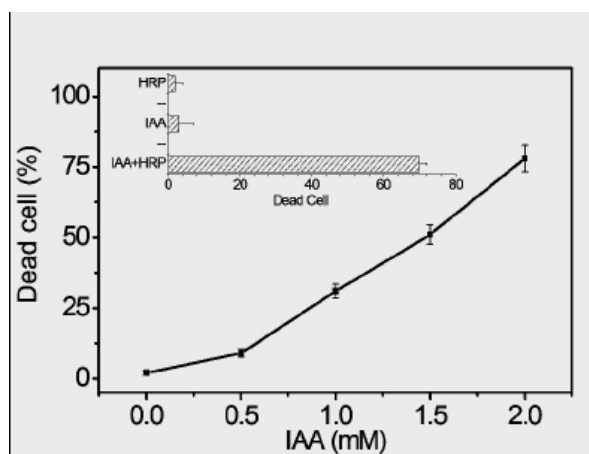

Figure 3- Cytotoxicity of IAA/HRP to McCoy cells assessed by trypan blue exclusion assay. Reaction conditions: McCoy cells (1 x 10⁶ cells/mL) and 1 μM HRP were incubated in PBS for 1 hour at 37°C. See methods for experimental details. The results are mean and SD of triplicates. (Inset) Controls with 2 mM IAA or 1μM HRP alone, compared to the complete system.
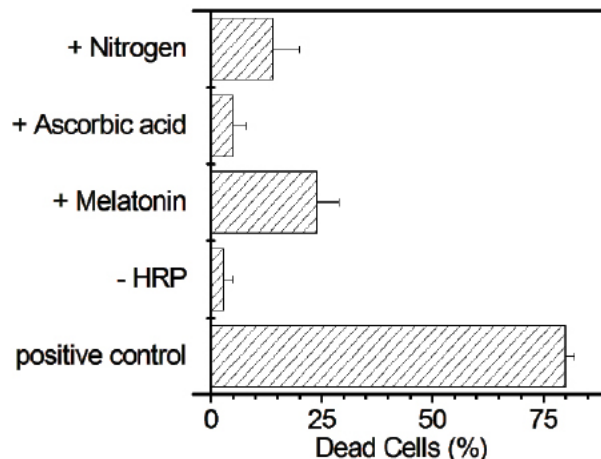

Figure 4 - Effect of antioxidants and absence of dioxygen on HRP/IAA cytotoxicity. Reaction conditions: McCoy cells (1 x 10⁶ cells/mL), 2 mM IAA, 1 μM HRP and, when present, 1 mM melatonin or 0.1 mM ascorbic acid were incubated in PBS for 1 hour at 37°C. See methods for experimental details. For anaerobic experiments, the reaction mixtures were purged with nitrogen. The results are mean and SD of triplicates
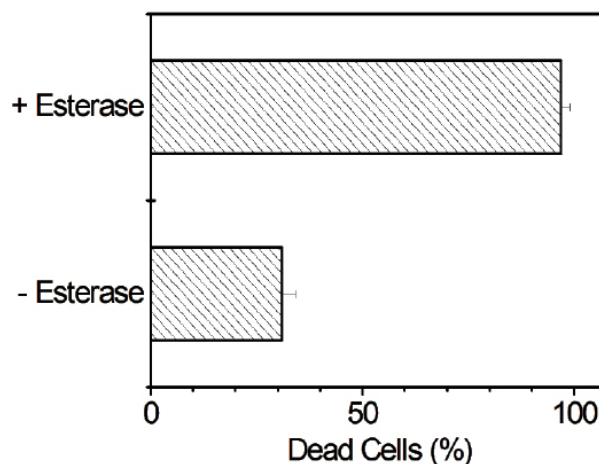

Figure 5 - Cytotoxicity of IAA-ethyl ester/esterase/HRP to McCoy cells assessed by trypan blue exclusion assay. Reaction conditions: McCoy cells (1 x 10⁶ cells/mL), 1 μM HRP, 2 mM IAA-ethyl ester and 5 units/mL esterase were incubated in PBS for 1 hour at 37°C. See methods for experimental details. The results are mean and SD of triplicates.

of IAA or IAA-ethyl ester and enzymes. Once again, a significant difference was observed between the IAA-ethyl ester alone and in combination with HRP and esterase. However, IAA-ethyl ester alone also exhibited some toxicity in this experimental model (Figure. 6). similar cytotoxic effects of IAA and IAA-ethyl ester in the absence of added esterase could be explained by a cellular esterase activity in the McCoy cells.
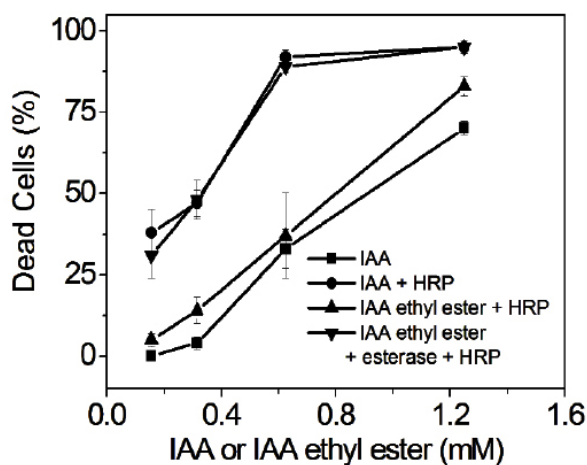
Figure 6 –Cytotoxicity of IAA/HRP or IAA ethyl ester/ esterase/HRP to McCoy cells assessed by neutral red assay. Reaction conditions: McCoy cells (1 x 10⁴ cells/mL), 1 μM HRP, 5 units/mL esterase. The results are mean and SD of triplicates.

