Enoxacin as a Small-Molecule Enhancer of microRNA

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Enoxacin is a second-generation quinolone with promising anticancer activity. In contrast to other members of the quinolone group, it exhibits an extraordinary cytotoxic mechanism of action. Enoxacin enhances RNA interference and promotes microRNA processing, as well as the production of free radicals. Interestingly, apart from its proapoptotic, cell cycle arresting and cytostatic effects, enoxacin manifests a limitation of cancer invasiveness. The underlying mechanisms are the competitive inhibition of vacuolar H^+-ATPase subunits and c-Jun N-terminal kinase signaling pathway suppression. The mentioned mechanisms seem to contribute to a safer, more selective and more effective anticancer therapy.

enoxacin miRNA RISC SMER

1. The miRNA Biogenesis

The miRNA biogenesis begins with transcribing a gene into a large primary transcript (pri-miRNA). The transcription is typically mediated by RNA polymerase II.[1] The pri-miRNAs are then cleaved by the microprocessor complex, composed of the RNA-binding protein DGCR8 and type III RNase Drosha, into a stem-loop structure called the precursor miRNA (pre-miRNA).[2] Following transportation by the Ran/GTP/Exportin5 complex from the nucleus to the cytoplasm, the pre-miRNAs are processed by another RNase III enzyme DICER into an miRNA/miRNA duplex. After the duplex is unwound, the mature miRNA is incorporated into a protein complex termed the RNA-induced silencing complex (RISC). The miRNA-loaded RISC mediates gene silencing via mRNA cleavage and degradation or translational repression, depending on the complementarity between the miRNA and the targeted mRNA transcript.[3] An important role in translational repression and mRNA degradation is played by GW182 protein. Its N-terminal domain is rich in glycine (G) and tryptophan (W) amino acids, while the C-terminus contains a silencing domain. GW182 directs proteins involved in deadenylation, decapping and exonucleolytic degradation of the target mRNA.[4] GW182 binds directly to AGO2, another member of the RISC[5] GW182, AGO2 and rest of the RISC can be found within GW processing bodies (GW/P-bodies).[6],[7] Interestingly, miRNAs may function as ligands directly binding the Toll-like receptor (TLR), triggering downstream signaling pathways[8] (**Figure 1**).

