



## Evaluation the Anti-proliferative Effect of NVP-AUY922 in Combination with Thymoquinone in Colorectal Cancer Cell Line

Attabak Toofani-milani<sup>1\*</sup>, Mahshid Mohammadian<sup>\*2</sup>, Roghayeh Paribanaem<sup>3</sup>

<sup>1</sup> Department of Medical Laboratory Sciences, Faculty of Medicine, Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran

<sup>2</sup> Department of Clinical Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

<sup>3</sup> Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

**\*Corresponding authors: Attabak Toofani-milani, Address:** Department of Medical Laboratory Sciences, Faculty of Medicine, Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran, **Email:** Toofani2020@yahoo.com, **Tel:** +989370715898

**Mahshid Mohammadian, Address:** Department of Clinical Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran, **Email:** Biochem426@gmail.com.

### Abstract

**Background & Aims:** Thymoquinone (TQ) is a natural component and the active herbal complex originate in *Nigella sativa* seed. TQ shows the anti-cancer effects in the previous studies. The effects of TQ, its mechanism on colorectal cancer, and its combination with other newly chemotherapeutic agents are unclear. Heat shock protein 90 (HSP90) has been upregulated in the numbers of malignancies. In this survey, we investigated the impacts of TQ and NVP-AUY922 (a HSP90 inhibitor) on HT-29 colorectal cancer cell line.

**Materials & Methods:** HT-29 cells were seeded and exposed to TQ and NVP-AUY922 for 24 hours in various concentrations. Cell viability (water-soluble tetrazolium-1) assay was performed. Moreover, in combination cases, various concentrations of both agents examined using cellular viability analysis.

**Results:** The TQ significantly inhibited cancer cell growth in colorectal cancer cell line in combination with various concentration of NVP-AUY922. Treatment with TQ could augment the cytotoxicity of NVP-AUY922 against the HT-29 as compared with that of NVP-AUY922 alone.

**Conclusion:** Our findings suggested the anti-proliferative effect of TQ and NVP-AUY922 through cytotoxic pathway to induce cell death.

**Keywords:** NVP-AUY922, Thymoquinone, Colorectal Cancer Cell Line.

Received 10 August 2021; accepted for publication 12 November 2021

### Introduction

Cancer is one of the main problems and cause of death after cardiovascular diseases in numerous countries (1, 2). Colorectal cancer (CRC) considered as

one of the common cancer among the others worldwide (3) 5-fluorouracil based chemotherapy remains the mainstay of treatment for CRC (3-5). Recently, chemotherapy agents including oxaliplatin, irinotecan,

and capecitabine have been developed (3, 6). Regardless of advances in cytotoxic and targeted therapy, ineffective chemotherapy remains as one of the major challenge in long-term managing of lethal metastatic disease and ultimately contributes to higher patient mortality (7). Heat shock protein 90 (HSP90) family are the family ubiquitous molecular chaperone proteins that engaged in folding, activation, maturation, and assembly of numerous proteins, and comprised essential mediators of signal transduction and cell cycle progression. HSP90 proteins have been upregulated in a number of malignancies. HSP90 inhibition can affect multiple oncogenic pathways and related proteins, and consequently could be considered as a smart target for drug development (8). The use of HSP 90 inhibitors has been recently introduced as an attractive anticancer therapy (9-13). NVP-AUY922 is a newly defined resorcinolic isoxazole-based HSP90 inhibitor which displayed potent preclinical efficacy in the treatment of cancer models (13).

Current study proposed the new insights into the usage of natural bioactive compounds to overcome chemoresistance in colon cancer chemotherapy (14). Thymoquinone (TQ) is the active herbal complex originated from *Nigella sativa* (NS) seed. According to previous surveys, numerous medical properties of TQ have been confirmed. In this regards NS has considerable phytochemical and pharmacological properties. Several animal and cell-based surveys have been performed, and some of them confirmed anti-inflammatory, and anti-cancer, effects of TQ (15, 16). However, specific impacts of exposure of CRC cells to TQ in combination with NVP-AUY922 have not been fulfilled in previous studies. This study was conducted to investigate the effects of various modulations of NVP-AUY922 and TQ on cellular toxicity and cellular viability of the HT-29 human colorectal cancer cells.