Discussion

The restriction of the action of toxic drugs to tumor sites is the major concern in cancer chemotherapy. ADEPT and related strategies such as gene-directed enzyme/prodrug (GDEPT) and virus-directed enzyme prodrug (VDEPT) therapies are among the most widely studied techniques to target tumor cells.[26] IAA is a promising candidate in this area, since it has low toxicity before its oxidation, catalyzed by HRP.[13]

The specificity of HRP towards IAA, in the absence of H_2O_2, is really remarkable, since peroxidases are among the most unspecific enzymes. Indeed, HRP belongs to a class of enzymes that use hydrogen peroxide as a co-substrate to oxidize a large number of phenol, aromatic amine and indole derivatives.[27-29] Another important peroxidase is myeloperoxidase, which comprises up to 5 % of the protein content in neutrophils and is responsible for the production of bactericidal hypochlorous acid when these cells are activated.[30] However, only HRP is able to catalyze the oxidation of IAA without hydrogen peroxide.[31] This high specificity has been confirmed here, since the ethyl ester of IAA, despite its structural similarity to IAA, was not recognized as a substrate by HRP. A reasonable explanation for the lack of reactivity between HRP and IAA-ethyl ester is the impossibility of oxidative decarboxylation while the protective ethyl group remains. This step is responsible for the production of skatolyl hydroperoxide which is essential for enzyme turnover and generation of cytotoxic species.[22,32] The necessity of oxidative decarboxylation during IAA-mediated generation of cytotoxic species was

the basis of our choice of IAA-ethyl ester as a potential prodrug. We suggest that the experimental design proposed here, in which two enzymes are necessary to trigger the generation of cytotoxic species, could confer an improved selectivity on a tumor site.

Certainly, one could argue that esterases are ubiquitous in mammalian cells and, consequently, not adequate for the ADEPT strategy. However, our proposal is just a model and could be further improved, for instance, by the synthesis of the β-galactoside ester of IAA. In this case, the cytotoxicity could be triggered by two non-mammalian enzymes, namely HRP and β-galactosidase, conjugated to specific antibodies against the tumor cell. Both enzymes have been used in the ADEPT strategy.[8-12] The proposal of using two enzyme-antibody conjugates to target a tumor cell seems more complicated compared to the usual ADEPT strategy; however, this approach could provide more selectivity since two specific antigens, in the tumor cell, should be recognized before triggering the cytotoxic effect by adding the nontoxic prodrug. Indeed, as well-known, systemic toxicity combined with lack of tumor selectivity is a major concern in cancer therapy. The scheme depicted in Figure 7 shows our proposal for the use of esters of IAA as two enzyme-dependent
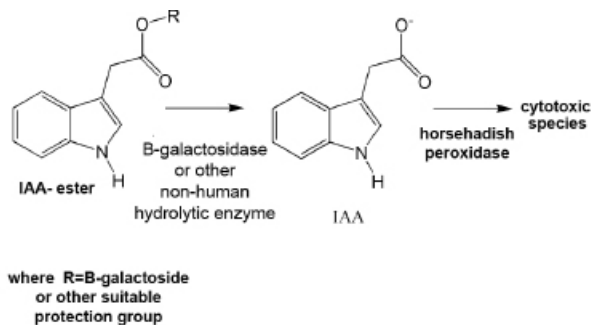

Figure 7- Proposal for the use of ester derivatives of IAA as a two-enzyme-dependent prodrug for ADEPT strategy.

prodrug in ADEPT or related strategies.

Another potential application of the IAA-ethyl ester derives from the fact that it is more liposoluble than IAA. For instance, in the GDEPT strategy, where engineered cells could express HRP.[25] In this case, after the diffusion of the IAA-ethyl ester to intracellular medium, endogenous esterases could hydrolyze it, releasing IAA as a substrate for the expressed HRP.

Our main focus in this work was to demonstrate a new chemical approach to producing cytotoxic species. The choice of the McCoy fibroblast cells was purely for our convenience, since this is the only readily available cell line in our laboratory. Unfortunately, IAA and the IAA-ethyl ester were toxic to McCoy cells in the absence of HRP and esterase when used at concentrations higher

than 1 mM. Further studies, using other tumor cells, should be carried out with this model.

Financial Supports

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