EXPERIMENTAL CELL RESEARCH 328 (2014) 87–98

Available online at www.sciencedirect.com

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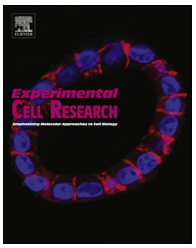

Research Article

Inhibition of autophagy by 3-MA potentiates purvalanol-induced apoptosis in Bax deficient HCT 116 colon cancer cells

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ARTICLE INFORMATION

Article Chronology:
Received 8 April 2014
Received in revised form
12 July 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
Colon cancer
Apoptosis
Autophagy
CDK inhibitors
Bax

ABSTRACT

The purine-derived analogs, roscovitine and purvalanol are selective synthetic inhibitors of cyclin-dependent kinases (CDKs) induced cell cycle arrest and lead to apoptotic cell death in various cancer cells. Although a number of studies investigated the molecular mechanism of each CDK inhibitor on apoptotic cell death mechanism with their therapeutic potential, their regulatory role on autophagy is not clarified yet. In this paper, our aim was to investigate molecular mechanism of CDK inhibitors on autophagy and apoptosis in wild type (wt) and Bax deficient HCT 116 cells. Exposure of HCT 116 wt and Bax$^{-/-}$ cells to roscovitine or purvalanol for 24 h decreased cell viability in dose-dependent manner. However, Bax deficient HCT 116 cells were found more resistant against purvalanol treatment compared to wt cells. We also established that both CDK inhibitors induced apoptosis through activating mitochondria-mediated pathway in caspase-dependent manner regardless of Bax expression in HCT 116 colon cancer cells. Concomitantly, we determined that purvalanol was also effective on autophagy in HCT 116 colon cancer cells. Inhibition of autophagy by 3-MA treatment enhanced the purvalanol induced apoptotic cell death in HCT 116 Bax$^{-/-}$ cells. Our results revealed that mechanistic action of each CDK inhibitor on cell death mechanism differs. While purvalanol treatment activated apoptosis and autophagy in HCT 116 cells, roscovitine was only effective on caspase-dependent apoptotic pathway. Another important difference between two CDK inhibitors, although roscovitine treatment overcame Bax-mediated drug resistance in HCT 116 cells, purvalanol did not exert same effect.

Abbreviations: AIF, apoptosis inducing factor; ABTS, 2, 2′-Azino-di-3-ethylbenzthiazoline sulfonate (6) diammonium salt; AO, acridine orange; BH, Bcl-2 homology; CDK, cyclindependent kinase; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DMSO, dimethylsulfoxide; HRP, horseradish peroxidase; MDC, monodansylcadaverine; MTT, 3–4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium Iodide; PI3K, phosphatidylinositol-3-kinase; POD, peroxidase; PVDF, polyvinyldifluoride; Rb, retinoblastoma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, tris-buffered saline; $\Delta\Psi$, mitochondrial membrane potential; 3-MA, 3-methyladenine

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http://dx.doi.org/10.1016/j.yexcr.2014.07.022
0014-4827/

Introduction

Colon cancer is a leading contributor to global cancer prevalence and mortality. It is the third most common cancer type and fourth leading cause of cancer-related death worldwide. According to 2008 global cancer statistics database, 1.24 million people were diagnosed with colon cancer all around the world and 610,000 people died because of this malignancy [1]. Surgical resection followed by adjuvant radiotherapy, and/or chemotherapy is the first option of therapy, whereas high recurrence rate and increasing incidence of metastatic cases renders the efficiency of therapy. Although several conventional chemotherapeutic agents; 5-FU, oxaliplatin *e.g.* are suggested in the treatment of metastatic colon cancer therapy, novel agents that specifically inhibit the molecular targets driving rapid cell proliferation are proving to be exciting drugs for the treatment of cancer.

Mammalian cell division is regulated by CDKs, which target different cyclin molecules at every step of cell cycle processes [2–4]. Since cell growth, proliferation and DNA replication are under control of different CDK molecules, inhibition of these kinase targets is essential to prevent carcinogenesis [5]. Purine-derived compounds; CDK inhibitors, have been designed to prevent cell proliferation, cell cycle arrest and induce apoptosis [6,7]. Roscovitine and purvalanol, novel CDK inhibitors, are referred as strong apoptotic inducers, which prevent the binding efficiency of CDKs to their cyclin partners. Therefore, these drugs effectively induced cell cycle arrest and apoptosis in prostate [8], breast [9], lung [10], cervix [11] and colon cancer [12,13] cell lines.