## Materials & Methods

### Chemicals and cell culture:

The CRC cell line; HT29 was obtained from the Pasteur Institute (Tehran, Iran). Cells were cultured in DMEM medium (Biowest, France) with the mixture of

10% fetal bovine serum (Biowest, France), 0.1% streptomycin, penicillin, and were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. TQ was obtained from Sigma and the stock solution prepared by dissolving TQ in pure water and diluted with the DMEM.

### Cell viability assay:

Cell proliferation was examined using the water-soluble tetrazolium-1 (WST-1) assay, according to the manufacturer's protocols.

Cells were mono-treated with NVP-AUY922 or TQ for 24 h, and co-treated with NVP-AUY922 and TQ in various combinations and concentrations. After 24 h, cellular viability of treated cells in response to TQ and NVP-AUY922 was investigated by WST-1. After plating, treatments were performed for 24 h in various groups (different concentrations) as described below;

I (TQ; 50 µM; NVP-AUY922;50 nM), II (TQ; 40 µM; NVP-AUY922;40 nM), III (TQ; 20 µM; NVP-AUY922; 20nM), IV (TQ; 10 µM; NVP-AUY922; 10nM) and V (TQ; 5 µM; NVP-AUY922; 5nM). VI group (control) was included the untreated control cells.

After treatment for 24 h, WST-1 assay performed as cellular viability test in all treatments. For this, 10 µl of WST-1 stock added to wells. After 3–4 hours at 37°C, cell viability was examined based on the cleavage of the tetrazolium salt, WST-1, to dark red formazan.

The absorbance was read at 420 nm with a reference wavelength > 650 by the enzyme-linked immunosorbent assay (ELISA) microplate reader.

Three or more independent experiments were performed for WST viability assay. The values were shown as the mean ± standard deviation. The statistical significance among different groups was determined using one-way ANOVA.

## Results

In this presented study, impacts of single treatments of NVP-AUY922 (1, 5, 10, 20, 50, and 100 nM)- or TQ (5, 10, 20, 25, 50, and 100 µM)- on the viability of HT29 were assessed. Both agents showed the weaker inhibitory impact in lower doses on HT-29, although the

inhibitory ratios for NVP-AUY922 in nM concentrations were higher than those for TQ ( $\mu\text{M}$ ) (Figure 1A and 1B).

The cytotoxicity of NVP-AUY922 and TQ in single treatments were increased in the dose dependent patterns (Figure 1A, 1B).

The weak cytotoxicity of NVP-AUY922 and TQ against the HT-29 cells at low concentrations were presented.

Consequently, the inhibitory impacts of various combination treatments with exposure to low concentrations of NVP-AUY922 and TQ in HT29 after 24 h were evaluated (Figure 2).