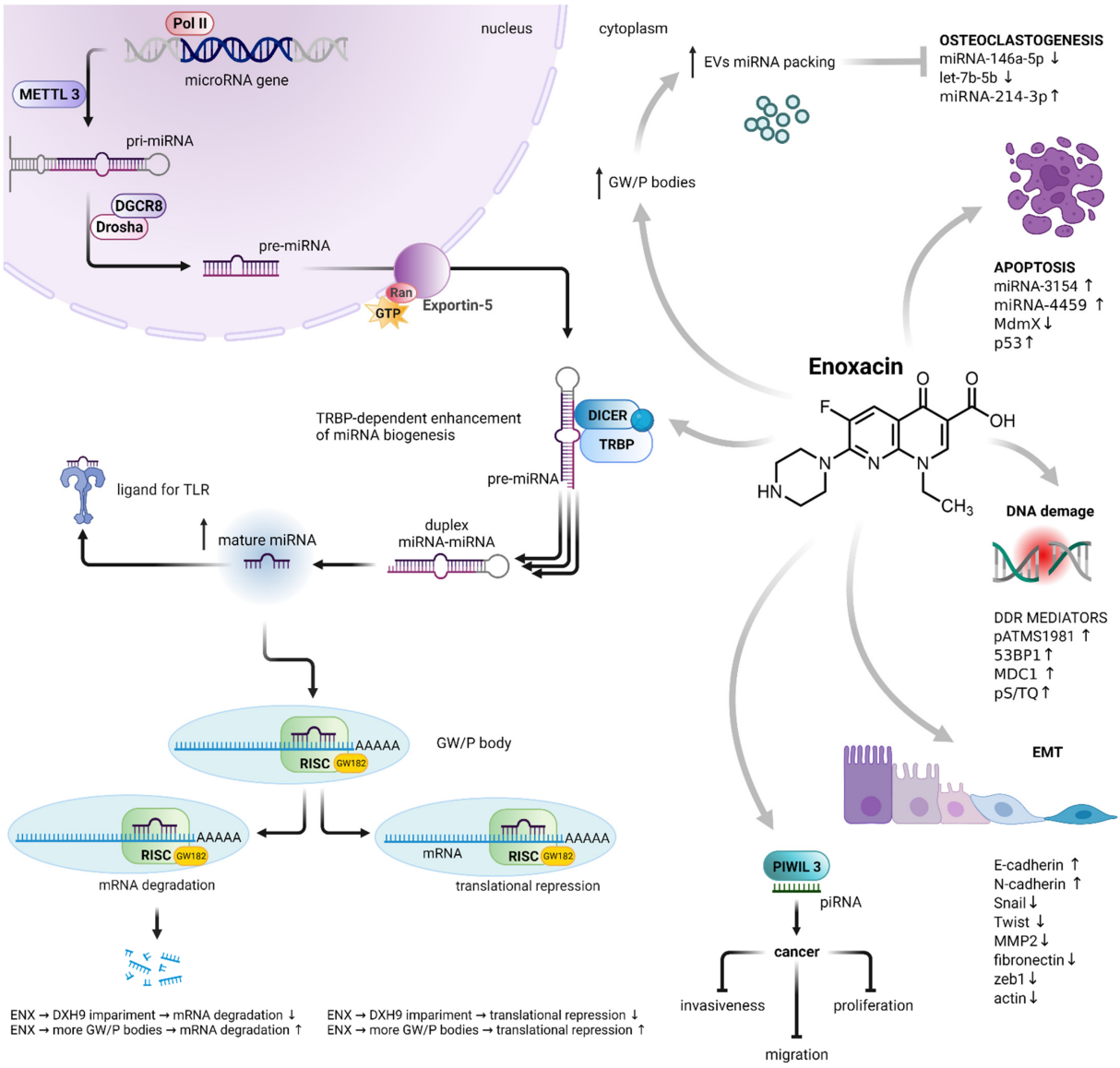

Figure 1. Enoxacin-induced dysregulation of miRNA biogenesis and its consequences. Enoxacin enhanced the activity of the RISC loading protein TRBP, resulting in an increase in the number of mature miRNAs. It also impairs the activity of DHX9 helicase, a member of the RISC, leading to the impairment of mRNA translational repression and degradation. On the other hand, it increases the number of GW/P bodies, the sites of RNA-mediated silencing, as well as the localization of miRNA packaging into EVs. The enoxacin-dysregulated biogenesis of miRNA is involved in osteoclastogenesis, apoptosis, DNA damage response, epithelial–mesenchymal transition, cancer cell proliferation, migration and invasiveness. Abbreviations: polymerase II (Pol II); methyltransferase-like 3 (METTL3); microprocessor complex subunit DGCR8 (DGCR8); type III RNase Drosha (Drosha); GTP-binding nuclear protein Ran (Ran); endoribonuclease DICER (DICER); TAR RNA-binding protein 2 (TRBP); Toll-like receptor (TLR); RNA-induced silencing complex (RISC); GW processing body (GW/P-body); Piwi-interacting RNA (piRNA); epithelial–mesenchymal transition (EMT); matrix metalloproteinase-2 (MMP2); DNA damage response (DDR); extracellular vesicles (EVs). MiRNA biogenesis adapted from[2] Created with BioRender.com.

It has been clear for almost twenty years that miRNA expression is dysregulated in human malignancies. The underlying mechanisms include chromosomal abnormalities,[9][10][11][12][13][14] transcriptional control changes,[15][16][17] epigenic changes and defects in the miRNA biogenesis machinery.[18][19][20]

2. The Effect on Cancer Cells

2.1. TRBP-Dependent Cytotoxicity

Small molecules can influence miRNA biogenesis. Enoxacin was reported as the first and unique small-molecule enhancer of microRNA (SMER) maturation.[21]

Jin et al. found that the increase in miRNAs was associated with high levels of their own precursors, thereby suggesting that enoxacin could promote the DICER processing activity without influencing the miRNA precursor expression.[21] The same authors established that the enoxacin activity was dependent on the TAR RNA-binding protein 2 (TRBP) and likely involved in the improvement of the TRBP-pre-miRNA affinity.[21]

