Leukemia Research 44 (2016) 17–24

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Research paper

Effects of the combination of decitabine and homoharringtonine in SKM-1 and Kg-1a cells

Suxia Geng[1], Han Yao[1], Jianyu Weng, Jiaqi Tong, Xin Huang, Ping Wu, Chengxin Deng, Minming Li, Zesheng Lu, Xin Du*

Department of Hematology, Guangdong General Hospital and Guangdong Academy of Medical Sciences, Guangzhou 510080, PR China

ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form 6 January 2016
Accepted 13 February 2016
Available online 16 February 2016

Keywords:
MDS
Decitabine
Homoharringtonine
Apoptosis
Methylation

ABSTRACT

The methylation inhibitor decitabine (DAC) has great therapeutic value for myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). However, DAC monotherapy is associated with relatively low rates of overall response and complete remission. Previous studies have shown promising results for combination treatment regimens including DAC. Homoharringtonine (HHT), an alkaloid from Chinese natural plants and Cephalotaxus, has demonstrated potential for leukemia treatment. Our studies have suggested that the combination of DAC and HHT has synergistic effects for inhibiting the viability of SKM-1 and Kg-1a cells. This combination leads to enhanced inhibition of colony formation and apoptosis induction compared with DAC alone in SKM-1 but not Kg-1a cells. Only high-dose DAC and HHT significantly up-regulate caspase-3 and caspase-9 and inhibit BCL-XL in the SKM-1 cell line. The combined effects of DAC plus HHT on apoptosis may not only depend on regulation of the apoptosis-related genes we examined but others as well. HHT had no demethylation effects, and HHT in combination with DAC had no enhanced effects on hypomethylation and DNMT1, DNMT3A and DNMT3B mRNA expression in SKM-1 cells. Overall, these results suggest that DAC used in combination with HHT may have clinical potential for MDS treatment.

1. Introduction

Myelodysplastic syndromes (MDS) are a group of hematologic disorders characterized by ineffective hematopoiesis, cytopenias, and an increased risk for the development of acute myeloid leukemia (AML) [1]. The underlying pathogenesis of MDS has not been fully elucidated, but there is considerable evidence that DNA methylation is altered in MDS, and it affects the expression of genes important for regulating hematopoietic stem cell proliferation and differentiation [2–4]. Decitabine (DAC), a potent hypomethylating agent, induced hypomethylation associated with the reactivation of multiple genes, and it is thought that this effect on gene expression contributes to the drug response mechanism. The use of DAC by MDS and AML patients had promising effects, leading to FDA approval for DAC for patients with MDS [5–7]. Although the clinical efficacy of DAC is superior to that of supportive therapy and conventional chemotherapy for MDS, it has become apparent that DAC is not curative and has shortcomings. DAC therapy leads to objective responses in only half of patients, including a modest complete remission rate of 9–39% [8–10]. There is a significant proportion of patients with MDS who do not respond to DAC and those who lose response or progress while on therapy. Patients with primary or secondary resistance to DAC have poor prognosis, and even responsive patients are uncured with most patients losing their response in less than two years [11]. In addition, pre-clinical studies and clinical trials have shown promising results for combination treatment regimens including DAC [12–14].

Homoharringtonine (HHT), an alkaloid from Chinese natural plants and Cephalotaxus, has shown potential for leukemia treatment [15]. HHT functions as a protein synthesis inhibitor and demonstrates strong growth-inhibiting activities in in vitro and animal experiments [16–18]. HHT has been widely used for the treatment of chronic myeloid leukemia (CML), AML and MDS [19,20]. HHT has relatively mild extramedullary toxicity and no anthracycline-like cardiac toxicity, making it a suitable candidate for the treatment of aged patients. A phase III clinical trial of HHT-based induction regimens in young patients with de novo AML performed in China indicated that HHT is the alternative treatment of choice [21]. In addition, combination therapy including HHT and

* Corresponding author at: Zhongshan Er Road #106, Guangzhou 510080, PR China.
E-mail address: miyadu@hotmail.com (X. Du).
[1] These authors equally contributed to this work.

http://dx.doi.org/10.1016/j.leukres.2016.02.002
0145-2126/

other anti-leukemia drugs for leukemia patients resulted in a high clinical response, and significantly improved survival was reported compared with those treated with HHT alone. The combination of HHT and ara-C has been widely used for AML patients in China [22,23]; however, the effects of DAC in combination with HHT have not yet been reported.

In this study, MDS-derived SKM-1 cells and AML-derived Kg-1a cells were used to assess the effects of DAC in combination with HHT on the induction of apoptosis and hypomethylation to explore the feasibility of the combination of DAC and HHT in vitro.

2. Materials and methods

2.1. Cell lines and cell culture

SKM-1 and Kg-1a cell lines were obtained from Tongji Medical College, and they were maintained in RPMI 1640 (Gibco-BRL) medium containing 10% heat-inactivated fetal bovine serum (Gibco-BRL) in a humidified atmosphere of 5% CO_2 at 37 °C with the medium refreshed every two days.