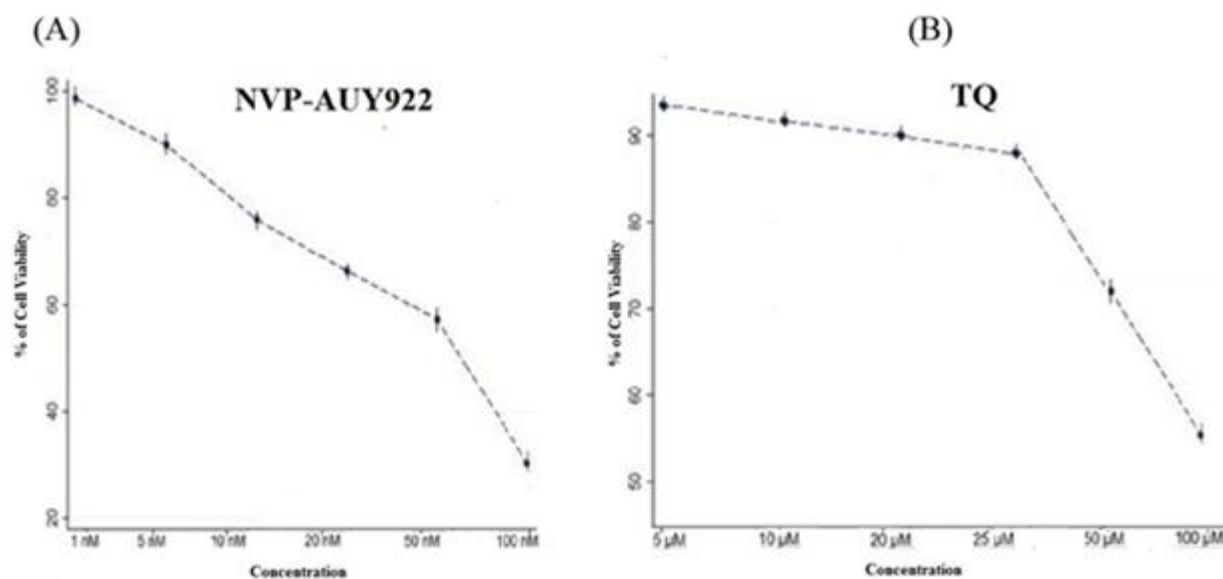
Comparison of inhibitory impacts of various combinations at the same time (TQ and NVP-AUY922), showed that there were more effective cellular inhibitory compared to single treatments (Figure 2). Data indicated that among combination groups [I (TQ; 50  $\mu\text{M}$ ; NVP-

AUY922;50 nM) II (TQ; 40  $\mu\text{M}$ ; NVP-AUY922;40 nM) III (TQ; 20  $\mu\text{M}$ ; NVP-AUY922; 20 nM), IV (TQ; 10  $\mu\text{M}$ ; NVP-AUY922; 10 nM) and V (TQ; 5  $\mu\text{M}$ ; NVP-AUY922; 5 nM). VI group (control)] there were significantly higher toxicity in group I compared to groups II, III, IV, V and VI ( $p < 0.05$ ).

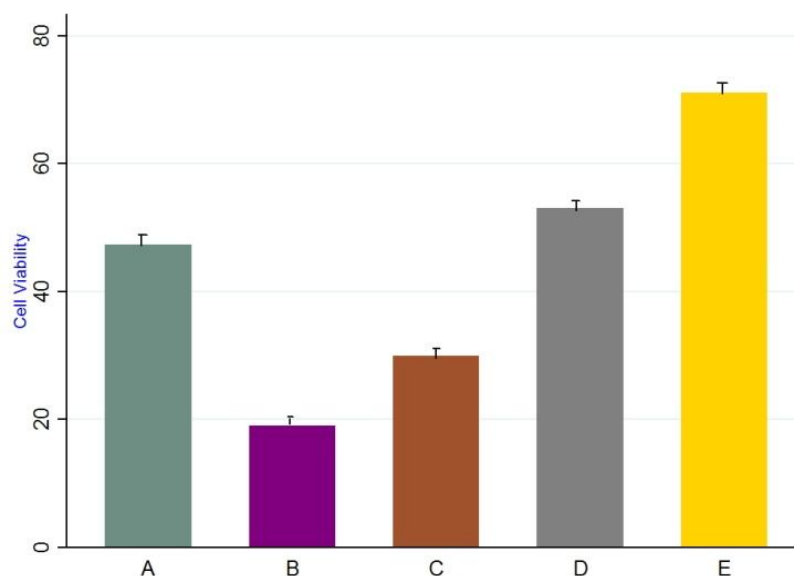
Also group II exerted significant toxic effects compared to groups III, IV, and V. Moreover, group II had lower cytotoxic effect versus group I. Besides, group III had more significant inhibitory effects compared to groups V.