The role of the TRBP in enoxacin-mediated cytotoxicity was confirmed in the three colorectal cancer cell lines (Co115, RKO and HCT-116) and their mutants with impaired TRBP expression. TRBP-impaired cells were more resistant to enoxacin, resulting in a 2-fold increase in the effective concentration (EC50). Mutant cells also did not undergo cell cycle arrest. Additionally, enoxacin induced a two-fold expression increase in 24 miRNAs in RKO and HCT-116 cells and from 1.5-fold up two five-fold increases in the expression of those miRNAs in RKO and HCT-116 xenograft mouse model. Enoxacin treatment also significantly decreased the number of lung and liver metastases in the HCT-116 xenograft.[22] Interestingly, ENX increased the TRBP-dependent miRNA expression in Ewing's sarcoma family tumor (ESFT) cell lines such as A673, TC252 and STA-ET-8.2, but not the expression of TRBP itself. It caused a 50% reduction in sphere formation; increased the expression of a panel of TRBP/DICER-dependent miRNAs; and decreased the expression of Oct-4, Nanog and Sox-2 proteins in primary ESFT spheres. This effect was similar to the introduction of exogenous TRBP. Thus, enoxacin can cause a decrease in the self-renewal of ESFT cancer stem-like cells (CSC).[23] However, this fluoroquinolone also significantly decreased TRBP and DICER protein expression levels in the prostate cancer cell lines DU145, LNCaP, VCaP, PC-3, 22Rv1 and Co115 via the induction of apoptosis. At the same time, it dysregulated the expression of a wide range of miRNAs involved in the development and progression of prostate cancer, e.g., miRNA-29b, which regulates the expression of the proteins E-cadherin, N-cadherin, Snail, Twist and matrix metalloproteinase-2 (MMP2) involved in the metastatic process.[24] The effect of the increased expression of tumor-suppressing miRNA was also notable in thyroid cancer cells lines (Cal62, TPC1, SW1736). This resulted in lower cell proliferation and cell invasiveness in vitro. The decreased expression of epithelial–mesenchymal transition (EMT) markers such as fibronectin, n-cadherin, zeb1, twist and actin was also observed. The upregulation of tumor suppressor miRNA, as well as the suppression of EMT markers, was also shown in the orthotopic mouse model of human thyroid cancer. The results were consistent with those using miRNA-restoring DICER1 silencing (miRNA-30a and miRNA-100). This suggests that enoxacin promotes the restoration of DICER1 activity in DICER1-impaired cells, e.g., human thyroid cancer cells.[25]

The role of enoxacin as an enhancer of DNA damage response (DDR) signaling was confirmed in cervical cancer HeLa cells. This antibiotic augmented the DDR mediator factors pATMS1981, 53BP1, MDC1 and pS/TQ without affecting their expression, whereas the activity of γH2AX levels was not affected. Based on the knowledge of the activation of TRBP by enoxacin, it is not surprising that TRBP silencing diminished ENX's stimulation of DDR. The knockdown of PACT or GW182 proteins (TNRC6A, B and C), which are effectors for miRNA-guided gene silencing, did not affect ENX-mediated DDR stimulation. These results seem to strengthen and confirm enoxacin's specificity towards TRBP.[26]

2.2. PIWIL-3-Dependent Cytotoxicity

Regarding the molecular target recognized by enoxacin, an additional protein has been proposed that merits mentioning. In 2017, Abell et al. determined that the Piwi-like protein 3 (PIWIL3) is a potential enoxacin target.[27] PIWIL3 belongs to the PIWI argonaut proteins involved in the maturation of the PIWI-interacting RNAs (piRNAs), small non-coding RNAs that differ from miRNAs.[28] Although mostly present in normal testis tissue, PIWIL3 has been reported to be aberrantly expressed in a variety of cancers, playing important roles in tumorigenesis.[29][30] An increase in miRNA-21 and miRNA-145 expression was observed in breast cancer MCF7 cells. Similar results were obtained in cells with small interfering RNA-mediated knockout of the PIWIL3. The staining with alkenox, a synthetic enoxacin analog, showed that PIWIL3 might be a mechanistic target of enoxacin.[27] Since PIWIL3 is more abundant in cancer cells, it might in part explain the specificity of ENX towards cancer cells.[27][30]

2.3. Other Consequences of Enoxacin-Mediated miRNA Dysregulation

Additionally, ENX affected RNA helicase DHX9, a member of the RISC. The expression of DHX9 was much higher in the small-cell lung cancer cell line H446 than in the non-small-cell lung carcinoma cell lines A549 and PC9. Enoxacin inhibited the proliferation of A549 in a dose-dependent manner. Similarly, it decreased the expression of DHX9 in A549 cells. However, silencing DHX9 impaired the cytotoxic effect of the drug.[31]

