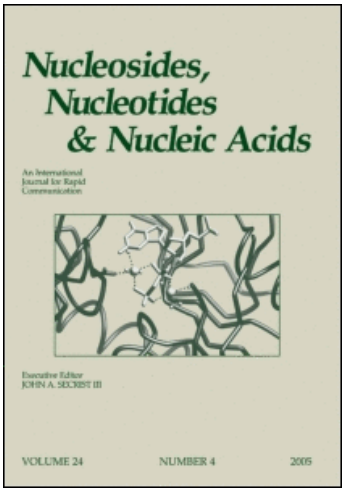

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 11 June 2010

To cite this Article Wang, X. and Albertioni, F.(2010) 'Effect of Clofarabine on Apoptosis and DNA Synthesis in Human Epithelial Colon Cancer Cells', Nucleosides, Nucleotides and Nucleic Acids, 29: 4, 414 — 418
To link to this Article: DOI: 10.1080/15257771003738568
URL: http://dx.doi.org/10.1080/15257771003738568

Nucleosides, Nucleotides and Nucleic Acids, 29:414–418, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003738568

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EFFECT OF CLOFARABINE ON APOPTOSIS AND DNA SYNTHESIS IN HUMAN EPITHELIAL COLON CANCER CELLS

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□ *Clofarabine, a new-generation purine nucleoside analogue, was thought to work via three mechanisms: incorporation into DNA; induction of apoptosis; and inhibition of ribonucleotide reductase, and showed significant efficacy in pediatric relapsed/refractory acute lymphoblastic leukemia (ALL) and hematologic malignancies in adults. By way of its unique metabolic properties, clofarabine is being explored in lymphoproliferative disorders and solid tumors. In this study, the effect of clofarabine on the DNA synthesis of human colon carcinoma cells (HCT116) was investigated by LigandTracer White which provides a simple and accurate method for investigating the uptake, phosphorylation, retention and DNA incorporation of nucleosides in cells. Clofarabine enters into HCT116 cells in a clearly detectable manner. At 100 nM, the interaction is visible and at 10 μM a high signal is achieved and approaches equilibrium after 1∼2 hours. The thymidine incorporation into the DNA synthesis was rapidly stopped by incubation with 10 μM clofarabine and a 3-fold increase in apoptosis induction in HCT116 cells by clofarabine was detected.*

Keywords Clofarabine; DNA synthesis; apoptosis; solid tumor; thymidine; nucleoside analogs

INTRODUCTION

Since the introduction of nucleoside analogs in the 1960s, they have become some of the most prevalent and active components of antitumor therapy, especially hematological malignancies. Clofarabine (2-chloro-2′-fluoro-2′-deoxy-9-β-D-arabinofuranosyladenine) is a new-generation purine nucleoside analogue designed to incorporate the best qualities of both cladribine and fludarabine. Clofarabine was thought to work via three mechanisms: incorporation into DNA to inhibit DNA polymerases; inducing apoptosis with potential therapeutic efficacy in the treatment of indolent lymphoproliferative disorders[1]; and inhibition of ribonucleotide reductase that provides deoxynucleotides for nuclear and mitochondrial (mtDNA) replication and repair. This broad range of activities explains the efficacy

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of the drug towards both rapidly growing and quiescent tumors. Preclinical studies indicated that intravenous clofarabine showed significant efficacy in pediatric leukemias, especially acute lymphoblastic leukemia (ALL) as well as in hematologic malignancies in adults. The current emphasis of clofarabine lies in the acute leukemias, but its activity in other disease groups, for example, lymphoproliferative disorders and solid tumors, is being investigated actively.

Human colorectal cancer, as a solid tumor, is a serious health problem in the Western world, and this cancer is commonly known as one of the most prevalent cancers and one of the leading causes of cancer-related death.[2] In the present study, we examined the effect of clofarabine on the DNA synthesis of human colon carcinoma cells (HCT116) through measuring the uptake of thymidine and clofarabine. The incorporation of ^{14}C-thymidine and ^{14}C-clofarabine was monitored using LigandTracer White.[3] We also investigated the degree of apoptosis in HCT116 cells upon treatment with clofarabine using Annexin V/PI staining.

