Novel Thiazolyl-Pyrazoline Analogs: Potential Role for Tyrosine Kinase Inhibition in Colorectal Cancer

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Abstract: Colorectal cancer (CRC) ranked the third and the second in the onset and mortality rates, respectively, among all types of cancer (GLOBOCAN 2020). In this study, we targeted epidermal growth factor receptor (EGFR) using two synthesized thiazolyl pyrazoline analogs in presence and absence of berberine as an adjuvant remedy. Preliminary results showed that these compounds exert anti-neoplastic and tyrosine kinase inhibitory effects against lung cancer cell lines. We assessed the anticancer activities of these compounds via quantitative determination of transcripts levels (qRT-PCR) of some apoptotic and proliferative markers in Caco-2 cell line. Our data showed a synergistic augmented effect between the tested compounds and berberine on the pro-apoptotic markers (BAX and p53). Furthermore, the oncogenes EGFR and c-MYC, as well as the anti-apoptotic gene ARC, exhibited downregulation trend. In conclusion, the novel compounds show promising results as potential TKI that inhibit malignant cell growth and restore cancer cells' apoptotic capacity.

Key words: Colorectal cancer, EGFR, tyrosine kinase inhibitors, thiazolyl-pyrazoline.

1. Introduction

Colorectal cancer is considered the third leading cause of cancer incidents since it accounts for 10% of annual worldwide cancer cases [1]. Geographically, the highest incidence rate is observed in developed countries while there is a continuous increase in developing countries. By this rate, approximately 2.5 million cases will be reached by 2035 [2]. Among the factors affecting the progression of colorectal cancer is epidermal growth factor receptor (EGFR) overexpression which results in a dysregulated signaling pattern that prevents apoptosis and promotes neoplastic cell growth, proliferation, and metastasis [3]. Once

epidermal growth factor (EGF) binds to EGFR through its extracellular region, dimerization and trans-autophosphorylation occur in the intracellular C-terminal tail at multiple tyrosine diverse residues. Consequently, these phosphorylated sites allow the docking and binding of substrate protein molecules, initiating a signaling cascade that triggers cell proliferation, angiogenesis, and metastasis [4].

Based on this, EGFR serves as an attractive target to constraint cell growth by using tyrosine kinase inhibitors (TKI) that prevent its phosphorylation and interrupt downstream pathways' activity [5]. It started from the early synthesized and clinically approved first-generation EGFR inhibitors that involve Gefitinib (Iressa®) and Erlotinib (Tarceva®). This was followed by second and third-generation TKI (Fig. 1) to overcome the acquired resistance in patients having mutations in the EGFR receptor, especially the most well-known point mutation in exon 20 that results in threonine substitution with methionine at position 790 [6]. However, drug resistance is constantly encountered, which urges the critical need to design novel safe and potent TKI to overcome this problem.

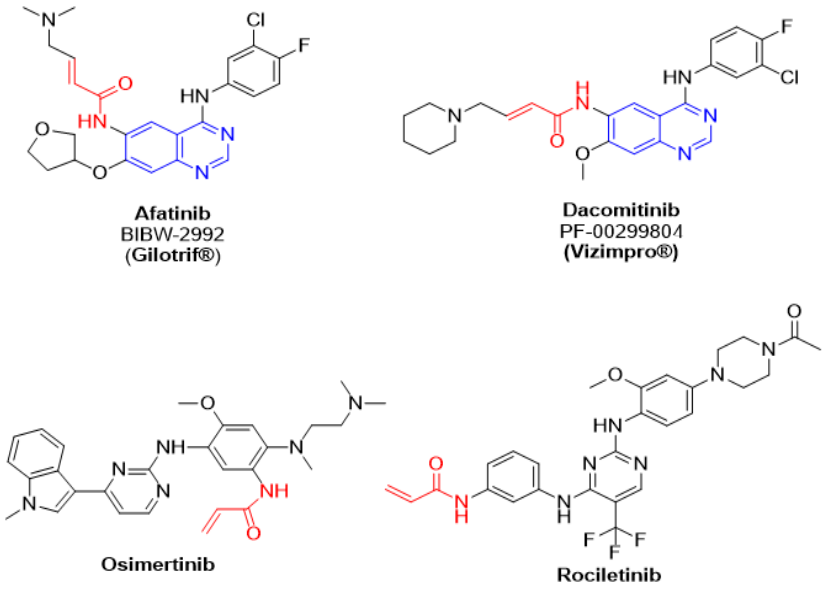

Fig. 1. Second and third generation EGFR inhibitors.