A strong inhibitory effect of enoxacin on the proliferation of human melanoma A375, Mel-Juso and Mel-Ho cell lines was observed. It dysregulated a set of 55 miRNAs in A375 cells (26 upregulated, 29 downregulated). Two upregulated miRNAs, miRNA-3154 and miRNA-4459, control the p53-Mdm2-MdmX network.[32] Interestingly, in many melanomas the overexpression of MdmX, a p53 negative regulator, was observed.[33] Enoxacin increased p53's activity without affecting its expression. At the same time, the expression of MdmX decreased in a dose-dependent manner. It alternates MdmX splicing by promoting exon 6 skipping. This process was observed in different cancer cell lines (A375, A2780 and MCF7).[34]

A different ENX antiproliferative mechanism without affecting apoptosis has been noted in 4T1 murine breast cancer cells. An increased level of GW/processing bodies, which are considered the surrogate markers for both the microRNA-mediated repression of translation and the extracellular vesicle (EV) packaging sites, was observed. MiRNA expression levels in the 4T1 cells' cytosol and their EV were compared. Enoxacin significantly increased only miRNA-214-3p and slightly increased miRNA-146a-5p, miRNA-290, miRNA-689 and let-7b-5p cytosolic levels.

In contrast, significant decreases in let-7b-5p, miRNA-146a-5p and miRNA-689 expression and an enormous 22-fold increase in miRNA-214-3p were observed. All mentioned miRNAs are involved in the regulation of bone remodeling and osteoclastogenesis. Additionally, EVs from enoxacin-treated 4T1 cells enhanced the proliferation of murine macrophage cells.[35] The effective concentration range affecting miRNA biogenesis (see **Table 1**) was from 50 to 124 µM; however, an achievable serum concentration for a standard clinical dose 400 mg two times a day was ca. 10 µM. Reduction of effective concentration could be obtained by structural modification of enoxacin. Significant decrease of IC50 was observed after structure modification of other fluoroquinolones, i.e., by fatty acid conjugation.[36][37] Moreover, it should be considered that tumor vessels are often more permeable compared to normal vessels, which could increase the ENX delivery and its intratumor concentration.[38]

Although it has been clear that enoxacin did not affect all existing miRNAs (e.g., 36 out of 22,000 tested in normal HEK293 cells[21] and 122 out 731 tested in cancer RKO cells[22]). It is worth mentioning that this may be partially explained by the existence of the alternative miRNA biogenesis pathways, which do not contain molecular targets of enoxacin.[39][40][41] Interestingly, the dysregulated miRNAs play an important role in cancer-related processes, e.g., miR-17* decreased the activity of mitochondrial antioxidative enzymes in PC3 cells;[42] miR-34a downregulated an oxidative-stress-induced silent information regulator 1 (SIRT1), a negative regulator of p53 protein, in HCT116 cells;[43] miR-30a-5p suppressed the epithelial–mesenchymal transition in SW480 cells by targeting integrin β3 (ITGB3);[44] and miR-212 was observed to inhibit the viability and invasion of HCT116 and SW620 cells via inhibition of the phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) expression.[45] An investigation of the role of the dysregulated miRNAs would definitely help to better understand the mechanism of enoxacin-mediated anticancer activity. However, the detailed molecular targets of particular tumor-suppressing or oncogenic miRNAs are beyond the scope of this entry and have been described elsewhere.[46][47][48][49][50]

3. The Effects on Non-Cancer Cells

It is worth mentioning that enoxacin affects not only cancer cells; it also increased the levels of miRNA related to the disease in the dominant negative TGF-β receptor (dnTGFβRII) CD8 cells from an autoimmune cholangitis mouse model. Despite the fact that enoxacin did not change the amount of CD8 T cells, it significantly decreased their proliferative response. Enoxacin also significantly decreased the level of interferon γ in mouse serum.[51] There is some research concerning enoxacin's impact on the neuronal system. Rats treated with 10 or 25 mg/kg enoxacin for 1 week were found to have elevated levels of miRNA related to the neuronal cell biology in their frontal cortex (**Table 2**). Those miRNAs include let-7, miRNA-124, miRNA-125 and miRNA-132. They are involved in the processes of neurogenesis (let-7, miR-124) and neuronal differentiation in human (miRNA-125) and mouse (miRNA-124) brains, the regulation of the dendritic spine length in mammalian neurons (miR-125NA), as well as neurite outgrowth (miRNA-132). The enoxacin-treated rats were also less likely to exhibit learned helplessness when they faced an inescapable shock.[52]