MATERIALS AND METHODS

HCT116 colon carcinoma cells (generously provided by Professor Stig Linder, Krolinska Institute, Stockholm, Sweden) were maintained in McCoy's 5A modified medium supplemented with 10% FCS, L-glutamine and penicillin at 37°C in 5% CO_2.

The cells were seeded in a tilted dish to achieve one target area and one reference area. The dishes were kept tilted in an incubator for 4–6 hours, until the cells had adhered, following change of medium to a volume of 10 ml. Then the dishes were kept horizontally to allow the cells to attach firmly to the dish surface for at least 24 hours. Synchronized cell dishes were seeded and handled exactly same, using the same cell concentration. Two LigandTracer White (Ridgeview Instruments AB, Uppsala, Sweden) were placed in an incubator (37°C in 5% CO_2) before the measurements started so that the instrument reached thermal equilibrium. In the ^{14}C-clofarabine measurements, two cell dishes with 3 ml cell culture medium and 100 nM or 10 μM ^{14}C-clofarabine were put on the cell dish holder in LigandTracer. In the ^{14}C-thymidine measurements, two cell dishes were incubated with 40kBq ^{14}C-thymidine in the presence or absence of 10 μM clofarabine. During the whole process, two dishes were kept in the LigandTracer.

For apoptosis, cell were cultured at a density of 6×10^3 cells/ml, then after 24 hours treated with or without 10 μM clofarabine. Cells were harvested after 24 hours and washed in PBS, were stained with fluorescein-conjugated annexin-V and PI (BD Biosciences, San Jose, CA, USA).

RESULTS

Clofarabine enters into HCT116 cells in a clearly detectable manner. At 100 nM the interaction was visible and at 10 μM a high signal was achieved and the interaction approached equilibrium after 1∼2 hours (Figure 1A). The clofarabine interrupted accumulation of thymidine and DNA synthesis is stopped rapidly in HCT116 cells (Figure 1B). The degree of apoptosis was investigated by Annexin V/PI staining upon clofarabine treatment. After the HCT116 cells were exposed to 0.1, 1, or 10 μM clofarabine for 24 hours, the apoptotic cells and necrotic cells were increased with the concentration of clofarabine. A 3-fold increase in apoptosis and necrosis was seen with 10 μM clofarabine (Figure 2).