Pyrazolines are compounds containing a five-membered ring with an endocyclic double bond and two adjacent nitrogen atoms. Various pyrazoline-based compounds have been proclaimed to have anticancer and chemo-preventive properties. In addition, thiazole compounds have an essential scaffold with both electron-donating and electron-accepting groups. Thiazole derivatives showed an antiproliferative effect through interaction and inhibition of multiple molecular targets, including; receptor tyrosine kinases, serine/threonine kinases, Bcl-2 family, and non-receptor tyrosine kinases. Thus, scaffolds containing both pyrazoline and thiazole active centers is a promising approach to develop effective anti-neoplastic drugs [7].

Berberine (BBR) is a natural active component that belongs to the class of isoquinoline alkaloids found in Ranunculaceae and Papaveraceae plant families (Fig. 2). It is a well-known Chinese herbal medicine used to treat viral and bacterial diarrhea, parasitic intestinal infections, hypertension, and type 2 diabetes. Several studies showed that berberine has a noticeable inhibitory effect on different cancer types, including; lung, liver, prostate, breast, and gastrointestinal tract. It is associated with induction of apoptosis and regulation of signaling pathways related to tumor progressions such as EGFR activity, MAPK, P53, and NF-kB [8], [9].

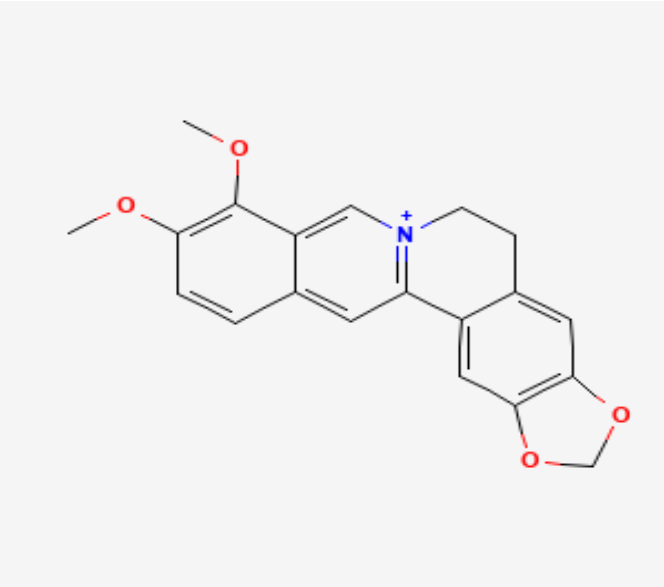

Fig. 2. Berberine chemical structure.

Based on the above-mentioned information, the synergistic effect of berberine with derivatives of thiazolyl-pyrazoline was tested to evaluate their effect on the EGFR and the apoptotic pathway in colorectal cancer. So, in our study, two novel potent thiazolyl-pyrazoline derivatives (**5B** and **5D**) were selected out of 14 compounds to test their anti-proliferative effect against colorectal cancer due to their superior potency and safety profiles as well as their dual activities on both EGFR and VEGFR-2. However, in this study we focused on assessing their EGFR inhibitory effect in presence of berberine as a natural adjuvant to enhance the effect of these synthetic compounds. Our aim is to test this combination on the expression of EGFR and several apoptotic markers.

2. Methods

2.1. Cell Culture Conditions

Dulbecco's Modified Eagle Medium (DMEM) (Biosera, France) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA) was used to culture colorectal adenocarcinoma (Caco2, HCT-116) cells and normal lung fibroblast (WI-38) in T-75 tissue culture flask (Corning, USA) at 37 ºC in the presence of 5% CO2 and 90% humidity.

2.2. MTT Assay

Ethyl-2-(3-(2,4-dichlorophenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-methylthiazole-5-carboxylate (**5B**) and 2-(3-(2,4-dichlorophenyl)-5-(3,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-methyl-N-phenylthiazole-5-carboxamide (**5D**) were synthesized according to a designed scheme (Abdelsalam *et al*., under publication) and dissolved in DMSO and Berberine was dissolved in water.

