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MicroRNA hsa-miR-29b potentiates etoposide toxicity in HeLa cells via down-regulation of Mcl-1

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ABSTRACT

Etoposide is commonly used as a monotherapy or in combination with other drugs for cancer treatments. In order to increase the drug efficacy, ceaseless search for novel combinations of drugs and supporting molecules is under way. MiRNAs are natural candidates for facilitating drug effect in various cell types. We used several systems to evaluate the effect of miR-29 family on etoposide toxicity in HeLa cells. We show that miR-29b significantly increases etoposide toxicity in HeLa cells. Because Mcl-1 protein has been recognized as a miR-29 family target, we evaluated downregulation of Mcl-1 protein splicing variant expression induced by miR-29 precursors and confirmed a key role of Mcl-1 protein in enhancing etoposide toxicity. Despite downregulation of Mcl-1 by all three miR-29 family members, only miR-29b significantly enhanced etoposide toxicity. We hypothesized that this difference may be linked to the change in Mcl-1L/Mcl-1S ratio induced by miR-29b. We hypothesized that the change could be due to miR-29b nuclear shuttling. Using specifically modified miR-29b sequences with enhanced cytosolic and nuclear localization we show that there is a difference, albeit statistically non-significant. In conclusion, we show that miR-29b has the synergistic effect with etoposide treatment in the HeLa cells and that this effect is linked to Mcl-1 protein expression and nuclear shuttling of miR-29b.

1. Introduction

Treatment of maladies using podophyllotoxins, present in wild chervil roots, dates back hundreds of years (Slevin, 1991). Etoposide, also known as VP-16-213, is a semisynthetic derivative of podophyllotoxin. FDA granted approval for etoposide use in 1983 (Hande, 1998), since then it has been used in various treatment combinations for cancer therapy, especially acute myeloid leukemia, non-Hodgkin's lymphoma, gastric or lung cancer (Hande, 1998). Its primary mechanism of action is topoisomerase II inhibition. It causes numerous single and double strand breaks in DNA in a dose dependent manner (Hande, 1998; Montecucco and Biamonti, 2007). Cell cycle checkpoints recognize this large number of DNA breaks and cause a cell cycle block in S and G2/M phases. Etoposide-affected cells usually choose a "fast and dirty" repair mechanism also called non-homologous end joining that causes chaotic joining of DNA. The result could be gene translocations usually producing non-functional genes. Cells often respond to the DNA damage and misrepair by initiating the programmed cell death. Another result is accumulation of mutations leading to carcinogenesis (Davis and Chen, 2013).

miRNAs are approximately 22 nucleotide long, non-coding RNA chains which modulate protein expression (He and Hannon, 2004; Ruegger and Grosshans, 2012; Wang et al., 2013). They are relatively stable small molecules capable of posttranscriptional modification of a gene's expression. This often results in changed phenotype of the cell. Not surprisingly, some of these miRNAs are very often dysregulated in tumors. On the other hand, many miRNAs are considered tumor suppressors. Using miRNA precursors in therapy may cause the level of the dysregulated miRNA to return to, or even exceed, its natural, endogenous level. Targeted modifications in miRNA levels could facilitate expression of important target proteins hence aiding in effectiveness of a drug.

miRNAs are usually transcribed from DNA by RNA polymerase II (Fig. 1). The resulting molecules of approximately a thousand nucleotides are called primary transcripts, some of them up to several kilobases in length (Lee et al., 2004). Moreover, Borchert et al. found that RNA polymerase III can transcribe small group of miRNAs (Borchert et al., 2006).

Abbreviations: Mcl-1, Myeloid cell leukemia protein; Mcl-1L, Myeloid cell leukemia protein long; Mcl-1S, Myeloid cell leukemia protein short; Bak, Bcl-2 homologous antagonist/killer; BCL-2, B-cell lymphoma; FDA, Food and Drug Administration; 3′UTR-region, 3′ untranslated region; RISC, RNA-induced silencing complex; DNMT, DNA methyltransferase; RTCA, Real time cell analyzer; SDS, Sodium dodecyl sulfate; PAGE, Polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride; BSA, Bovine serum albumin; TBS, Tris-buffered saline.

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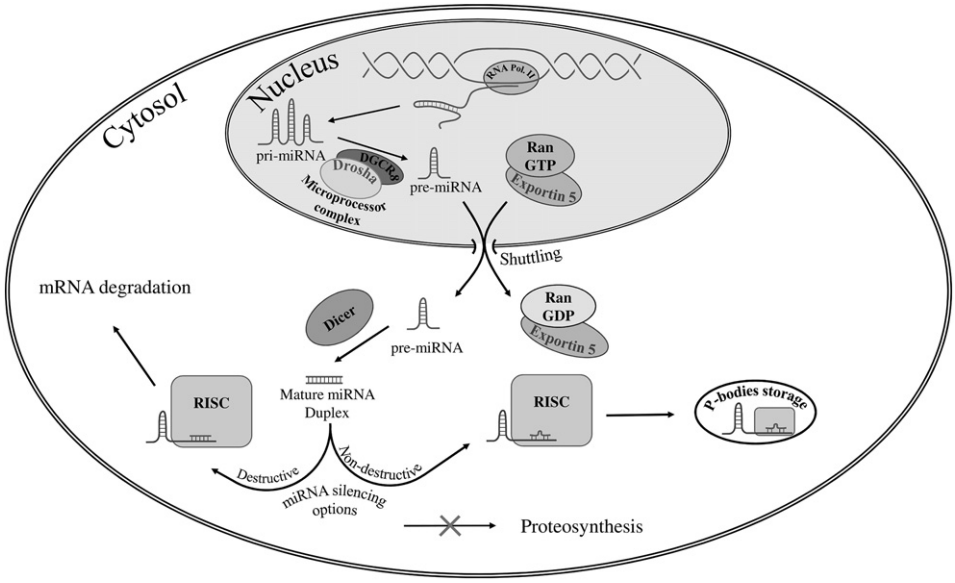

Fig. 1. Schematic representation of microRNA biogenesis.

