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Modulation of 5-fluorouracil cytotoxicity through thymidylate synthase and NF-κB down-regulation and its application on the radiolabelled iododeoxyuridine therapy on human hepatoma cell

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Abstract

The inhibition of thymidylate synthase (TS) by 5-fluorouracil (5-FU) was known to increase the incorporation of radiolabelled iododeoxyuridine (IdUrd) into DNA. The relatively non-toxic compounds such as thiol-containing antioxidant pyrrolidinodithiocarbamte (PDTC) or aromatic fatty acid phenylbutyrate (PB) had been reported to enhance the cytotoxic efficacy of 5-FU. We designed a novel strategy through triplet combination of PB, PDTC and 5-FU to increase the radiolabelled IdUrd uptake and investigated the underlying mechanisms. The growth inhibition and [^{125}I]IdUrd-DNA incorporation by PB, PDTC, 5-FU in different combinations were tested on parent or p21^{Waf1} transfected Hep3B cells. The combination of PB and PDTC was more effective in enhancing 5-FU cytotoxicity than either drug alone. The combination of PB/PDTC and 5-FU blocked cells in S-phase and resulted in 8.5-fold increase of radiolabelled IdUrd-DNA incorporation. The transfection of p21^{Waf1} did not change the general pattern of enhancement. Intriguingly, the combination of PB and PDTC effectively down-regulated NF-κB and TS and prevented their up-regulation from 5-FU treatment than either drug alone through a p21^{Waf1}-independent mechanism. Based on this strategy, the 3-drug combination offered potential for improved radiolabelled IdUrd molecular radiotherapy for hepatoma treatment.

Keywords: Iododeoxyuridine; Thymidylate synthase; 5-FU; PDTC; PB; NF-κB

1. Introduction

IdUrd is not targeting on TS, clinically ineffective, and only used for radiosensitizer [1]. We and many others have demonstrated that radiolabelled IdUrd can produce much higher killing efficacy than cold IdUrd [2,3]. IdUrd is a carrier for targeting high-killing power of radioisotope to DNA. It was estimated that Auger electrons from single molecule ^{125}I may cause un-repairable double strand breaks. ^{131}I produce β-ray although less efficient which has wider range of cell killing. The combination of [^{125}I/^{131}I] IdUrd has proved to be most effective in targeting tumor in vivo [3–5]. Increasing radiolabelled IdUrd incorporation into DNA is the key of success.

Specific TS inhibitor like fluorodeoxyuridine, thymitaque or non-specific TS inhibitor like 5-FU can increase radiolabelled IdUrd uptake through inhibition of de novo thymidine synthesis [6,7]. The higher the TS inhibition, the higher the IdUrd-DNA incorporation can be seen [3]. 5-FU has been widely used in cancer therapy for many decades. Radiolabelled IdUrd is a representative drug taking the advantage of inhibition of TS from 5-FU which results in high cell kill through Auger electrons from ^{125}I labeled

Abbreviations: IdUrd, iododeoxyuridine; TS, thymidylate synthase; 5-FU, 5-fluorouracil; PDTC, pyrrolidinodithiocarbamte; PB, phenylbutyrate; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; dUMP, deoxyuridine monophosphate; dTMP, thymidine monophosphate

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IdUrd and the cross-fire effect from ^{131}I-IdUrd. The combination of relatively non-toxic cytostatic drugs to enhance the cytotoxicity of 5-FU has long been a promising clinical research area like leucovorin as a good example. We are particular interested in aromatic fatty acid, and antioxidant on the modulation of 5-FU cytotoxicity.

PB, an aromatic fatty acid, induced cytostasis and apoptosis on a variety of cancer types. PB has been reported to enhance the cytotoxic effect of 5-FU through an increased and sustained expression of p21^{Waf1} [8,9]. PDTC, a thiol-containing anti-oxidant decreased the tumorigenic properties of colon cells administered alone or together with 5-FU through a p53-independent p21^{Waf1} induction [10,11]. Although p21^{Waf1} induction was associated with their chemo-sensitization effect on 5-FU, there was no direct evidence that overexpression of p21^{Waf1} increased the 5-FU sensitivity. But there had direct evidences that overexpression of TS and NF-κB decreased the 5-FU sensitivity [12,13]. We are interested in how the TS and NF-κB activity changed under the concomitant use of PB, PDTC during 5-FU treatment.