Further analysis showed the lower cellular viability in group V versus control group ( $p < 0.05$ ).

Treatment with TQ could augment the cytotoxicity of NVP-AUY922 against the HT-29 as compared with that of NVP-AUY922 alone.



**Fig 1:** Results of cellular viability assay in HT-29 cells treated with NVP-AUY922 or TQ in single treatments. Growth-inhibitory curves of HT-29 cells treated to gradient concentrations of NVP-AUY922 (A) or TQ (B) in single treatments. Data presented as mean  $\pm$  standard deviation. TQ: Thymoquinone.



**Fig 2:** Results of cell viability assay of combination treatments with TQ and NVP-AUY922 in HT-29 cells. Concentrations of NVP-AUY922 in combination with TQ were chosen based on the initial tests described in the methods to assess the cytotoxic impacts of combinations on the HT-29 cell line.

A: (TQ 20  $\mu$ M; NVP-AUY922 20 nM), B: (TQ 50  $\mu$ M; NVP-AUY922 50 nM), C: (TQ 40  $\mu$ M; NVP-AUY922 40 nM), D: (TQ 10  $\mu$ M; NVP-AUY922 10 nM) and E: (TQ 5  $\mu$ M; NVP-AUY922 5 nM). Data presented as mean  $\pm$  standard deviation. TQ: Thymoquinone.

## Discussion

Traditional medicines are usually without harmful side effects, and are generally are low-priced. TQ, an ingredient of *Nigella sativa* (NS), considered as one of this safe and efficacious agents (15, 16).

TQ interferes in a wide range of tumorigenic processes and counteracts carcinogenesis, malignant growth, invasion, migration, and angiogenesis. Moreover, TQ can specifically sensitize tumor cells toward conventional cancer treatments (e.g., radiotherapy, chemotherapy, and immunotherapy), and simultaneously minimize therapy-associated toxic effects in normal cells (17).

To better explain its functions, we aimed to assess the anti-proliferative effects of TQ as a natural component in combination with NVP-AUY922 after 24h exposure in HT-29 cell line. For this, we assessed whether treatment with TQ and NVP-AUY922 may increase the cellular toxicity in HT-29 CRC cell line.

Based on results of our study using cellular viability assay, the TQ displayed strong cytotoxicity impacts

when combined with low concentration of NVP-AUY922 in nM range.

The results showed that the few ranges of concentrations, from 5 to 50 nM of NVP-AUY922 and 5-50  $\mu$ M of TQ, gained the considerable possible toxicity and these ranges can be utilized to find the effective concentrations of these agents for treatments of CRC. In these concentrations, significant cytotoxic effects in compared to control group were presented in HT-29 cancer cell line. In this regards, the cellular viability in the TQ and NVP-AUY922 treated cells were lower than the untreated group and single treatments. Therefore, it could be understood that these combinations might had considerable effect in the CRC cell death induction.

In accordance to our data, previous studies showed that after administration of TQ, the significant growth inhibition has been displayed in the breast, gastric, and colon cancer (18-20).

In a study done by Pazhouhi et. al. it was confirmed that TQ synergistically increased the anti-cancer effects

of temozolomide in the glioblastoma cell line (21). Also, Khazaei et. al. described synergistic apoptotic cell death of glioblastoma cells upon treatment with TQ in combination with chemotherapy (22). Likewise, TQ in combination with 5-FU inhibited the expression of pro-cancerous NF- $\kappa$ B, iNOS, VEGF, Wnt,  $\beta$ -catenin, COX-2, and TBRAS with a concomitant elevation in anti-tumorigenic TGF- $\beta$ 1, TGF- $\beta$ RII, DKK-1, CDNK-1A, Smad4, and GPx expression (23). Similarly, Fröhlich et. al. displayed the elevated ROS production and induction of DNA damage in human colon cancer cells that exposed to TQ and artemisinin (24).