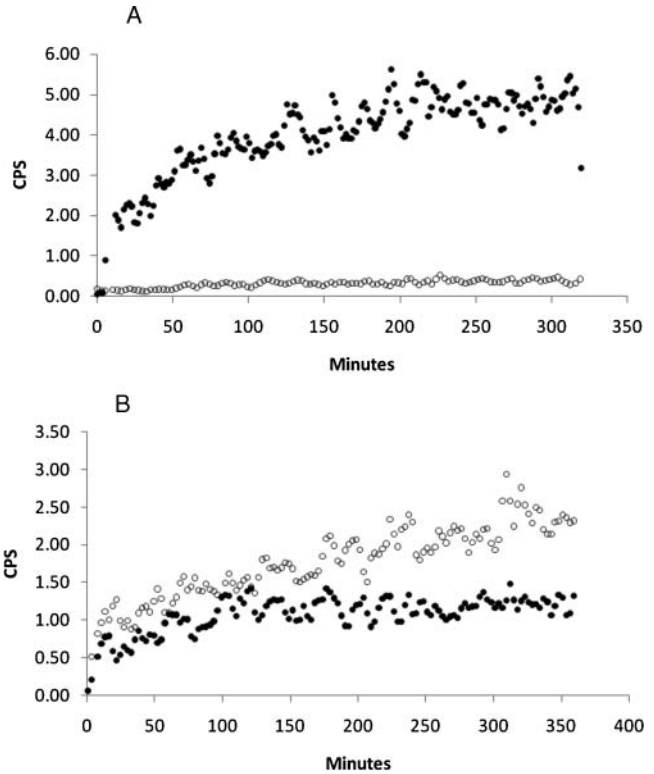

FIGURE 1 A) Uptake of ^{14}C-clofarabine by HCT116 cells. Clofarabine enters the cells with significantly higher signal using 10 μM clofarabine. The interaction approaches equilibrium after 1–2 hours using 10 μM ^{14}C-clofarabine. Briefly, after 24 hours, two synchronized cell dishes were incubated with 0.1 μM (○) and 10 μM (●) ^{14}C-clofarabine, then the incorporation of ^{14}C-clofarabine was monitored by two LigandTracer White operated in an incubator (37°C, 5% CO_2). B) Uptake of ^{14}C-thymidine in HCT116 cells in presence and absence of clofarabine. Synchronized cell dishes were incubated with 40 kBq ^{14}C-thymidine in the presence (●) or absence (○) of 10 μM clofarabine. The incorporation of ^{14}C-thymidine was monitored by two LigandTracer White. Same setting and operation were performed with panel A. Study shows the clofarabine interrupt ^{14}C-thymidine incorporation in HCT116 cells.

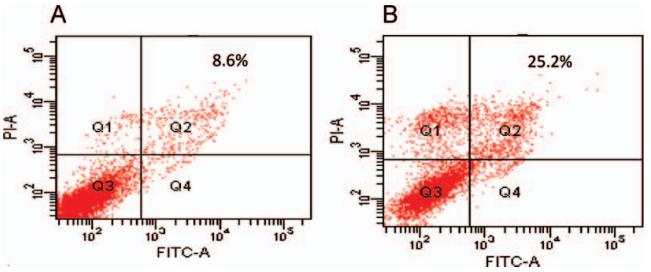

FIGURE 2 Clofarabine induces apoptosis in HCT116 cells. Cells were incubated A) without or B) with 10 μM clofarabine for 24 hours and subjected to fluorescence-activated cell sorting analysis after Annexin V and PI staining. A 3-fold increase in apoptosis and necrosis was seen upon 10 μM clofarabine treatment.

DISCUSSION

As a derivative of cladribine and fludarabine, clofarabine is found to be effective against various subtypes of leukemia and is currently being tested as an oral formulation combination therapy of both leukemias and solid tumors. The conversion of clofarabine to its phosphorylated form is initiated by the generation of monophosphates from nucleosides. This reaction can be catalyzed by either deoxycytidine kinase or deoxyguanosine kinase. DNA damage caused by nucleoside triphosphates, induces expression of p53, leading to induction of pro-apoptotic proteins. However, in resting cells high levels of nucleotides interferes with repair of DNA strand breaks which activates PARP resulting in NAD depletion and decrease levels of ATP and finally apoptosis. Clofarabine-TP (active form of clofarabine nucleotide) may substitute for deoxyadenosine-TP and together with cytochrome c binds to Apaf-1, formation of the apoptosome-complex, and resulting in DNA fragmentation. The capacity of different nucleoside triphosphates to induce apoptosis through the Apaf-1-mediated pathway in a cell-free system was tested demonstrating that clofarabine-TP is an efficient agent in triggering apoptosis.[4,5]

Resistance to nucleoside analogues in cell lines has been linked to deoxycytidine kinase deficiency. Deoxycytidine kinase is expressed in a constitutive manner throughout the cell cycle, and expressed predominantly in lymphoid tissues, indicating cell type specific regulation. Since many solid tumor cells have relatively low deoxycytidine kinase activity, so it is important to investigate the relation between this enzyme and the efficacy of clofarabine in different human tumor cell lines.

In summary we showed for the first time here using LigandTracer that clofarabine might lead solid tumor cells to apoptosis via an incorporation that interferes with the DNA synthesis. We conclude that clofarabine might provide a useful new avenue for certain solid cancer cells.

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