HCT116, Caco2 cells, and WI-38 cells were collected by trypsin (Biosera, France) and seeded in 96-multiwell culture plates (Corning, USA) upon 80% confluency is achieved. Different concentrations of the compounds were prepared by diluting the stock using a culture medium to treat the cells for 24 hours. The media was discarded, and cells were incubated for 4 hours with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Bio Basic, Canada) using a final concentration of 0.5mg/ml in each well. The formed formazan is dissolved in DMSO (VWR, USA), and the absorbance is read at 570 nm using an M965+ microplate reader (Metertech Inc., Taiwan).

2.3. Gene Expression Analysis

2.3.1. RNA extraction

Total RNA was extracted from drug-treated and untreated cultured cells by Trizol reagent (Thermofisher, USA) using the manufacturer's recommended instructions. The RNA purity and concentration were checked using the nanodrop (Thermofisher, USA) after dissolving in DEPC treated water.

2.3.2. cDNA synthesis

cDNA synthesis was performed to convert 2 µg of RNA using random primers using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, USA).

2.3.3. Primer design

Primers targeting the genes of interest were manually designed with the aid of NCBI. The following primers were used: EGFR, c-MYC, BAX, ARC, BAG-1, TGF-B, P53, and FAS (Table 1). The obtained results were normalized using the HPRT housekeeping gene. These primers have a length of 21-23 nucleotides and give a product length ranging from 105 bp to 190 bp. Also, they are located on different exons to avoid undesirable genomic amplification with an annealing temperature of approximately 60°C.

Table 1. Primer Sequence of Targeted Genes

Primer name	Forward primer	Reverse primer	Product size
EGFR	5'-CCTATCAAGTGGATGGCATTGG-3'	5'-GTGGCTGAGGGAGGCGTTCT-3'	181
c-MYC	5'-CTCGGATTCTCTGCTCTCCTC-3'	5'-CCTCATCTTCTTGTTCCTCCTC-3'	122
BAX	5'-GGTTGTCGCCCTTTTCTACTTTG-3'	5'-AGGAAGTCCAATGTCCAGCCCA-3'	105
ARC	5'-AAAGGGACGAGTCCGAAGATTC-3'	5'-GGAGTTTATTCACTTCCAGCGGT-3'	149
BAG-1	5'-AAGAGATGAATCGGAGCCAGGA-3'	5'-TGGGAGGTAACATGAAGGTCG T-3'	145
TGF-B	5'-AATTCCTGGCGATACCTCAGCA-3'	5'-TGAACCCGTTGATGTCCACTT G-3'	190
P53	5'-TTCGAGATGTTCCGAGAGCTGAA-3'	5'-GGAGGTAGACTGACCCTTTTT G-3'	123
FAS	5'-CTTTCACTTCGGAGGATTGCTCA-3'	5'-AGTTGATGTCAGTCACTTGGGCA-3'	123
HPRT	5'-CTGGCGTCGTGATTAGTGATGAT-3'	5'-AGCACACAGAGGGCTACAATGT-3'	187

2.3.4. Real-Time PCR

Quantitative real-time PCR was performed in a 10 µl reaction volume using GoTaq qPCR master mix (Promega INC, USA), equal volumes of cDNA, and the specific primer pairs using QuantStudio 3 real time PCR (ThermoFisher, USA). The amplification was done for 40 cycles by denaturation at 95º for 30 sec then annealing and extension at 60º for 1 min in addition to a preceding initial denaturing step for 2 mins at 95º. Melting curves data were collected after the amplification is done to ensure the specificity of the reactions. Livak method was applied to determine the fold change in gene expression of each marker in the treated cells compared to negative control cells. The calculated fold change was regarded as an upregulation for values above 1 and as a downregulation in case of values below 1.

3. Results

3.1. Cytotoxicity Assay Using MTT

The anti-proliferative potential of **5B** and **5D** was estimated by MTT assay by applying various concentrations of each drug on colorectal cell lines and the percentage of growth inhibition (GI%) was

measured after 24 h incubation. A higher inhibitory effect was observed in cells treated by **5D** compared to those treated by **5B**. Additionally, these drugs showed a good safety on normal WI-38 cell line with an IC-50 of 72.40±7.18 for 5B and 51.43±3.46 for **5B** and **5D** respectively.