2.2. Cell viability assay

Cells were plated at a density of 1×10^5 cells/mL in 5 mL of medium and split 24 h prior to treatment. Different concentrations of DAC (Sigma) and HHT (Minsheng pharmacy, Hangzhou, China) alone or in combination were added to medium in 96-well plates and incubated for 72 h. MTS mixed with PMS (Promega) was added, and after a 1 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance at 490 and 690 nm was recorded using an ELISA plate reader. Doses inhibiting 50% proliferation (IC50) were analyzed by the median-effect method (CalcuSyn software; Biosoft). In vitro cytotoxicity was assayed in triplicate under the following experimental conditions: DAC alone, HHT alone, and DAC plus HHT. The effects of the combinations were estimated using CalcuSyn software, which was developed based on the median-effect method created by Chou and Talalay [24,25].

2.3. Methylcellulose colony-forming assay

After washing, the media was replaced on the cells, and they were incubated overnight prior to treatment in a 6-well plate. After a 3-day drug treatment, an equal number of viable cells (500 per plate) were plated in a 24-well plate in triplicate on methylcellulose at a final concentration of 0.9%. Colonies were counted after 10–16 days using an inverted microscope. A colony was defined as a cluster of >40 cells [26,27].

2.4. Apoptosis assay

Cells were washed twice in phosphate-buffered saline, resuspended in 1 mL binding buffer containing annexin V, and they were then analyzed by flow cytometry after the addition of propidium iodide (PI). Annexin V binds to cells that express phosphatidylserine on the outer layer of cell membranes, and PI stains the cellular DNA of cells with compromised membranes. This staining allows for the discrimination of live (unstained with either fluorochrome), apoptotic (stained only with annexin V) and necrotic (stained with both annexin V and PI) cells. The Annexin V apoptosis detection kit APC (E09658-1645) was purchased from eBioscience (San Diego, USA).

2.5. RNA extraction and cDNA synthesis

RNA was extracted using the TRIzol reagent (Invitrogen). The RNA quality was analyzed in a 0.8% agarose gel stained with ethidium bromide. The RNA (~1 μg) was synthesized into first-strand cDNA with random hexamer primers using the PrimeScript™ RT Reagent Kit (TaKaRa).

Table 1
Primer sequences used for Q-PCR detection.

Primer name	Sequence (5′–3′)
Caspase-3-F	TGTGAGGCGGTTGTAGAAGTT
Caspase-3-R	TCTGTTGCCACCTTTCGGTT
Caspase-9-F	AACAGGCAAGCAGCAAAGTT
Caspase-9-R	TCCATCTGTGCCGTAGACAG
BCL2-F	CCAAGAATGCAAAGCACATCC
BCL2-R	CCCAGCCTCCGTTATCCTG
BCL-XL-F	GTCTCCGGCCTTCAACATCA
BCL-XL-R	CGAAAGCACCAGTGGACTCT
LINE-1-M-F	TTATAAAGTTGTATGTTATTGTCGG
LINE-1-M-R	AAAATTAAATTAATTCTAAATTCGAA
DNMT1-F	TACCTGGACGACCCTGACCTC
DNMT1-R	CGTTGGCATCAAAGATGGACA
DNMT3A-F	TATTGATGAGCGCACAAGAGAGC
DNMT3A-R	GGGTGTTCCAGGGTAACATTGAG
DNMT3B-F	GGCAAGTTCTCCGAGGTCTCTG
DNMT3B-R	TGGTACATGGCTTTTCGATAGGA
β-Actin-F	CCTGGCACCCAGCACAAT
β-Actin-R	GCCGATCCACGGAGTACT

2.6. DNA extraction and bisulfite conversion

DNA was extracted using the TaKaRa genomic kit (Dalian, China) and measured at 260/280 nm to determine its concentration and purity. Bisulfate DNA conversion was performed using the EZ DNA Methylation-Gold Kit™, and gel electrophoresis was used to determine whether the conversion was complete.

2.7. Quantitative PCR

PCR primers were designed using Primer Premier 5, and they were synthesized by Invitrogen Shanghai Trading Co., Ltd. (Table 1). The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression and LINE-1 gene methylation. The cDNA or DNA was then used as a template for comparative quantitative PCR with SYBR Premix EX TaqII (TaKaRa), and each sample was detected in duplicate. β-actin was an internal control gene. Assembled plates were then covered and run using the following conditions: an initial denaturation step at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 min with a final step at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

2.8. Statistical analysis

Data are presented as the mean ± SD. P values were calculated using Student's *t*-test, and $P < 0.05$ was considered statistically significant. CalcuSyn software (Biosoft, Ferguson, MO, USA) was used to evaluate the synergistic effects of the drug combination and generate combination index (CI) plots. SPSS version 13.0 was used to analyze the data, which was plotted with GraphPad Prism.

3. Results

3.1. Synergism between DAC and HHT in SKM-1 and Kg-1a cells

We first measured the IC50s of DAC and HHT (Table 2) and then examined cell viability. As shown in Fig. 1, DAC and HHT alone demonstrated concentration-dependent inhibition of the cell viability of the SKM-1 and Kg-1a cells. At high doses, HHT resulted in 90% inhibition, whereas only 70% inhibition was observed with the highest DAC dose.