Apoptosis is referred as type I cell death and it is characterized by DNA fragmentation, nuclear condensation, apoptotic body formations [14]. Second type of cell death is referred as autophagy, an evolutionary conserved process, characterized by massive degradation of cytosolic contents [15]. The autophagy process is finalized by fusion of autophagosome to endosomes and lysosomes, which engulf cytoplasmic contents within a double-membrane vacuole [16]. Autophagolysosomes are specific structures in cytoplasm to degrade futile organelles or proteins to recycle into other necessary components of organelles, proteins or energy source. This important physiological process is carried out through interaction of various molecules such as Bcl-2, Beclin-1, Atg5 and Atg12 [17,18]. Autophagic marker, Beclin-1, has Bcl-2 homology (BH) 3 domain and it is a linker protein between apoptosis and autophagy due to its interaction with anti-apoptotic Bcl-2 family members. It is suggested that overexpression of Bcl-2 might also prevent autophagy when cells were exposed to death inducers [19]. In addition, LC3 is a cytosolic soluble protein which is cleaved during autophagic induction and involved in the autophagic vacuole membrane formation. When autophagic process starts, LC3-I (16 kDa) is converted to LC3-II (14 kDa) [20]. Recent studies showed that another autophagic key molecule; p62 is integrated in the autophagosome complexes during autophagy and reduced level of cytoplasmic free p62 level could be accepted as an autophagic marker in the cells. Although autophagy is classified as the second type of cell death, a number of reports showed that drug-induced apoptosis mechanism could be postponed in cancer cells by activating autophagy [21]. Recent reports showed that inhibition of autophagy by the treatment of specific inhibitors for autophagic regulators; 3-MA or suppression of autophagy regulatory pathways may provoke apoptotic efficiency of chemotherapeutic agents in prostate [22], breast [23], colon [24], lung [25], HeLa [26] cancer cells. In order to avoid the cancer recurrence after chemotherapy, many attempts have been made to overcome chemotherapeutic effectiveness. For this reason, the determination of the autophagic effect of novel drug candidates as well as their apoptotic molecular targets in cancer cells may increase their therapeutic potential. For this reason, in this study our aim was to figure out the molecular determinants between autophagy and apoptosis crosstalk after purvalanol or roscovitine treatment in Bax deficient HCT 116 colon cancer cells.

In the present study, we found that CDK inhibitors; roscovitine and purvalanol caused a significant increase in apoptotic cell population by inducing cell cycle arrest and activate mitochondrial apoptotic cell death cascade in HCT 116 wt and apoptosis deficient HCT 116 $Bax^{-/-}$ colon carcinoma cells. Moreover, although HCT 116 $Bax^{-/-}$ colon cancer cells were resistant against purvalanol-induced apoptosis, inhibition of autophagy by 3-MA co-treatment augmented purvalanol-induced apoptotic cell death.

Material and methods

Drugs, chemical and antibodies

Roscovitine was purchased from Sigma (St. Louis, MO, USA), dissolved in DMSO to prepare 10 mM stock solution and aliquots were kept at −20 °C. Purvalanol was purchased from Tocris Bioscience (Bristol, United Kingdom) and dissolved in DMSO at an initial stock concentration of 10 mM. MDC and AO were purchased from Sigma (St. Louis, MO, USA), each was dissolved in DMSO to prepare 10 mM stock solution and aliquots were kept at −20 °C. CDK1, CDK 2, CDK4, CDK7, CDK9, cyclin A, cyclin B1, cyclin D3, cyclin E, phospho Rb (Ser795), β-actin, cleaved PARP, pro-caspase-9, pro-caspase-7, pro-caspase-3, p53, p21, Mcl-1, Puma, Atg3, Atg5, Atg7, Atg12 and LC3 (1:1000 dilution) rabbit antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Beclin-1, Bcl-2, Bak, Bid, p62 (1:1000 dilution) mouse antibodies were purchased from Becton Dickinson Biosciences (BD Biosciences, New Jersey, USA). HRP-conjugated secondary anti-rabbit and anti-mouse antibodies (1:5000) were from CST.

Cell lines and culture conditions

HCT 116 (CCL-247) cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). HCT 116 $Bax^{-/-}$ human colon cancer cells were kindly provided by Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University, USA). Both cell lines were maintained in McCoy's medium (PAN Biotech, Aidenbach, Germany) with 2 mM L-glutamine, 10% fetal calf serum (PAN Biotech), 1% non-essential amino acids (Biological Industries, Kibbutz Beit-Haemek, Israel), and 100 U/100 mg ml^{-1} penicillin/streptomycin (Biological Industries) and grown in the presence of 5% CO_2 in humidified air at 37 °C.

MTT cell viability assay

The dose dependent effect of each CDK inhibitor on HCT 116 colon cancer cells was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Roche, Indianapolis, IN,