Enoxacin is also capable of affecting artificial miRNAs (amiRNAs), especially by enhancing the amiRNA-mediated reversible inhibition of the CRISPR-Cas9 system in both in vitro and in vivo studies. Interestingly, amiRNA alone did not show an inhibitory effect towards the CRISPR-Cas9 system, indicating the crucial role of enoxacin. In

contrast, some of the naturally occurring miRNAs were able to inhibit CRISPR-Cas9 activity by binding to single-guide RNA (sgRNA), a part of the sgRNA/Cas9 complex, in the absence of enoxacin. Surprisingly, the presence of enoxacin in concentrations up to 50 µM did not affect the natural miRNAs' influence on the CRISPR system. The proposed explanation of these differences in the impacts of enoxacin on the inhibition of natural and artificial miRNAs is based on the low binding capacity of amiRNAs towards RISC and the high binding capacity towards the RISC of natural miRNAs. Another factor could be the difference in the amounts of amiRNAs vs. natural miRNAs. The amiRNAs are believed to outnumber natural miRNAs. Taking the above into account, the amiRNA/enoxacin system turned out to be specific and reversible, making it a convenient tool for CRISPR-Cas9 regulation.[53]

In 2008, Shan et al. investigated the ability of microRNA processing to enhance some fluoroquinolones, including enoxacin and its three derivatives. They identified the structural elements of enoxacin responsible for its activity, such as a carboxyl group at the 3rd carbon atom, fluorine at the 7th carbon atom, as well as nitrogen at the 8th position of naphyridine.[21]

Table 1. The miRNA-regulating activity levels in in vitro studies depending on the different enoxacin concentrations.

miRNA	Conc. [µM]	Effect [↑/↓]	Expression Change Change-Fold	Cell Line	Ref.
Cancer cells					
let-7b-5p, miR-146a-5p, miR-689	50	↓	0.5–1	4T1 (miRNA from EV),	[35]
miR-100	124	↓	0.5–1	primary ESFT spheres	[23]
miR-141, miR-191	124	↓	1.5–2	DU145, LNcap,	[24]
miR-21-5p, miR-30a-3p, miR-30a-5p, miR-100-5p, miR-204-5p, miR-221-3p	124	↑	<1.5	Cal62, STA-ET-8.2, TPC1	[25]
Let-7f, miR-26a,	124	↑	<1.5	A673, SW1736	[23]
miR-21	100	↑	1.5–2	MCF7	[27]
miR-16, miR-18a*, miR-21, miR-26a, miR-29b, miR-29c, miR-31, miR-193a,	124	↑	1.5–2	HCT-116	[22]
let-7f, miR-26a, miR-99a, miR-100, miR-143, miR-145,	124	↑	1.5–2	A673, STA-ET-8.2, TC252, primary ESFT spheres	[23]
miR-21-5p, miR-30a-3p, miR-100-5p, miR-146b-5p, miR-221-3p,	124	↑	1.5–2	Cal62, SW1736, TPC1	[25]
miR-17 *, miR29b, miR-132, miR-146a, miR-191 miR-449a,	124	↑	1.5–2	DU145 LNcap,	[24]

