Ind. J. Pharm. Edu. Res., 2026; 60(3):1224-1232.
https://www.ijper.org

Original Article

A Glimpse of the Interaction between N-Nitrosamines and HPV E5 in Rodent Cell Transformation

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ABSTRACT

Background: A few epidemiologic surveys and experimental studies observed the synergistic carcinogenic effect of nitrosamines and High-Risk Human Papillomavirus (HPV) in partial digestive tract cancers. They mainly focused on the co-carcinogenesis of HPV E6 and E7 in cancer development. HPV E5 plays an important role in early-stage cancer but has limited capacity as an independent carcinogen. Objective: To investigate whether nitrosamines enhance the carcinogenesis of E5, a rapid in vitro rodent cell transformation assay was established. **Materials and Methods:** We have successfully established BALB/c 3T3-E5 cells expressing HPV16 E5 using a lentivirus vector. Six foodborne N-nitroso Compounds (NOCs) are selected to induce malignant cell transformation. **Results:** We have screened two compounds with strong transformation capability, N-nitrosodimethylamine and N-nitrosodiethylamine alone. The combined action of NOCs and E5 is highly efficient, with more transformed foci than a single NOC or E5 oncoprotein, and the synergistic effect becomes more evident with increasing concentrations of NOCs, especially N-nitrosodipropylamine. Furthermore, we also confirm the working concentration range of 0.05-0.1 ng/mL of NOCs to meet the demands for cell viability in carcinogenicity tests. **Conclusion:** In conclusion, we provide cell models for studying the interaction mechanism of HPV16 E5 and NOCs, as well as assessing preventive or therapeutic candidates for HPV-positive tumors in the future.

Keywords: Human Papillomavirus16, E5 oncoprotein, N-nitrosamines, BALB/c 3T3 cell, Synergy effect, Carcinogenicity.

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Received: 22-12-2025;
Revised: 19-02-2026;
Accepted: 06-04-2026.

INTRODUCTION

Cancer development is a complicated process in which multiple tumor initiators and promoters participate and act together, such as chemical, biological, and physical carcinogens. Some high-risk factors, such as air pollution,[1] smoking,[2] diet,[3] and pathogens,[4,5] have an etiological relationship with upper digestive tract tumors. Many epidemiological surveys discovered that diet is a major contributor to esophageal cancer in China.[6,7] Various foods such as salted dried fish, fermented pickled vegetables, and salted meat, etc., are prevalent in some regions with specific dietary cultures in China. These foods are rich in nitrate and nitrite, which are risk factors for digestive tract cancer. Nitrate and nitrite as precursors are endogenously metabolized into nitrosamine and nitrosamide, well-known potent carcinogens.[8] Investigators detected the accumulation of nitrosamine in the food and gastric environments of residents from high-incidence areas of tumors, indicating a positive correlation between nitrosamine levels and the incidence of cancers, like esophageal cancer. In addition, the high levels of disinfection byproducts in drinking water[9] and tobacco-specific nitrosamines[10] are related to a high risk of cancer. Hence, it is evident that nitrosamine is one of the direct causes of digestive tract tumors.

However, most research mainly focus on the role of nitrosamines as independent risk factors. The studies on the cooperative effect of nitrosamines with other carcinogenic factors are relatively scarce. The interactions between environmental and biological carcinogens are primary. For instance, aflatoxin B1 enhances carcinogenicity in conjunction with the hepatitis B virus,[11] and polycyclic aromatic hydrocarbons also exhibit a synergistic carcinogenic effect with the Epstein-Barr virus.[12] High-risk HPVs, especially types 16 and 18, have been demonstrated as a pathogen of cervical and other genital tract cancers. HPV has been detected in a subset of head and neck and esophagus tumors, and HPV infection is suggested as a risk factor for these tumors.[13] However, the causal role of HPV, especially in esophageal tumors, has been

DOI: 10.5530/ijper.20263287

inconclusive because HPV has limited carcinogenic potency as an independent cause. At present, rare epidemiological studies have reported that smoking and HPV infection can increase the risk of esophageal cancer.[14] There is little evidence about the synergistic effects of foodborne nitrosamines and HPV in esophageal cancer. The E6 and E7 oncoproteins encoded by HPV are well characterized by their oncogenic activities.[15,16] The E6 and E7, in cooperation with nitrosamine chemicals, promote the malignant transformation of human esophageal epithelial cells.[17,18] HPV E5 oncoprotein plays an important role in tumor onset[19,20] and displays weak transforming activity *in vitro*.[21,22] There have been no reports about whether nitrosamine chemicals can enhance the transformation potency of E5 in cell and animal models.