Biogenesis continues with Drosha (RNAse III enzyme) and DGCR8 enzymatic splicing. Drosha and DGCR8 are often called the microprocessor complex. The precursor miRNAs are approximately 60–70 hundred nucleotides long (Han et al., 2006; Lee et al., 2003). After shuttling pre-miRNAs from nucleus to cytosol by proteins Exportin 5 and RanGTP (Kohler and Hurt, 2007; Yi et al., 2003), they are cleaved by endonuclease Dicer (RNAse III) (Bernstein et al., 2001; Hutvagner et al., 2001). The Dicer produces miRNA duplexes. The last step is formation of mature miRNA that is ready to modulate protein expression via an RNA-induced silencing complex (RISC) (Bernstein et al., 2001). The modulation is posttranscriptional with two possible options, each a result of the complementarity of miRNA to the target mRNA. Full complementarity of a miRNA to 3′ UTR region of mRNA target results in the cleavage of the mRNA by RISC. The other case, in which miRNA does not fully complement the target mRNA, the result is association of the RISC to the target mRNA without its degradation. Therefore, ribosomal transcription is blocked. mRNA-RISC can be stored in p-bodies (Valencia-Sanchez et al., 2006). The miR-29 family are well known tumor suppressor miRNAs, which are important players in malignant hematopoiesis (Kollinerova et al., 2014). This family contains three miRNA isoforms miR-29a, miR-29b and miR-29c (Jiang et al., 2014; Wang et al., 2013; Yan et al., 2015). The literature discusses their impact on down-regulation in several tumors (Wang et al., 2015), epigenetic regulation through DNMT3A or DNMT3B regulation, and regulation of the extracellular matrix (Wang et al., 2013).

Protein Mcl-1 (full name - myeloid cell leukemia 1 protein) is a protein that belongs to the BCL-2 protein family. The protein was discovered by Kozopas et al. in 1993 during a study of programmed myeloid cell differentiation (Kozopas et al., 1993). There are three known splicing variants of the Mcl-1 protein: Mcl-1L (long variant), Mcl-1S (short variant) and Mcl-1ES (extrashort variant). Of these Mcl-1L is generally considered anti-apoptotic and the Mcl-1S a pro-apoptotic (Bae et al., 2000). Mcl-1L protein is localized primarily in the mitochondria (Thomas et al., 2012) and its half-life is up to 4 h (Yang-Yen, 2006). The Mcl-1L protein figures in signal cascades as an example of counterpoise against pro-apoptotic proteins, including its short splicing variant Mcl-1S. The action is mediated by Mcl-1/Bak protein interaction resulting in partial pro-apoptotic signal inhibition (Cuconati et al., 2003) or by the Mcl-1L/Mcl-1S interaction, which is associated with apoptotic cell death (Morciano et al., 2016). Mcl-1 may be classified as an oncogene because its gene is usually mutated and overexpressed in many cancers (Belmar and Fesik, 2015). It has been demonstrated that Mcl-1 is one of the miR-29 protein targets (Mott et al., 2007).

We evaluated the effect of miR-29 family members on etoposide toxicity in HeLa cells and found that only one of the isoforms, miR-29b, significantly increases the toxicity of etoposide, and that the increase is linked to the change in Mcl-1L/Mcl-1S ratio and miR-29b nuclear shuttling.

2. Materials and methods

2.1. Chemicals

Etoposide, siRNA (six different siRNA against Mcl-1 and Bak proteins (for more information see Table 1)), Minimal essential medium Eagle, penicillin (10,000 units/ml)/streptomycin (10 mg/ml) solution, RNAse Zap, TRIzol solution for RNA extraction and purification, chloroform, nuclease-free water, isopropanol, IGEPAL® CA 630, ethanol 96%, Tris, NaCl, $MgCl_2$, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St-Louis, MO, USA). pre-miR-29a, pre-miR-29b, pre-miR-29c, pre-miR negative control 1, Opti-MEM, non-essential amino acids, TaqMan reverse transcription reaction kit, TaqMan reverse and forward mirR-29 primers for reverse transcriptase reaction, TaqMan reverse and forward miR-29 family primers, TaqMan miR-29 family probes for RT-PCR analysis and TaqMan universal PCR master mix, No AmpErase UNG and Lipofectamine 2000 were purchased from Life-Technologies (Prague, Czech Republic). Fetal bovine serum was ordered from Bio-Tech (Prague, Czech Republic) and miR-29b artificial analogs with improved sequences (5′uagcaccauuugaaauaguguuc3′ and 5′uagcaccauuugaaauaagaguu3′ according to (Hwang et al., 2007) were bought from GE Dharmacon (Lafayette, CO, USA).

Table 1
IDs of the siRNAs that were used in our experiments.

Protein	Ranking	siRNA ID
Mcl-1	1	SASI_Hs01_00162656
	2	SASI_Hs01_00162657
	3	SASI_Hs01_00162658
Bak	1	SASI_Hs02_00331326
	2	SASI_Hs01_00075044
	3	SASI_Hs02_00331327