3.2. Effect of Different Treatments on Gene Expression

We estimated the change in the transcript level of several selected genes in Caco-2 cells after 24 h treatment. Fig. 3 shows the fold change of signaling and apoptosis-related genes when cells were treated with **5D** (10 µM) and in combination with berberine (200 µM). We noticed that berberine enhanced the activity of **5D,** yielding a higher downregulating effect on EGFR (1.3-fold), TGF-B (1.3-fold), BAG-1 (1.5 fold), and the anti-apoptotic gene ARC (2.5-fold). Additionally, there is a notable increase in the expression of the pro-apoptotic gene BAX (2.2-fold), tumor suppressor p-53 gene (2.2-fold), and Fas (1.4-fold).

Furthermore, Fig. 4 illustrates the alteration in gene expression after treatment with **5B** (10 µM) individually and with berberine (200 µM). The collected data shows that the combination between **5B** and BBR gave a similar effect as that with **5D** but slightly increased the effect on Fas (1.75-fold increase) and ARC expression (2.7-fold decrease). Interestingly, c-MYC expression was only downregulated when **5B** and **5D** were mixed with berberine scoring a 1.19 and 1.32-fold change, respectively.

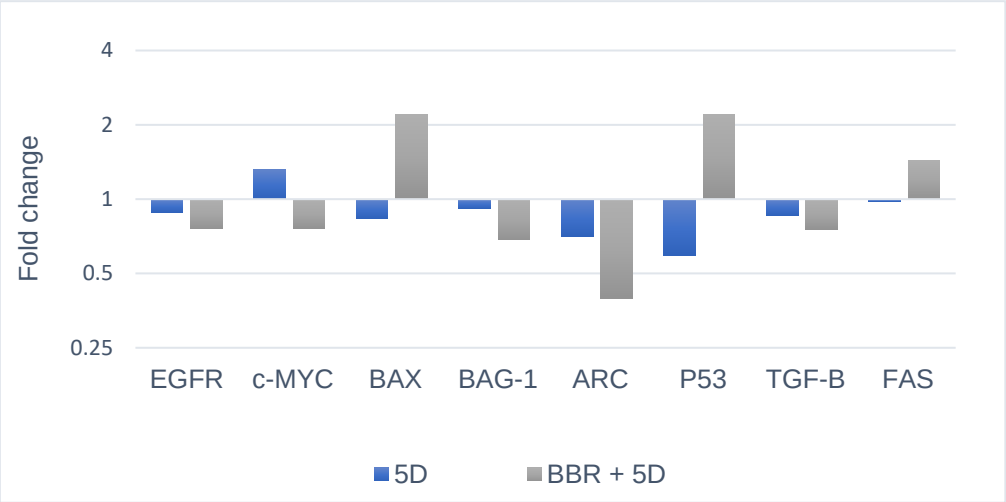

Fig. 3. Treatment by 5D and BBR.

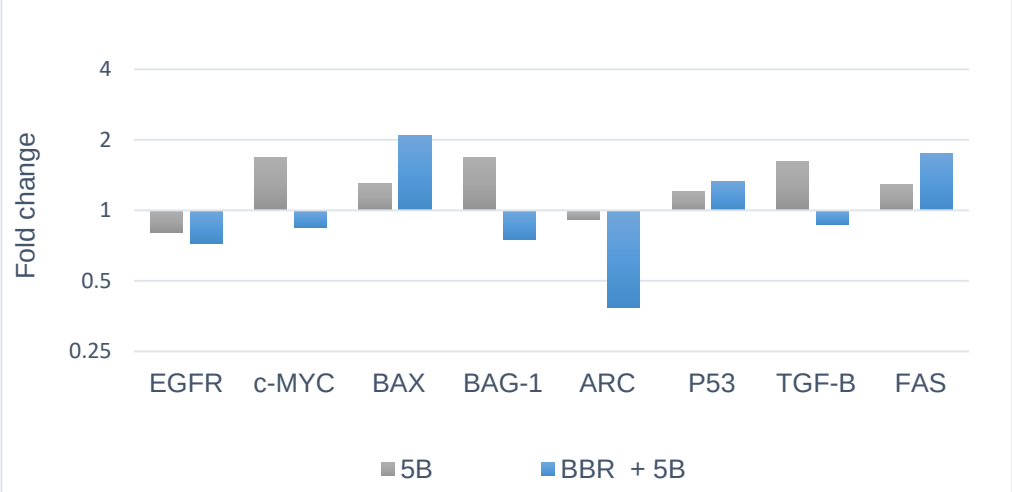

Fig. 4. Treatment by 5B and BBR.