The purpose of this study is to propose a novel strategy through triplet-combination of PB, PDTC with 5-FU to increase radiolabelled IdUrd uptake. The strategy may be useful in hepatoma treatment due to the relatively quiescent surrounding normal tissues as well as both 5-FU and radiolabelled IdUrd are suitable for intra-arterial infusion. We also found for the first time that PB and PDTC inhibit the expression of TS and NF-κB during 5-FU treatment through a p21^{Waf1} independent mechanism.

2. Materials and methods

2.1. Chemicals

PB, PDTC, and 5-FU were purchased from Sigma and freshly prepared 10 × working solution in PBS before experiments. IdUrd were purchased from Sigma and [^{125}I] IdUrd were synthesized from our laboratory with methods as previously published with some modifications [14]. Briefly, 100 μL of oxidizing agent (H_2O_2:1N HCl:H_2O = 4:1:95) was added to a 300 μL v-vial coated with 50 μg (0.1 μM) of 5-tributylstannyl-2′-deoxyuridine and containing 20 μL ethanol and 3.7–37 MBq (0.1–1 mCi) sodium [$^{125/131}$I]iodide. The reaction mixture was set aside and vortexed intermittently. After 8 min, the mixture was frozen in liquid nitrogen, lyophilized under vacuum for about 1 h to give the final product as a ''hot kit''. Unreacted [$^{125/131}$I] iodide (in form of I_2 in the presence of oxidizing agent), HCl, solvents (ethanol and H_2O) and oxidizing agent (H_2O_2) were removed during lyophilization. The lyophilized [$^{125/131}$I]IdUrd hot kit was redissolved in ethanol and the radiochemical purity was determined using TLC and HPLC. The retention time of [$^{125/131}$I]IdUrd was 7.2–7.4 min. The labeling yield was >95% and the radiochemical purity was >98%. The lyophilized [^{125}I]IdUrd product was stable up to 3 weeks. The lyophilized [$^{125/131}$I]IdUrd hot kit, if dissolved in physiological saline and eluted through a 0.22 μm apyogenic disk, was ready for biological or clinical application.

2.2. Growth inhibition assay comparing PB, PDTC and 5-FU against hepatoma cell line

Hep 3B hepatoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 × non-essential amino acids, 2 mM sodium pyruvate and 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies). To evaluate the combination effect of PB and/or PDTC on the cytotoxicity of 5-FU, we determined the growth inhibition effect of each drugs in the presence of PDTC 20 μM or PB 2 mM or both with or without 5-FU 20 μM for 72 h on Hep3B cells. Hep3B cells were seeded in 100 mm tissue culture dishes at a cell density of 1×10^6 cells per dishes, and cultured at 37 °C in a 5% CO_2 incubator. Indicated drugs were added 4 h after seeding. Viable cells were washed three times with phosphate buffered saline (PBS), trypsinized and counted by the trypan blue dye exclusion method. Each condition was expressed as an average of three determinations for the concentration of drugs. In order to understand whether p21^{Waf1} expression is responsible for the 5-FU-enhancing effect from PB and PDTC treatment, we use transient p21^{Waf1}-overexpressed Hep3B cell for experiments. The transfection was performed by liposome with cytomegalovirus-derived expression vector pCR3.1/p21^{Waf1}, containing the human p21^{Waf1} cDNA.

2.3. Growth inhibition of [^{125}I]IdUrd in combination with PB, PDTC and 5-FU

To evaluate the combination effect of [^{125}I]IdUrd on the 3-drug combination, Hep3B cells were pre-treated PB 2 mM, PDTC 20 μM and 5-FU 20 μM for 45 h before adding [^{125}I]IdUrd 0.37 MBq/mL (10 nmole/L), and cold IdUrd 10 μM for another 3 h incubation, then washed the cells with PBS twice and changed with fresh DMEM medium for another 24 h. Cells were washed three times with PBS, trypsinized and counted by the trypan blue dye exclusion method.

2.4. Cell cycle analysis

Hep3B cells treated with or without PB/PDTC/5-FU in different combinations for 48 h were trypsinised, washed twice with PBS. Cell pellets were suspended in 1 mL 70% ethanol for 30 min at −20 °C. 1×10^6 cells were centrifuged and resuspended in 1 mL of propidium iodide staining solution (0.04 mg/mL propidium iodide, 100 μg/mL DNase-free RNase A) and incubated at 37 °C for 20 min.