Therefore, we intend to establish an E5-expressed rodent cell model and an *in vitro* transformation assay to examine the synergistic effect of nitrosamines and HPV E5. Six N-nitroso compounds, especially N-Nitrosodiethylamine (NDEA), N-Nitrosodimethylamine (NDMA), which are common in the diet in China's regions, are applied to rapidly test the cytotoxicity and carcinogenicity at less than 100-fold actual concentrations in drinking water.[23]

MATERIALS AND METHODS

Cell line and *in vitro* culture

The Tumor Room of the Institute for Viral Disease Control of the Chinese Center for Disease Control and Prevention provided HEK293 cells and BALB/c 3T3 cells. These cells were fed Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S), and 1% Glutamine (Gln) and maintained at 37°C with a 5% CO_2 atmosphere. DMEM medium, FBS, and agents were purchased from Hyclone. The HEK293 cells and BALB/c 3T3 cells were authenticated by Short Tandem Repeat (STR) genotyping. The reports of the STR genotype were submitted as supplementary data. All experiments were performed with mycoplasma-free cells. The detection results of mycoplasma were provided as supplementary data.

Construction of recombinant lentiviral vector HPV16 E5-pLVX-Puro

The HPV16 E5 Open Reading Frame (ORF) was amplified with primers: forward, 5'-CCGGAATTCGCCACCATGACAAAT

CTTGATACTGCATCC-3', 16E5 reverse, 5'-CGCGGATCCTTAATGATGATGATGATGATGTGTA

ATTAAAAAGCGTGCATGT-3'. The amplicon was tagged with C-His sequence (ATGATGATGATG

ATGATG) and cloned into the pLVX-Puro plasmid using EcoR I (GAATTC) and BamH I (GGATCC) restriction endonucleases (New England Biolabs). The HPV16 E5-pLVX-Puro recombinant plasmid was constructed. HEK293 cells were co-transfected with recombinant and helper plasmids using FuGENE^R HD transfection reagent (Promega, Madison, WI, USA). The Tumor Laboratory of the Chinese Center for Disease Control and Prevention gifted SL2, SL3, and SL4 helper plasmids and pLVX-Puro plasmid. The culture supernatant was collected and concentrated after 48 hr of transfection. The lentivirus stocks were obtained and stored at -80°C for long-term storage.

Establishment of BALB/c 3T3-E5 cells

BALB/c 3T3 cells were plated in a 6-cm dish and maintained for 24 hr. The cells were transduced with HPV16 E5-pLVX-Puro and cultured for 48 hr. Subsequently, the transduced cells were selected with 1 μg/mL puromycin to obtain the BALB/c 3T3-E5 cells stably expressing His-tagged E5. The BALB/c 3T3 cells transduced with empty pLVX-Puro are named as mock BALB/c 3T3.

Total RNA was isolated from the transduced cells using the TRIzol agents (Sigma Aldrich, St. Louis, MO, USA). The cDNA was synthesized using a two-step RT-PCR kit SuperScriptTM (Promega, A3500, Madison, WI, USA). The cDNA as a template was amplified using PCR with HPV16 E5 primers. The amplicons were visualized using gel electrophoresis to analyze the gene expression. Total proteins were isolated from transduced cells. The expression levels of His-tagged E5 fusion protein were subsequently analyzed by Western blotting, using His tag antibody (ZSGB-BIO, TA-02) and GAPDH antibody (ZSGB-BIO, TA-08).

Toxicity test of N-Nitroso compounds

An online platform, ADMETlab 2.0, was introduced to predict the toxicity of N-Nitroso Compounds. Detailed operation procedures can be found in the references.[24] Simplified Molecular Input Line Entry System (SMILES) of N-Nitrosodipropylamine (NDPA), N-Nitrosopyrrolidine (NPYR), N-Nitrosodiethylamine (NDEA), N-Nitrosodimethylamine (NDMA), N-Nitrosomorpholine (NMOR), and N-Nitrosopiperidine (NPIP) were input into ADMETlab 2.0. The Toxicity parameters were exported into a sheet. The output value of AMES (Ames Bacterial Reverse Mutation Assay) is the probability of being genotoxic. The output value of Carcinogencity is the probability of being carcinogenic.

The six NOCs were purchased from J&K Scientific. The chemical structures are shown in Figure 1. These compounds were reconstituted to a 1 mg/mL stock solution in DMSO and then diluted into the indicated working concentrations with a culture medium. BALB/c 3T3 and BALB/c 3T3-E5 cells were seeded at 2×10^2 cells/well in a 96-well plate, respectively, and maintained for 24 hr. The cells were transferred into the culture medium containing 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng/mL NOCs and maintained for 4 days. The cell viability was detected using a CCK-8 kit (Sigma-Aldrich, 96992, St. Louis, MO, USA). The cells treated with DMSO were designated as a blank control.