4. Discussion

Recently, the number of patients developing colorectal cancer has increased, which raises a significant concern towards this disease. Our study selected the epidermal growth factor receptor as a target to aid in the treatment of CRC due to its prominent role in cell proliferation. We hypothesized that utilization of berberine as a natural adjuvant with thiazolyl pyrazoline derivatives would interrupt the aberrant signaling of the overexpressed EGFR. Additionally, the effect of treatment on some of the proapoptotic and anti-apoptotic markers was evaluated. When Caco-2 cells were treated with BBR mixed with either compound **5D** or **5B** for 24 hours, we noticed an additional downregulatory effect in EGFR expression compared to when thiazolyl-pyrazoline derivatives were administered alone. This can be attributed to the ability of **5B** and **5D** as thiazolyl-pyrazoline analogs to inhibit the tyrosine kinase activity of EGFR, especially that this class of drugs showed in previous research an inhibitory effect towards EGFR using enzyme assay [7].

Additionally, previous studies on gastric, prostate, and IMCE colorectal cancer cell lines, showed a significant decline in EGFR activity post-treatment with BBR [8], [10], [11]. Moreover, the observed downregulation of c-MYC could be attributed to the deactivation of the downstream MAPK-ERK pathway. A study revealed that knocking down the ternary complex transcription factors (TCF) gene would stop the expression of immediate early gene c-MYC. This supports the possible relation between ERK and c-MYC since ERK activates TCF transcriptional capability [12]. Furthermore, A study done on small cell lung cancer cell line, used a qRT-PCR analysis and revealed that when the transcription level of c-MYC is depleted, the mRNA levels of BAG1 decreases. This relation may be due to the capability of c-MYC to bind to the BAG-1 gene locus inducing its transcription and preventing the cell from undergoing apoptosis [13]. As a result, in our study, the observed downregulation of both c-MYC and BAG-1 is an indication of hindrance of cell proliferation and initiation of apoptosis. intriguingly, the decline in EGFR expression was accompanied by a decrease in TGF-β, which could be due to the presence of a direct relationship between them, as speculated by a study done on breast cancer. In that study, they showed an increase in the gene expression of EGFR when cells were treated with TGF-β. Also, this crosstalk enhanced the TGF-β-driven migration and invasion capability of cells [14].

P53 is an essential tumor suppressor that can control the transcription of the pro-apoptotic gene BAX that interrupts the anti-apoptotic proteins Bcl-2 and Bcl-xL, causing the release of cytochrome c from mitochondria [15]. Additionally, Fas involves a P53 responsive element that activates Fas transcription once P53 binds to it. Another research confirmed their relationship when Fas was upregulated four times upon transduction of cells with P53 [16]. This explains the upregulation of BAX and Fas pro-apoptotic genes in response to the upregulation of P53. Meanwhile, Arc is involved in intrinsic apoptotic pathways disruption through interaction with BAX impeding its ability to undergo conformational changes and movement to mitochondria. Additionally, it has a region rich with proline and glutamic acid that binds to a domain in P53 that is responsible for its tetramerization, which adversely affects its transcription capability and promotes its translocation to the cytoplasm due to exposure of the nuclear export signal of P53. A previous study that screened the expression of ARC in colon cell lines and tumor colon tissue found a noticeable level of ARC in approximately all tumor samples compared to their benign margins and in most tested cell lines. These pieces of information indicate that ARC could be implicated in anti-apoptosis in colorectal cancer and the observed decline supported the cells to undergo apoptosis [17]. The data obtained in our study shows that the combination of berberine and thiazolyl pyrazoline derivatives has a strong synergistic effect towards apoptosis induction and inhibition of proliferation.

5. Conclusion

EGFR overexpression in colorectal cancer plays a vital role in its progression since it enables the cells to proliferate and escape apoptosis. As a result, tyrosine kinase inhibitors represent a possible treatment

strategy to hinder the signaling pathways associated with EGFR by binding to its ATP binding sites. We aimed to investigate the potential of novel thiazolyl pyrazolines to inhibit EGFR expression and apoptosis induction when added with the natural isoquinoline berberine. Our results showed a good synergism where P53 was upregulated with its downstream transcription targets BAX and Fas, indicating apoptosis activation. Furthermore, EGFR and TGF-b were downregulated post-treatment suggesting a decline in cell proliferation and migration. Overall, we recommend combining berberine and thiazolyl pyrazoline derivatives to enhance colorectal cancer treatment.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Ayat Fayez conducted the experimental work, performed the data analysis, and initiated manuscript writing; Sherif F. Hammad and Doaa A. Ghareeb helped in supervision of the experimental work, data analysis, and manuscript revision; Maha Adel El Demellawy and Ahmed Osman contributed in conceptualization of the study, interpretation of the data, and manuscript revision; all authors had approved the final version.