miRNA	Conc. [μM]	Effect [↑/↓]	Expression Change Change-Fold	Cell Line	Ref.
miR-214-3p	50	↑	2–2.5	4T1 (cytosolic miRNA),	[35]
miR-145	100	↑	2–2.5	MCF7	[27]
miR-7, miR-16, miR-18a*, miR-29c, miR-101, miR-128, miR-181a, miR-212	124	↑	2–2.5	HCT-116, RKO	[22]
miR-100-5p, miR-146b-5p	124	↑	2–2.5	SW1736, TPC1	[25]
miR-34a, miR-449a	124	↑	2–2.5	DU145, LNcap	[24]
let-7f, miR-99a, miR-100, miR-145	124	↑	2–2.5	A673, STA-ET-8.2, TC252, primary ESFT spheres	[23]
miR-7, miR-26a, miR-29b, miR-30a, miR-101, miR-122, miR-125a, miR-125b, miR-126, miR-128, miR-143, miR-181b, miR-205	124	↑	2.5–3	HCT-116, RKO	[22]
miR-100, miR-145	124	↑	2.5–3	A673, TC252	[23]
miR-29b	124	↑	2.5–3	LNcap	[24]
let-7a, let-7b, miR-30a, miR-31, miR-126, miR-181b, miR-193a, miR-193b,	124	↑	3–3.5	HCT-116, RKO	[22]
let-7f, miR-143, miR-181a,	124	↑	3–3.5	A673, STA-ET-8.2, primary ESFT spheres	[23]
miR-181a, miR-193b	124	↑	3.5–4	HCT-116	[22]
let-7b, miR-143, miR-205	124	↑	4–4.5	HCT-116, RKO	[22]
miR-143	124	↑	4–4.5	TC252	[23]
miR-125a	124	↑	ca. 5	HCT-116	[22]
miR-214-3p	50	↑	ca. 22	4T1 (miRNA from EV)	[35]
Non-cancer cells					
miR-128-1	60	↓	0.5–1	dnTGFβRII T cells	[51]
let-7i, miR-128	50	↓	1.5–2	HEK293	[21]
let-7b, miR-23a, miR-30e, miR-96, miR-99a, miR-125a, miR-146, miR-190, miR-199a*,	50	↑	1.5–2	HEK293	[21]

miRNA	Conc. [μM]	Effect [↑/↓]	Expression Change Change-Fold	Cell Line	Ref.
miR-124a, miR-139, miR-152, miR-199b	50	↑	2–2.5	HEK293	[21]
miR-29b-1, miR-145a-5p, miR-326-3p	60	↑	2–2.5	dnTGFβRII T cells	[51]
miR-181a	60	↑	2.5–3	dnTGFβRII T cells	[51]

miRNA	Dose	Effect [↑/↓]	Expression Change: Change-Fold	Tissue	Ref.
miR-124	10 mg/kg 25 mg/kg	↑	ca. 4. ca. 6	rat frontal cortex	[52]
let-7a, miR-125a-5p	10 mg/kg 25 mg/kg	↑	ca. 11. ca. 20		
miR-132	10 mg/kg 25 mg/kg	↑	ca. 19 (for both doses)		
miR-30a-5p, miR-146b-5	15 mg/kg	↑	1.5–2	human orthotopic thyroid tumor from Cal62-luc mouse	[25]
mIR-100-5p, miR-30-3p, miR-204-5	15 mg/kg	↑	2–2.5	human orthotopic thyroid tumor from Cal62-luc mouse	[25]
miR-16, miR-18a*, miR-21, miR-26a, miR-29b, miR-29c, miR-31, miR-101, miR-193a	10 mg/kg	↑	1.5–2	tumor from HCT-116 mouse xenograft	[22]
miR-16, miR-29c, miR-31, miR-101, miR-181a	10 mg/kg	↑	1.5–2	tumor from RKO mouse xenograft	[22]
miR-128, miR-212	10 mg/kg	↑	2–2.5	tumor from HCT-116 mouse xenograft	[22]
miR-18a*, miR-21, miR-26a, miR-29b, miR-30a, miR-128	10 mg/kg	↑	2–2.5	tumor from RKO mouse xenograft	[22]
let-7b, miR-7, miR-143, miR-181b, miR-125b	10 mg/kg	↑	2.5–3	tumor from HCT-116 mouse xenograft	[22]
let-7a, miR-7, miR-122, miR-125a, miR-125b, miR-126, miR-181b, miR-193a,	10 mg/kg	↑	2.5–3	tumor from RKO mouse xenograft	[22]

miRNA	Dose	Effect [↑/↓]	Expression Change: Change-Fold	Tissue	Ref.
miR-193b, miR-205, miR-212					
let-7a, miR-30a, miR-122, miR-126	10 mg/kg	↑	3–3.5	tumor from HCT-116 mouse xenograft	[22]
miR-143	10 mg/kg	↑	3–3.5	tumor from RKO mouse xenograft	[22]
miR-125a, miR-181a, miR-193b	10 mg/kg	↑	3.5–4	tumor from HCT-116 mouse xenograft	[22]
let-7b	10 mg/kg	↑	4.5–5	tumor from RKO mouse xenograft	[22]
miR-205	10 mg/kg	↑	4.5–5	tumor from HCT-116 mouse xenograft	[22]

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