Two-stage cell transformation assay

1×10^4 BALB/c 3T3-E5 and BALB/c 3T3 cells, respectively, were seeded in a 25mm bottle, cultured in DMEM for 24 hr, and then exposed to six individual NOCs as tumor initiators at different concentrations for 3 days, followed by incubation in free-NOCs DMEM for an additional 4 days. Then, cells were transferred to DMEM/F12(1:1) containing 0.2% ITES supplement (Sigma-Aldrich, St. Louis, MO, USA) and 2% FBS. The cells were exposed to 0.1 μg/mL 12-O-Tetradecanoyl-Phorbol-13-Acetate (TPA) as a promoter for 7 days. Following this, the cells were continuously cultured in DMEM/F12 (1:1) for an additional 28 days until transformation occurred. The protocol was modified from the previous study.[25] Following a 6-week culture period, the cells were subjected to methanol fixation and stained with Giemsa. The morphologically aberrant foci can be identified and scored as a photo catalogue.[26] The transformation foci were counted and presented as mean±standard error of the mean.

RESULTS

Construction of the recombinant lentivirus vector HPV16 E5-pLVX-Puro

After cloning the HPV16 E5 ORF to the pLVX-Puro plasmid (Figure 2A). PCR was performed using primers targeting the E5 gene ORF, and an approximately 250-bp amplification band was observed in the DNA sample (Figure 2B), demonstrating that the HPV16 E5 gene ORF was correctly inserted into pLVX-Puro. The sequence of the E5 ORF was confirmed by Sanger sequencing (data not shown).

Establishment of stably transduced cells

An approximately 250-bp amplification band was observed in the RNA sample from BALB/c 3T3-E5 cells (Figure 2C). A band of 8 kDa was detected in BALB/c 3T3-E5 cells using the WB assay in Figure 2D. These results indicate that the His-tagged E5 gene could be successfully expressed.

Cell transformation assay

The BALB/c 3T3 cell transformation assay was conducted with HPV E5 alone. Blank BALB/c-3T3 cells and the empty pLVX-puro-transduced BALB/c 3T3 cells exhibit normal morphology with sensitive contact inhibition and monolayer growth; Giemsa-stained cells have lighter staining and clear structures of the nucleus and cytoplasm (Figure 3A and B). The transformation foci in E5-pLVX-puro-transduced cells exhibit aberrant morphology characterized by deep basophilic staining, dense multilayer growth, loss of contact inhibition, disordered arrangement, and invasion towards surrounding monolayer cells (Figure 3C). After statistical analysis of the three groups, no transformation foci are observed in the normal and pLVX-puro-transduced cells. There is an average of 2 transformation foci in E5-pLVX-puro-transduced cells. These results demonstrate that the E5 oncoprotein has a weak transforming ability. The establishment of the BALB/c 3T3 cells transformation assay will contribute to further study the synergistic effects of the HPV16 E5 gene and NOCs.

Synergistic carcinogenic effect of NDMA and HPV16 E5

As shown in Supplementary Table 1, NDMA has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

As seen in Figure 4A, cell viability decreases as the concentration of NDMA increases. The viability of the two types of cells is higher than 60% at below 0.1 ng/mL. The viability of BALB/c 3T3 cells decreases to a minimum of 58.8%, while the viability of BALB/c 3T3-E5 cells decreases to 24.8% at 1ng/mL. The viability of BALB/c 3T3-E5 cells is lower than that of BALB/c 3T3 at all concentrations.

As seen in Figure 4B, the average number of transformation foci in BALB/c 3T3 cells induced by NDMA increases with increased concentration. The foci number in BALB/c 3T3-E5 cells is slightly

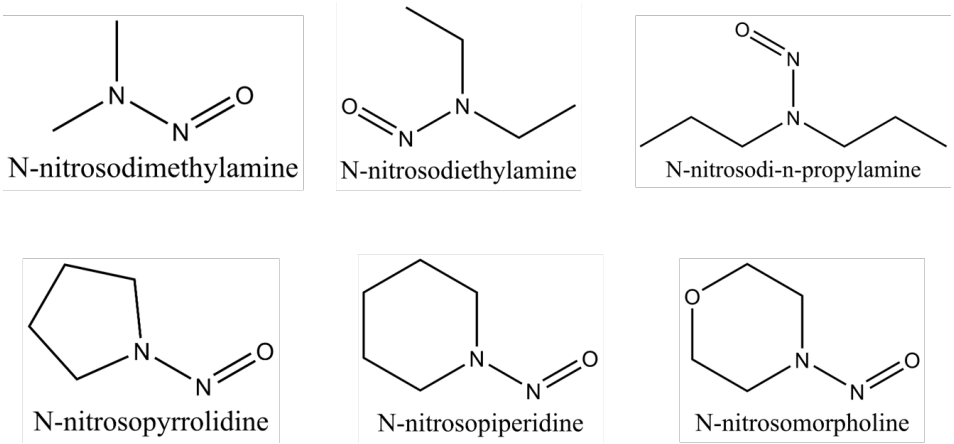

Figure 1: The chemical construction of six NOC compounds.