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References

[1] Sung, H., Ferlay, J., Siegel, R. L., *et al.* (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 71(3)*, 209-249.

[2] Dekker, E., Tanis, P. J., Vleugels, J. L. A., *et al.* (2019). Colorectal cancer. *The Lancet, 394(10207)*, 1467–1480.

[3] Wee, P., & Wang, Z. (2017). Epidermal growth factor receptor cell proliferation signaling pathways. C*ancers, 9(5)*, 1-45.

[4] Seshacharyulu, P., Ponnusamy, M. P., Haridas, D., *et al.* (2012). Targeting the EGFR signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets, 16(1)*, 15-31.

[5] Xie, Y. H., Chen, Y. X., & Fang, J. Y. (2020). Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction and Targeted Therapy, 5(1)*, 1-30.

[6] George, R. F., Samir, E. M., Abdelhamed, M. N., *et al.* (2019). Synthesis and anti-proliferative activity of some new quinoline based 4,5-dihydropyrazoles and their thiazole hybrids as EGFR inhibitors. *Bioorganic Chemistry, 83*, 186-197.

[7] Sever, B., Altıntop, M. D., Radwan, M. O., *et al.* (2019). Design, synthesis and biological evaluation of a new series of thiazolyl-pyrazolines as dual EGFR and HER2 inhibitors. *European Journal of Medicinal Chemistry, 182*, 111648.

[8] Wang, L., Cao, H., Lu, N., *et al.* (2013). Berberine inhibits proliferation and down-regulates epidermal growth factor receptor through activation of Cbl in colon tumor cells. *PLoS ONE, 8(2)*, e56666.

[9] Xie, J., Xu, Y., Huang, X., *et al.* (2015). Berberine-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species generation and mitochondrial-related apoptotic pathway. *Tumour Biology, 36(2)*, 1279-1288.

[10] Huang, Z., Zheng, H., Wang, W., *et al.* (2015). Berberine targets epidermal growth factor receptor signaling to suppress prostate cancer proliferation in vitro. *Molecular Medicine Reports, 11(3)*, 2125-2128.

[11] Wang, J., Yang, S., Cai, X., *et al.* (2016). Berberine inhibits EGFR signaling and enhances the antitumor effects of EGFR inhibitors in gastric cancer. *Oncotarget, 7(46)*, 76076-76086.
[12] Gualdrini, F., Esnault, C., Horswell, S., *et al.* (2016). SRF co-factors control the balance between cell proliferation and contractility. *Molecular Cell, 64(6)*, 1048–1061.
[13] Zhang, X. Y., Pfeiffer, H. K., Mellert, H. S., *et al.* (2011). Inhibition of the single downstream target BAG1 activates the latent apoptotic potential of MYC. *Molecular and Cellular Biology, 31(24)*, 5037-5045.
[14] Zhao, Y., Ma, J., Fan, Y., *et al.* (2018). TGF-β transactivates EGFR and facilitates breast cancer migration and invasion through canonical Smad3 and ERK/Sp1 signaling pathways. *Molecular Oncology, 12(3)*, 305-321.
[15] Abraha, A. M., & Ketema, E. B. (2016). Apoptotic pathways as a therapeutic target for colorectal cancer treatment. *World Journal of Gastrointestinal Oncology, 8(8)*, 583-591.
[16] Munsch, D., Watanabe-Fukunaga, R., Bourdon, J. C., *et al.* (2000). Human and mouse fas (APO-1/CD95) death receptor genes each contain a p53-responsive element that is activated by p53 mutants unable to induce apoptosis*. *Journal of Biological Chemistry, 275(6)*, 3867-3872.
[17] Mercier, I., Vuolo, M., Jasmin, J. F., *et al.* (2008). ARC (apoptosis repressor with caspase recruitment domain) is a novel marker of human colon cancer. *Cell Cycle, 7(11)*, 1640-1647.

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