Also, TQ was established to chemosensitize 5-FU in gastric cancer treatment by inducing apoptosis and (19).

We suggest that the ability of TQ to effectively increase the cytotoxic effects of NVP-AUY922 denotes the probable efficacy of these combinations in inhibiting cellular viability of CRC cells. TQ has been shown to have higher toxicity in combined to NVP-AUY922 in different doses.

As TQ significantly decreases the viability of human colon cancer cells in a concentration- and time-dependent manner, and TQ increased apoptosis induction is related to the upregulation of Bax and inhibition of Bcl-2 and Bcl-xl expression, so it has been indicated that TQ also activated caspase-9,-7, and -3 (25).

It seems that it would be an ideal agent to utilize in combination with chemotherapy to increase the efficacy of chemotherapy in CRC.

In conclusion, chemo-sensitizing effects of TQ in CRC cells proposed the potential treatment to the success of CRC chemotherapy via increase of cancer cell growth inhibition.

Indeed, TQ which effectively increased the cytotoxic effects of NVP-AUY922 is likely to inhibit the growth of the chemo-resistance cells. Further studies proposed to evaluate various combinations of NVP-AUY922 and TQ and their effects on reduction of subsequent relapse following chemotherapy.

### Acknowledgments

Thanks to Dr.Sadegh Feizollah Zadeh who helped us with this research.

### Conflict of interest

The authors have no conflict of interest in this study.

### References

1. Mohamadi N, Kazemi SM, Mohammadian M, Milani AT, Moradi Y, Yasemi M. Toxicity of cisplatin-loaded poly butyl cyanoacrylate nanoparticles in a brain cancer cell line: Anionic polymerization results. *Asian Pac J Cancer Prev* 2017;18(3):629.
2. Arshad Z, Rezapour-Firouzi S, Mohammadian M, Ebrahimifar M. The sources of essential fatty acids for allergic and cancer patients; a connection with insight into mammalian target of rapamycin: A narrative review. *Asian Pac J Cancer Prev* 2018;19(9):2391.
3. Van der Jeught K, Xu H-C, Li Y-J, Lu X-B, Ji G. Drug resistance and new therapies in colorectal cancer. *World J Gastroenterol* 2018;24(34):3834.
4. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, et al. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000;6(4):1322-7.
5. Showalter SL, Showalter TN, Witkiewicz A, Havens R, Kennedy EP, Hucl T, et al. Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil: Is it time to move forward? *Cancer Biol Ther* 2008;7(7):986-94.
6. Yaffee P, Osipov A, Tan C, Tuli R, Hendifar A. Review of systemic therapies for locally advanced and metastatic rectal cancer. *J Gastrointest Oncol* 2015;6(2):185.
7. Hammond WA, Swaika A, Mody K. Pharmacologic resistance in colorectal cancer: a review. *Ther. Adv. Med. Oncol* 2016;8(1):57-84.
8. Usmani SZ, Bona R, Li Z. 17 AAG for HSP90 inhibition in cancer-from bench to bedside. *Curr. Mol. Med* 2009;9(5):654-64.
9. Mohammadian M, Zeynali S, Azarbaijani AF, Ansari MHK, Kheradmand F. Cytotoxic effects of the newly-developed chemotherapeutic agents 17-AAG in combination with oxaliplatin and capecitabine in colorectal cancer cell lines. *Research in pharmaceutical sciences* 2017;12(6):517.

10. Mohammadian M, Zeynali-Moghaddam S, Ansari MHK, Rasmi Y, Azarbayjani AF, Kheradmand F. Dihydropyrimidine dehydrogenase levels in colorectal cancer cells treated with a combination of heat shock protein 90 inhibitor and oxaliplatin or capecitabine. *Adv. Pharm. Bull* 2019;9(3):439.
11. Zeynali-Moghaddam S, Mohammadian M, Kheradmand F, Fathi-Azarbayjani A, Rasmi Y, Esna-Ashari O, et al. A molecular basis for the synergy between 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan in human colorectal cancer cells through VEGF and MMP-9 gene expression. *Gene* 2019;684:30-8.
12. Moradi Z, Mohammadian M, Saberi H, Ebrahimifar M, Mohammadi Z, Ebrahimpour M, et al. Anti-cancer effects of chemotherapeutic agent; 17-AAG, in combined with gold nanoparticles and irradiation in human colorectal cancer cells. *DARU J. Pharm. Sci* 2019;27(1):111-9.
13. Mayor-López L, Tristante E, Carballo-Santana M, Carrasco-García E, Grasso S, García-Morales P, et al. Comparative study of 17-AAG and NVP-AUY922 in pancreatic and colorectal cancer cells: are there common determinants of sensitivity? *Transl. Oncol* 2014;7(5):590-604.
14. Zhang P, Lai Z-L, Chen H-F, Zhang M, Wang A, Jia T, et al. Curcumin synergizes with 5-fluorouracil by impairing AMPK/ULK1-dependent autophagy, AKT activity and enhancing apoptosis in colon cancer cells with tumor growth inhibition in xenograft mice. *J. Exp. Clin. Cancer Res* 2017;36(1):1-12.
15. Motaghd M, Al-Hassan FM, Hamid SS. Cellular responses with thymoquinone treatment in human breast cancer cell line MCF-7. *Pharmacogn. Res* 2013;5(3):200.
16. Gali-Muhtasib H, Roessner A, Schneider-Stock R. Thymoquinone: a promising anti-cancer drug from natural sources. *Int. J. Biochem. Cell Biol* 2006;38(8):1249-53.
17. Mostofa A, Hossain MK, Basak D, Bin Sayeed MS. Thymoquinone as a potential adjuvant therapy for cancer treatment: evidence from preclinical studies. *Front pharmacol* 2017;8:295.
18. Gali-Muhtasib H, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, et al. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med* 2008;12(1):330-42.
19. Lei X, Lv X, Liu M, Yang Z, Ji M, Guo X, et al. Thymoquinone inhibits growth and augments 5-fluorouracil-induced apoptosis in gastric cancer cells both in vitro and in vivo. *Biochem. Biophys. Res. Commun* 2012;417(2):864-8.
20. Woo CC, Hsu A, Kumar AP, Sethi G, Tan KHB. Thymoquinone inhibits tumor growth and induces apoptosis in a breast cancer xenograft mouse model: the role of p38 MAPK and ROS. *PloS one* 2013;8(10):e75356.
21. Pazhouhi M, Sariri R, Rabzia A, Khazaei M. Thymoquinone synergistically potentiates temozolomide cytotoxicity through the inhibition of autophagy in U87MG cell line. *Iran. J. Basic Med. Sci* 2016;19(8):890.
22. Khazaei M, Pazhouhi M. Temozolomide-mediated apoptotic death is improved by thymoquinone in U87MG cell line. *Cancer Invest* 2017;35(4):225-36.
23. Kensara OA, El-Shemi AG, Mohamed AM, Refaat B, Idris S, Ahmad J. Thymoquinone subdues tumor growth and potentiates the chemopreventive effect of 5-fluorouracil on the early stages of colorectal carcinogenesis in rats. *Drug Des. Devel. Ther* 2016;10:2239.
24. Fröhlich T, Ndreshkjana B, Muenzner JK, Reiter C, Hofmeister E, Mederer S, et al. Synthesis of novel hybrids of thymoquinone and artemisinin with high activity and selectivity against colon cancer. *Chem Med Chem* 2017;12(3):226-34.
25. Kundu J, Choi BY, Jeong C-H, Kundu JK, Chun K-S. Thymoquinone induces apoptosis in human colon cancer HCT116 cells through inactivation of STAT3 by blocking JAK2-and Src-mediated phosphorylation of EGF receptor tyrosine kinase. *Oncol. Rep* 2014;32(2):821-8.