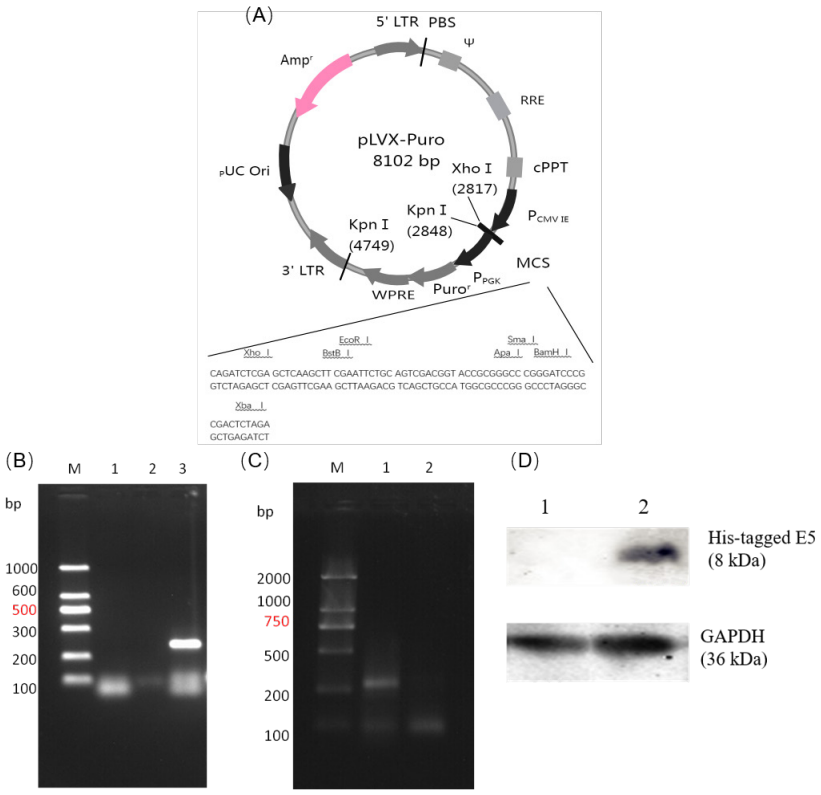

Figure 2: Construct of the recombinant lentiviral vector E5-pLVX-puro. A: The schematic diagram of the pLVX-puro backbone plasmid. B: PCR detection of a 250-bp E5 gene fragment in blank (lane 1), empty pLVX-puro plasmid (lane 2), and recombinant E5-pLVX-puro plasmid (lane 3) using the primer set. M: DL1000 DNA marker. C: RT-PCR detection of a 250-bp fragment in the mRNA sample from E5-pLVX-puro (lane 1) and empty pLVX-puro (lane 2) transduced cells using the primer set targeting E5. M: DL2000 DNA marker. (D) Western blot analysis of His-tagged E5 fusion protein from empty pLVX-puro-transduced (lane 1) and E5-pLVX-puro-transduced 3T3 cells (lane 2) using antibodies recognizing His and GAPDH, respectively.

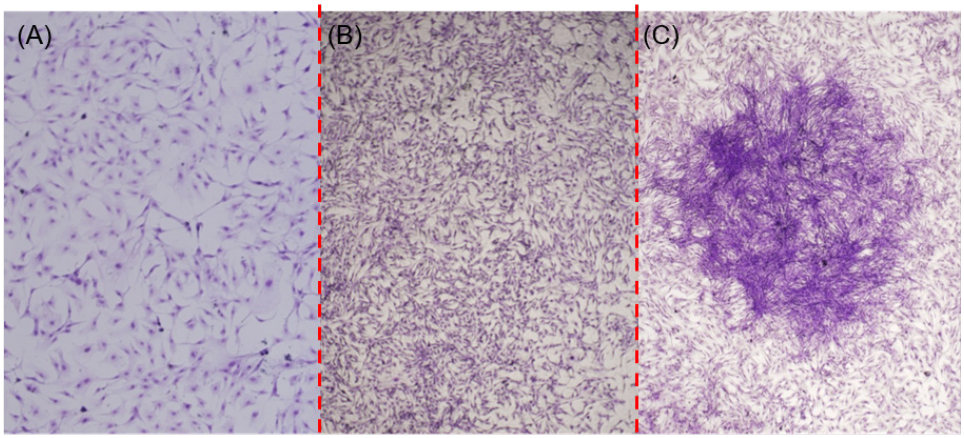

Figure 3: Microscopic image of cell morphology in transformation experiments. A: normal cells. B: empty pLVX-puro-transduced cells. C: E5-pLVX-puro-transduced cells after Giemsa staining (×100).

higher than BALB/c 3T3 cells at all concentrations, indicating no significant synergistic effect between HPV16 E5 and NDMA. The strong transformation of NDMA possibly hides the contribution of HPV E5.

Synergistic carcinogenic effect of NDEA and HPV16 E5

As shown in Supplementary Table 2, NDEA has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

As seen in Figure 5A, with increasing concentrations of NDEA, the cell viability of BALB/c 3T3 cells and BALB/c 3T3-E5 cells gradually decreases. The viability is over 60% in BALB/c 3T3-E5 and BALB/c 3T3 cells at 0.05 ng/mL concentration. The viability of BALB/c 3T3-E5 cells is lower than that of BALB/c 3T3 cells at any concentration of NDEA.

As shown in Figure 5B, the number of transformation foci in BALB/c 3T3 cells treated with less than 0.1 ng/mL NDEA is less than 4. However, when the concentration is over 0.1 ng/mL, the number significantly increases in a dose-dependent manner. The number in BALB/c 3T3-E5 cells is higher than BALB/c 3T3 cells at all concentrations of NDEA, especially when the concentration is 0.1 ng/mL, the number in the former is about three times that of the latter. The number in the combined treatment group is also higher than the sum of HPV16E5 alone and NDEA alone. Therefore, these results supported that HPV16 E5 and NDEA exhibit a synergistic effect, and the synergistic effect is more evident when the concentration of NDEA is 0.1 ng/mL.

Synergistic carcinogenic effect of NDPA and HPV16 E5

As shown in Supplementary Table 3, NDPA has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

As shown in Figure 6A, NDPA has a low cytotoxicity effect on cells with higher than 70% viability at all concentrations. As seen in Figure 6B, the average number of transformation foci in BALB/c 3T3 cells increases with the increase in the concentration of NDPA. However, NDPA has a relatively weak transformation capacity compared to NDMA and NDEA. When acting alone, the maximum foci number is only 6 at a 1 ng/mL concentration. When NDPA, especially at a concentration below 0.5 ng/mL, acts in combination with HPV16 E5, the number of transformation foci is two to three times that of NDPA alone. These results suggest that HPV16 E5 synergizes with NDPA, exhibiting the strongest synergistic effect among the NOCs tested.

Synergistic carcinogenic effect of NPYR and HPV16 E5

As shown in Supplementary Table 4, NPYR has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

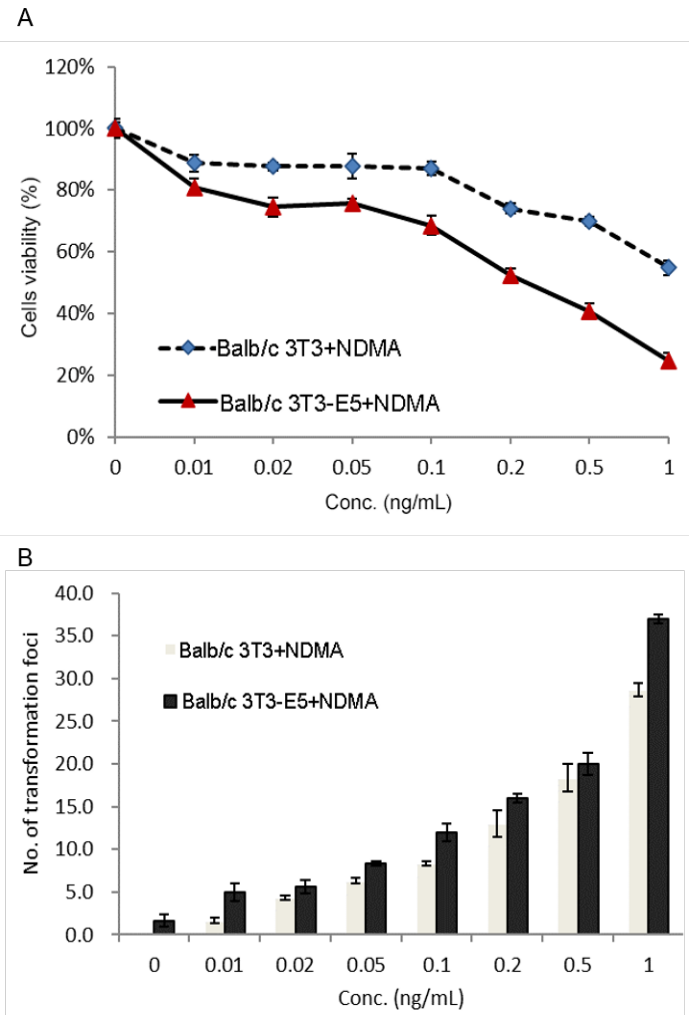

Figure 4: The cytotoxicity and transformation assay in NDMA-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

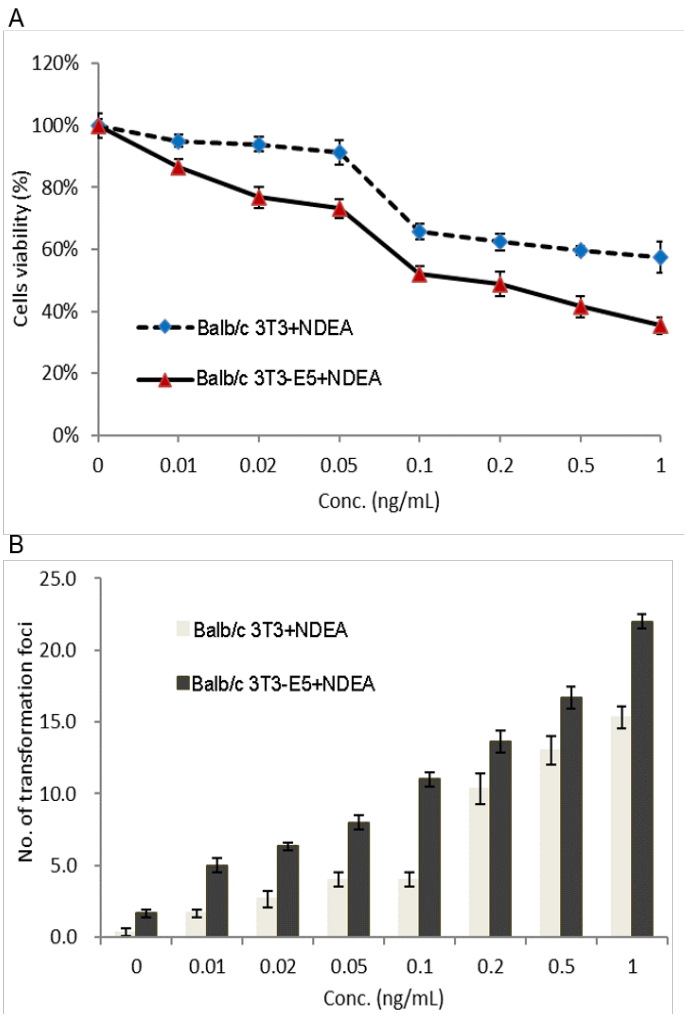

Figure 5: The cytotoxicity and transformation assay in NDEA-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

As shown in Figure 7A, NPYR has a high cytotoxicity on BALB/c 3T3 cells in a concentration-dependent manner. When the concentration of NPYR is 0.05 ng/mL, the viability of BALB/c 3T3 cells is lower than 60%. The viability of BALB/c 3T3-E5 cells gradually declines and is over 60% at all concentrations.

As shown in Figure 7B, the average number of malignant transformation foci in BALB/c 3T3 cells showed a dose-dependent increase with higher NPYR concentrations. The number of foci in BALB/c 3T3-E5 cells is greater than that of BALB/c 3T3 cells across all tested concentrations of NPYR. Therefore, these results show that HPV16 E5 and NPYR have a synergistic carcinogenic effect with a stable trend in all concentrations.

Synergistic carcinogenic effect of NMOR and HPV16 E5

As shown in Supplementary Table 5, NMOR has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

As shown in Figure 8A, the viability of BALB/c 3T3 and BALB/c 3T3-E5 cells gradually decreases with the increased concentration of NMOR. When the concentration is 0.05 ng/mL, the viability of these cells is nearly 60%.

As shown in Figure 8B, the number of transformation foci in BALB/c 3T3 cells increases with the increase in concentration of NMOR. However, the transformation potency is weaker than that of other NOCs. The average number in combined treatments of E5 and NMOR is higher than that of NMOR alone.

Synergistic carcinogenic effect of NPIP and HPV16 E5

As shown in Supplementary Table 6, NPIP has strong genotoxicity with a high output value of AMES test (+++) and carcinogenicity with a high output value of Carcinogencity (+++) in ADMET evaluation models.

As shown in Figure 9A, the viability continuously decreases with the increase in concentration of NPIP. The viability of BALB/c 3T3 and BALB/c 3T3-E5 is about 60% at 0.1 ng/mL. Almost 90% of cells were dead at 1 ng/mL NPIP, showing high cytotoxicity. When the concentration is below 0.5 ng/mL, the number of transformation foci in BALB/c 3T3 cells increases in a dose-dependent manner, as shown in Figure 9B. When the concentration of NPIP is below 0.5 ng/mL, the number in the

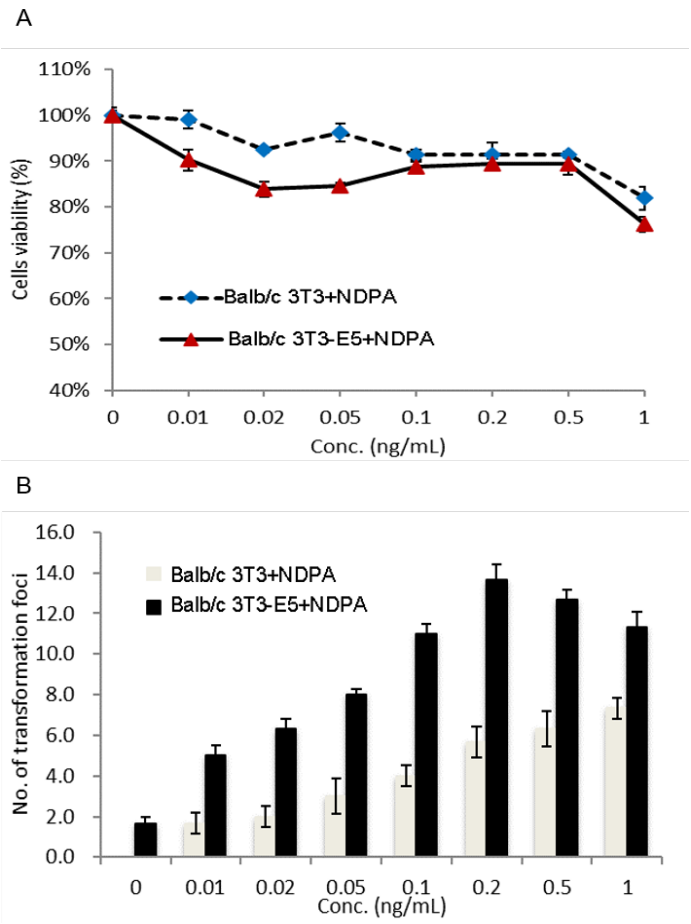

Figure 6: The cytotoxicity and transformation assay in NDPA-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

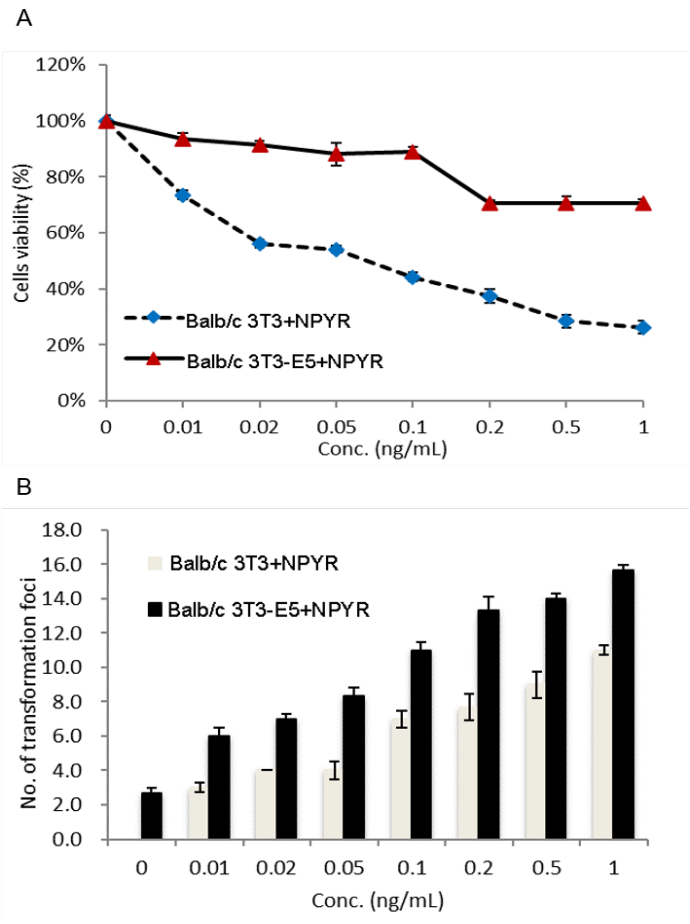

Figure 7: The cytotoxicity and transformation assay in NPYR-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

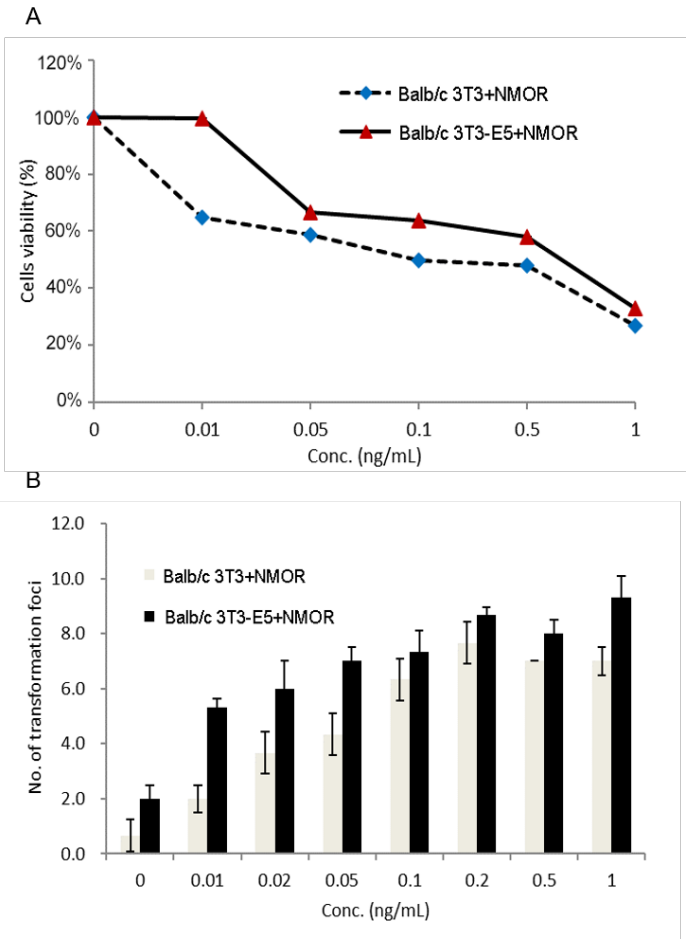

Figure 8: The cytotoxicity and transformation assay in NMOR-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

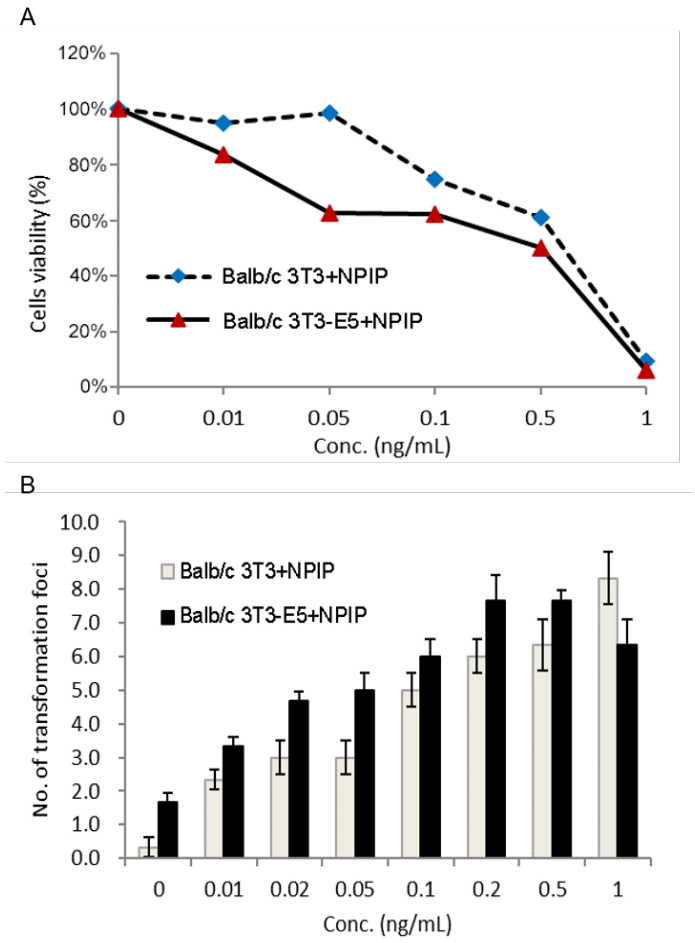

Figure 9: The cytotoxicity and transformation assay in NPIP-induced cells *in vitro*. The cell viability (A) and transformation foci number (B) in BALB/c 3T3 and BALB/c 3T3-E5 cells.

combined treatment is higher than that of NPIP alone. High concentrations of NPIP may exert a modest influence on the formation of transformed foci in 3T3-E5 cells.

DISCUSSION

Most NOCs have cytotoxicity and genotoxicity according to the empirical evidence and experimental observation.[27-30] The primary mechanism is that NOCs are endogenously metabolized by the cytochrome P450 family, and the metabolic products react with DNA to form alkylated DNA adducts, resulting in gene mutations. The accumulated gene mutations and chromosomal aberrations in cells lead to alterations in the gene expression profile and the ultimate changes in the cell phenotype.[30] The irreparable DNA damage triggers a series of signaling pathways resulting in cell death and senescence.[28] Likewise, these NOCs undergo different CYP-mediated metabolism pathways and form various DNA alkylation adducts, leading to various carcinogenic potencies.[28,30]

We have observed that the NOCs, especially NDMA and NDEA, have a potent transformation potency, and the effect becomes more evident with increased concentration. Although NOCs exhibit certain cytotoxic effects at high concentrations, they may mildly interfere with cell transformation, because these transformed cells may be resistant to apoptosis induced by NOCs. The parental cells sensitive to apoptosis undergo death. These inferences were supported by the observations in other compound testing experiments.[31] Nevertheless, the cytotoxic effect of NOCs was transient, owing to the limited exposure duration (3 days) of NOCs in the transformation assay. To minimize the impact of cytotoxicity of NOCs on their transformation ability, we also confirm the working concentration range of NOCs to meet the demands for cell viability in the carcinogenicity tests. In the BALB/c 3T3 transformation model, cell viability exceeding 60% is consistently observed across almost all groups at a concentration of less than 0.05 ng/mL. