OTJHS
Online Turkish Journal of Health Sciences

e-ISSN: 2459-1467

OTSBD
Online Türk Sağlık Bilimleri Dergisi

Online Turkish Journal of Health Sciences 2022;7(1):117-122 *Online Türk Sağlık Bilimleri Dergisi 2022;7(1):117-122*

Traf2 ve Nck Etkileşimli Protein Kinaz (TNIK) Inhibitörünün Metastatik Köpek Meme Tümör Hücrelerinde Apoptotik Etkisinin Belirlenmesi

Determination of the Apoptotic Effect of Raf2 and Nck-Interacting Protein Kinase (TNIK) Inhibitor on Metastatic Canine Mammary Gland Tumor Cells

[1]Asuman DEVECİ ÖZKAN, [2]Ayten HACIEFENDİ, [2]Fatih ÖZKAN, [1]Gamze GÜNEY ESKİLER, [1]Süleyman KALELİ, [1]Ecir Ali ÇAKMAK, [3]Özge TURNA

[1] Department of Medical Biology, Faculty of Medicine, Sakarya University, Sakarya, Turkey
[2] Department of Medical Biology, Institute of Health Science, Sakarya University, Sakarya, Turkey
[3] Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa, Istanbul, Turkey

Asuman Deveci Özkan: https://orcid.org/0000-0002-3248-4279
Ayten Haciefendi: https://orcid.org/0000-0001-7071-5624
Fatih Özkan: https://orcid.org/0000-0002-4658-8968
Gamze Güney Eskiler: https://orcid.org/0000-0002-2088-9914
Süleyman Kaleli: https://orcid.org/0000-0002-6043-2521
Ali Ecir Çakmak: https://orcid.org/0000-0003-2735-2105
Özge Turna: https://orcid.org/0000-0002-7638-0519

ÖZ

Amaç: Köpek meme tümörleri (KMT) dişi köpeklerde en sık görülen tümörlerdir ve kullanılan başlıca tedavi seçeneği olarak cerrahi ciddi komplikasyonlara neden olmaktadır. Bu nedenle KMT için yeni tedavi seçeneklerine ihtiyaç duyulmaktadır. Traf2 ve Nck ile etkileşime giren serin protein kinaz (TNIK) WNT hedef genlerinin transkripsiyonel bir düzenleyicisidir ve meme kanseri gelişiminde yüksek oranda eksprese edilmektedir. TNIK'in inhibisyonu, anormal WNT sinyali olan kanserlerde yeni bir terapötik hedef olabilir. Bu nedenle, bu çalışmada NCB-0846'nın metastatik sarkom KMT alt tipi üzerindeki potansiyel terapötik etkisini ilk kez araştırılması amaçlanmıştır.

Materyal ve Metot: NCB-0846'nın KMT hücreleri üzerindeki sitotoksik ve apoptotik etkileri, WST-1, Annexin V, hücre döngüsü, akridin oranj (AO) ve DAPI boyama ile analiz edilmiştir.

Bulgular: Elde edilen verilere göre NCB-0846, doza ve zamana bağlı anlamlı bir şekilde KMT hücre canlılığını azaltmış ve nükleer hasara sebep olmuştur. Ayrıca, NCB-0846, G0/G1 fazında hücrelerin birikmesi yoluyla apoptotik hücre ölümünü indüklemiştir.

Sonuç: Sonuç olarak elde ettiğimiz bulgular, NCB-0846'nın potansiyel olarak KMT için yeni bir terapötik anti-kanser ajan olabileceğini göstermektedir. Bununla birlikte KMT hücreleri üzerinde NCB-0846'nın TNIK ve Wnt sinyalinin inhibe edici aktivitesinin aydınlatılmasına yönelik daha ileri araştırmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Köpek meme tümörleri, apoptoz, TNIK inhibitörü

ABSTRACT

Objective: Canine mammary gland tumors (CMGTs) are the most common tumors in female dogs and the main treatment options used in CMGTs are surgery caused some complications. Therefore, new treatment options are needed for the CMGTs. Traf2 and Nck-interacting serine protein kinase (TNIK) as a transcriptional coregulator of Wnt targeted genes is highly expressed in breast cancer development. The inhibition of TNIK may be a new therapeutic target in cancers with abnormal WNT signaling. Therefore we aimed to investigate the potential therapeutic effect of NCB-0846 on metastatic sarcoma CMGTs subtype, for the first time.

Materials and Methods: The cytotoxic and apoptotic effects of NCB-0846 on CMGT cells were analyzed by WST-1, Annexin V, cell cycle, acridine orange (AO) and DAPI staining.

Results: NCB-0846 significantly inhibited cell viability in a dose and time dependent manner ($p<0.05$) and induced nuclear damage in CMGT cells. Furthermore, NCB-0846 caused apoptotic cell death through the accumulation of cells in the G0/G1 phase.

Conclusion: Our findings demonstrated that NCB-0846 could be potentially a new therapeutic anti-cancer agent in the treatment of CMGTs. However, further investigations need to be performed in order to elucidate the inhibitory activity of TNIK and Wnt signaling by NCB-0846 on CMGT cells.

Keywords: Canine mammary gland tumor cells, apoptosis, TNIK inhibitor

Sorumlu Yazar / Corresponding Author:
Asuman Deveci Ozkan
Department of Medical Biology, Faculty of Medicine, Sakarya University, 54290, Korucuk, Adapazarı, Sakarya, Turkey.
Tel: +902642954297
E-mail: deveci@sakarya.edu.tr

Yayın Bilgisi / Article Info:
Gönderi Tarihi/ Received: 15/12/2021
Kabul Tarihi/ Accepted: 10/01/2022
Online Yayın Tarihi/ Published: 01/03/2022

Atıf / Cited: Deveci Özkan A et al. Determination of the Apoptotic Effect of Raf2 and Nck-Interacting Protein Kinase (TNIK) Inhibitor on Metastatic Canine Mammary Gland Tumor Cells. *Online Türk Sağlık Bilimleri Dergisi 2022;7(1):*117-122. doi: 10.26453/otjhs.1036628

INTRODUCTION

Canine mammary gland tumors (CMGTs) are the most common tumors after skin tumors in female dogs and account for approximately 52% of all tumors.[1-4] CMGTs are subclassified (sarcomas, carcinomas, and carcinosarcomas) according to histopathological differentiation.[5] Mammary gland sarcomas with clear borders, hard consistency and a large area in dogs account for approximately 10-15% of all CMGTs.[6] The main treatment options used in CMGTs are surgery, radiotherapy, chemotherapy and immunotherapy whereas surgery that causes some complications is the first choice.[7] Therefore, new treatment options are needed for the increasing incidence of CMGTs, which have very similar properties with human breast tumors.

Traf2 and Nck-interacting serine protein kinase (TNIK) as a transcriptional coregulator of Wnt targeted genes is a center kinase that encodes 1360 amino acids and highly expressed in breast cancer development.[8,9] Studies have shown that an increase in TNIK expression and the abnormal activation of the Wnt/β-catenin signaling pathway plays an important role in the formation and development of breast cancer.[10-12] Therefore, the inhibition of TNIK may be a new therapeutic target in cancers with abnormal WNT signaling.[13]

Furthermore, increased TNIK expression in various types of cancer (pancreatic, colorectal and hepatocellular carcinoma) is associated with poor prognosis in patients.[10,14,15] Based on these findings, TNIK inhibition is important and various classes of TNIK inhibitors have been developed for this purpose.[13,16] In this context, many studies have been conducted to develop new TNIK inhibitors, recently.[8,13,17] Padgaonkar et al. report that ON108600 is a dual inhibitor of CK2 and TNIK and shows inhibitory activity in MDA-MB-231 cells.[17] Additionally, Masuda et al. demonstrate that NCB-0001 induces the expression of LC3 that is a marker of autophagy in PAMC82 human gastric cancer and T47D human breast cancer cell lines.[13] As a new TNIK inhibitor NCB-0846 inhibits the TGFβ1-induced EMT in NSCLC cells.[8] However, there is no study in the literature evaluating the effects of NCB-0846 as a TNIK inhibitor on CMGT cells.

Therefore, in our study, the potential therapeutic effect of NCB-0846, a new type of TNIK inhibitor, was investigated for the first time on metastatic sarcoma CMGTs subtype. CMGT cells were diagnosed as liposarcoma with high metastatic capacity based on the criteria defined by Goldschmidt et al.[18]

MATERIALS AND METHODS

Ethical Statement: The female dog was operated for cancer treatment, not for experimental procedure. Tissue material removed by operation was used in the study. This was in conformity with the "Regulation on Working Procedures and Principles of Animal Experimental Ethics Committees Prepared by the Turkish Ministry of Environment, Urbanisation and Climate Change published in the Official Gazette of the Republic of Turkey dated February 2014 and numbered 28914".

***Cell Culture Conditions*:** CMGT which was used in this study was a 22-year-old intact French Bulldog female dog, weighing 16 kg with an 8-month history of mammary mass (>5 cm) and diagnosed as metastatic liposarcoma. Primary cell isolation and culture from CMGT tissue was conducted as described by Turna et al.[19]

Cell Viability Assay: To determine the cytotoxic effects of NCB on CMGT cell, we performed WST-1 assay. For this purpose, the equal number of cells ($2x10^4$ cell/well) were cultured in 96 well plate. Then the cells were incubated with different concentrations of NCB-0846 (1, 2, 2.5, 3, 4 and 5 μM) as a TNIK inhibitor for 24 and 48h. After treatment of NCB-0846, WST-1 dye was added into each well and incubated for 45 min at 37 °C in the dark and the absorbance was obtained with the microplate reader (Allsheng, China) at 450 nm. According to WST-1 results, the most effective NCB-0846 concentrations and incubation time (reduced viability by approximately 50%) were determined for further experiments. Each experiment was performed in triplicate.

Annexin V Assay: To determine the apoptotic effects of NCB-0846 on CMGT cell, we performed Annexin V assay. For this purpose, the equal number of cells ($1x10^5$ cell/well) were cultured in 6 well plate. Then the cells were incubated with the most effective concentrations of NCB-0846 (2.5 and 5 μM) for 48h according to the WST-1 assay results. After treatment of NCB-0846, the cells were stained with Annexin V reagent (Millipore, Germany) and incubated for 30 min. Stained cells were analyzed with Muse Cell Analyzer (Millipore, Germany). Each experiment performed was in triplicate.

Cell Cycle Assay: To further analyze the effects of NCB-0846 on cell cycle arrest in CMGT cells, the equal number of cells ($5x10^5$ cell/well) were cultured in 6 well plate. Then the cells were incubated with the most effective concentrations of NCB-0846 (2.5 and 5 μM) for 48h according to the WST-1 assay results. After treatment of NCB-0846, the cells were fixed with 70% ethyl alcohol and fixed cells were stained with Cell Cycle Kit (Millipore, Germany). After incubation for 30 min, the stained cells were analyzed with Muse Cell Analyzer (Millipore, Germany). Each experiment was performed in triplicate.

Acridine Orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) Staining: To determine the morphological changes of cell and nucleus on CMGT cells, we performed AO and DAPI staining. For this purpose the equal number of cells ($5x10^5$ cell/well) were cultured in 6 well plate. Then the cells were incubated with the most effective concentrations of NCB-0846 (2.5 and 5 µM) for 48h according to the WST-1 assay results. After treatment of NCB-0846, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min and fixed cells were stained with AO or DAPI dye for 30 min in the dark, separately. Stained cells were visualized by EVOS Floid Cell Imaging System (Thermo Fisher Scientific).

Statistical Analysis: All statistical analysis was performed by GraphPad Prism Version 8. One-way ANOVA with the Post-hoc Tukey test was used for multiple comparisons. $p < 0.05$ was considered significant.

RESULTS

According to the WST-1 analysis results, the viability of cells were decreased in a dose and time dependent manner and shown in Figure 1. The viability of CMGT cells significantly reduced to 76.05±1.93%, 77.34±0.82%, 59.08±0.21%, 58,69±0.74%, 55.73±1.69% and 39.48±1.23% at concentration of 1, 2, 2.5, 3, 4 and 5 µM NCB-0846, respectively for 48 h as shown in Figure 1. Thus, we selected 2.5 and 5 µM NCB-0846 within 48 h for further experiments, in CMGT cells.

To determine the apoptotic effect of NCB-0846 on CMGT cells, we conducted Annexin V assay and shown in Figure 2.

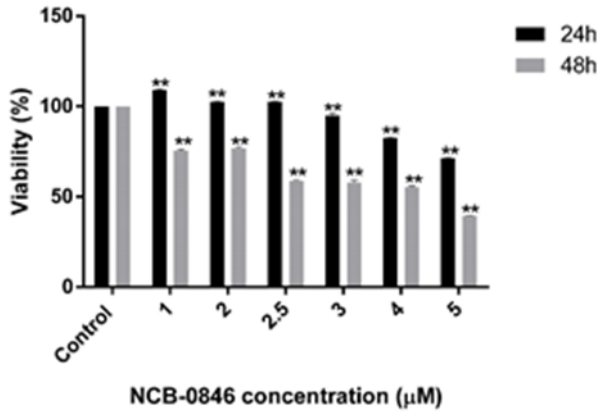

Figure 1. The cytotoxic effects of NCB-0846 was determined by WST-1 analysis on CMGT cells and the cells were treated with different concentrations of NCB-0846 (1, 2, 2.5, 3, 4 and 5 µM) for 24 and 48h ($p < 0.01$**).

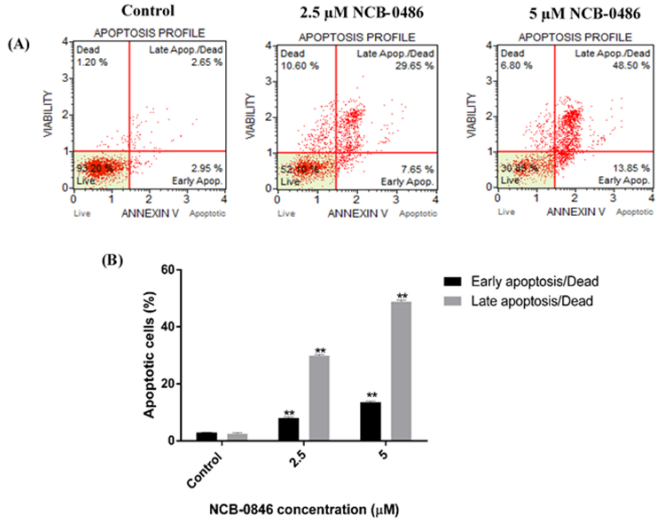

Figure 2. The apoptotic effect of NCB-0846 on CMGT cells was determined by Annexin V analysis (A) Annexin V histograms of CMGT cells treated with 2.5 and 5 µM concentration of NCB-0846, (B) Statistical comparison of the NCB-0846-induced apoptotic cell death ($p<0.01$**).

Our results demonstrated that NCB-0846 increased the proportion of total apoptotic cells especially late apoptotic cells as shown in Figure 2A. Following administration of 2.5 and 5 µM NCB-0846, the late apoptotic cells were significantly increased from 2.65±1.87%, to 29.65±1.53% and 48.50±2.13% respectively for 48h as shown in Figure 2B. Therefore, these results were consistent with the WST-1 results. Our results indicated that NCB-0846 treatment resulted in G0/G1 arrest for 48 h in CMGTs cells as shown in Figure 3A. The accumulation of CMGT cells in the G0/G1 phase increased significantly from 61.6±1.47% to 65.2±0.79 % and 73.7±1.86%, at 2.5 and 5 µM NCB-0846 for 48 h as shown in Figure 3B. Therefore, the NCB-0846 treatment resulted in G0/G1 phase arrest and apoptotic cell death.

After treatment with NCB-0846, typical apoptotic morphological changes were observed such as membrane blebbing and cell shrinkage in the cell especially at 5 µM concentration for 48h as shown in Figure 4. The DAPI staining results demonstrated that irregular nuclear shrinkage and condensation were observed in CMGT cells after treatment with both 2.5 and 5µM NCB-0846. Therefore our findings were consistent with the Annexin V and cell cycle assay results.

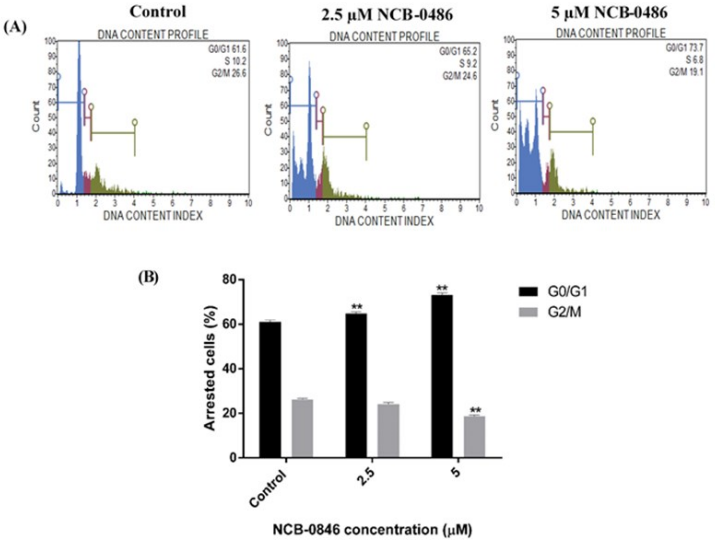

Figure 3. The effect of NCB-0846 on cell cycle arrest was determined by cell cycle analysis (A) Cell cycle histograms of CMGT cells treated with 2.5 and 5 µM concentration of NCB-0846, (B) Statistical comparison of the NCB-0846-induced cell cycle arrest level ($p<0.01$**).

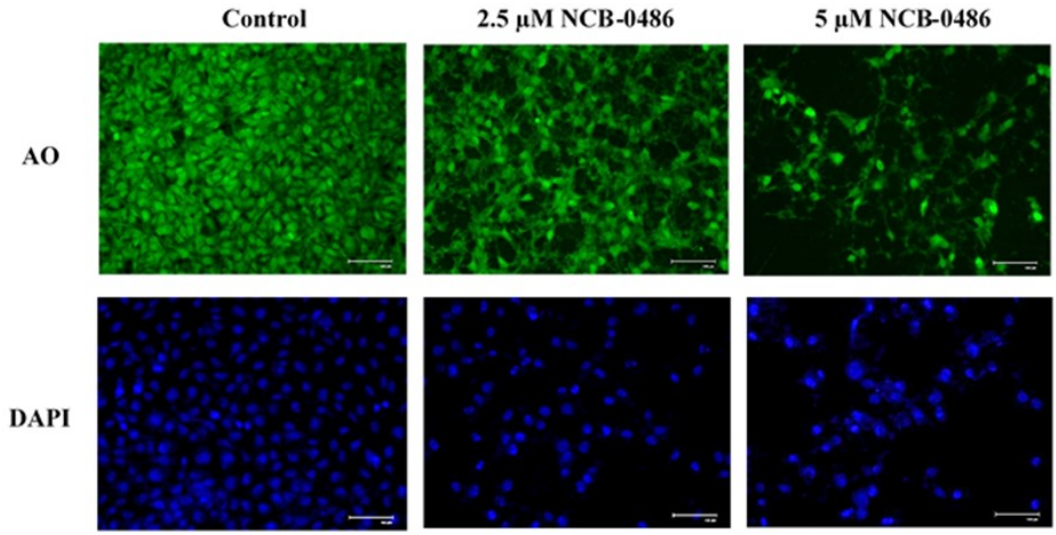

Figure 4. To determine the morphological changes of cell and nucleus on CMGT cells, we performed AO and DAPI staining. CMGT cells were treated with 2.5 and 5 µM concentration of NCB-0846 and stained with AO and DAPI dye (Scale bar: 100 µm).

DISCUSSION AND CONCLUSION

In this study, the potential cytotoxic and apoptotic effects of NCB-0846 were revealed in CMGT cells. According to our findings, NCB-0846 inhibited cell viability in a dose and time dependent manner and exhibited nuclear damage on CMGT cells. Furthermore, NCB-0846 induced apoptotic cell death through the accumulation of cells in the G0/G1 phase. Therefore, our findings demonstrated that NCB-0846 could be potentially a new therapeutic anti-cancer agent on CMGTs.

CMGTs are more aggressive than tumors in humans, and therefore chemotherapy is insufficient as a treatment option and only surgical intervention can be performed. For this reason, chemotherapy used in the treatment of CMGTs is not preferred due to the excess of side effects and thus there is an urgent need to new treatment options for better outcome. According to our results, NCB-0846 showed anticancer properties via the inhibition of cell proliferation, the induction of apoptosis, G0/G1 cell cycle arrest and nuclear damage in CMGT cells. The therapeutic effects of TNIK inhibition with small-molecule compounds have been demonstrated in the literature.[8,20-22] Kim et al.[22] state that NCB-0005 inhibits TGFβ1-induced activation of Wnt signaling in A549 lung adenocarcinoma cells and Sawa et al.[21] demonstrate that ON108600 which is a CK2 (casein kinase-2)/TNIK dual inhibitor targets stem-like cancer cells. On the other hand, NCB-0846 is a newly identified small molecule compound with high inhibitory activity against TNIK and shows antitumoral properties by inhibiting Wnt signaling.[20] Sugano et al.[8] shows that NCB-0846 inhibits TGFβ/SMAD signaling and EMT induction in A549 cells. Additionally, NCB-0846 suppresses the expression of Wnt targeted genes and Wnt-driven tumorigenesis in colorectal cancer cells. Therefore, our results were inconsistent with the literature.

In conclusion, we evaluated the therapeutic effect of NCB-048 at different concentrations on metastatic CMGT cells, for the first time. Our results indicated that the administration of NCB-0846 in CMGT cells induced early apoptosis and G0/G1 arrest. Consequently, as a new TNIK inhibitor, NCB-0846 has an anti-cancer potential agent on CMGT cells. However, further investigations need the elucidation of the inhibitory activity of TNIK and Wnt signaling by NCB-0846 on CMGT cells as well as human breast cancer.

Ethics Committee Approval: The study does not need an ethical approval. The bitch was operated for cancer treatment, not for experimental procedure. Tissue material removed by operation was used in the study. This was in conformity with the "Regulation on Working Procedures and Principles of Animal Experimental Ethics Committees Prepared by the Turkish Ministry of Environment, Urbanisation and Climate Change published in the Official Gazette of the Republic of Turkey dated February 2014 and numbered 28914".

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – ADO, AH, FO; Supervision – GGE, OT, AEC, SK; Materials – ADO, AH, FO; Data Collection and/or Processing – ADO, GGE, OT, AEC, SK; Analysis and/or Interpretation – ADO, AH, FO, GGE; Writing – ADO, OT, AEC, SK.

Peer-review: Externally peer-reviewed.

REFERENCES

1. Moulton JE. Tumors of the Mammary Gland, Moulton JE (ed), Tumors in Domestic Animals, 3 rd Ed. California; University of California Press; 1990.
2. Doré M, Lanthier I, Sirois J. Cyclooxygenase-2 expression in canine mammary tumors. Vet Pathol. 2003;40(2):207-212. doi:10.1354/vp.40-2-207
3. Hellmén E. Complex mammary tumours in the female dog: a review. J Dairy Res. 2005. doi:10.1017/s002202990500124x
4. Saba CF, Rogers KS, Newman SJ, Mauldin GE, Vail DM. Mammary gland tumors in male dogs. J Vet Intern Med. 2007;21(5):1056-1059. doi:10.1892/0891-6640(2007)21[1056:mgtimd]2.0.co;2
5. Sleeckx N, de Rooster H, Veldhuis Kroeze EJ, Van Ginneken C, Van Brantegem L. Canine mammary tumours, an overview. Reprod Domest Anim. 2011;46(6):1112-1131. doi:10.1111/j.1439-0531.2011.01816.x
6. Serin G, Aydoğan A. Chondrosarcoma in the mammary gland of a bitch: A case report, Veterinarni Medicina. 2009;54(11):543-546.
7. Soderstrom MJ, Gilson SD. Principles of surgical oncology. Vet Clin North Am Small Anim Pract. 1995;25(1):97-110. doi:10.1016/s0195-5616(95)50007-5
8. Sugano T, Masuda M, Takeshita F, et al. Pharmacological blockage of transforming growth factor-β signalling by a Traf2- and Nck-interacting kinase inhibitor, NCB-0846. Br J Cancer. 2021;124(1):228-236. doi:10.1038/s41416-020-01162-3
9. Sun Y, Gao X, Wu P, et al. Jatrorrhizine inhibits mammary carcinoma cells by targeting TNIK mediated Wnt/β-catenin signalling and epithelial-mesenchymal transition (EMT). Phytomedicine. 2019;63:153015. doi:10.1016/

j.phymed.2019.153015
10. Jin J, Jung HY, Wang Y, et al. Nuclear expression of phosphorylated TRAF2- and NCK-interacting kinase in hepatocellular carcinoma is associated with poor prognosis. Pathol Res Pract. 2014;210(10):621-627. doi:10.1016/j.prp.2013.10.007
11. Shitashige M, Satow R, Jigami T, et al. Traf2- and Nck-interacting kinase is essential for Wnt signaling and colorectal cancer growth. Cancer Res. 2010;70(12):5024-5033. doi:10.1158/0008-5472.CAN-10-0306
12. Mahmoudi T, Li VS, Ng SS, et al. The kinase TNIK is an essential activator of Wnt target genes. EMBO J. 2009;28(21):3329-3340. doi:10.1038/emboj.2009.285
13. Masuda M, Sawa M, Yamada T. Therapeutic targets in the Wnt signaling pathway: Feasibility of targeting TNIK in colorectal cancer. Pharmacol Ther. 2015;156:1-9. doi:10.1016/j.pharmthera.2015.10.009
14. Zhang Y, Jiang H, Qin M, Su X, Cao Z, Wang J. TNIK serves as a novel biomarker associated with poor prognosis in patients with pancreatic cancer. Tumour Biol. 2016;37(1):1035-1040. doi:10.1007/s13277-015-3881-5
15. Takahashi H, Ishikawa T, Ishiguro M, et al. Prognostic significance of Traf2- and Nck- interacting kinase (TNIK) in colorectal cancer. BMC Cancer. 2015;15:794. doi:10.1186/s12885-015-1783-y
16. Yamada T, Masuda M. Emergence of TNIK inhibitors in cancer therapeutics. Cancer Sci. 2017;108(5):818-823. doi:10.1111/cas.13203
17. Sato K, Padgaonkar AA, Baker SJ, et al. Simultaneous CK2/TNIK/DYRK1 inhibition by 108600 suppresses triple negative breast cancer stem cells and chemotherapy-resistant disease. Nat Commun. 2021;12(1):4671. doi:10.1038/s41467-021-24878-z
18. Goldschmidt MH, Shofer FS, Smelstoys JA. Neoplastic lesions of the mammary gland. In: Mohr U (ed) Pathobiology of the aging dog. Iowa State University Press; 2001:168–178.
19. Turna O, Baykal A, Sozen Kucukkara E, et al. Efficacy of 5-aminolevulinic acid-based photodynamic therapy in different subtypes of canine mammary gland cancer cells. Lasers Med Sci. 2021. doi:10.1007/s10103-021-03324-y
20. Masuda M, Uno Y, Ohbayashi N, et al. TNIK inhibition abrogates colorectal cancer stemness. Nat Commun. 2016;7:12586. doi:10.1038/ncomms12586
21. Kim J, Moon SH, Kim BT, Chae CH, Lee JY, Kim SH. A novel aminothiazole KY-05009 with potential to inhibit Traf2- and Nck-interacting kinase (TNIK) attenuates TGF-β1-mediated epithelial-to-mesenchymal transition in human lung adenocarcinoma A549 cells. PLoS One. 2014;9(10):e110180. doi:10.1371/journal.pone.0110180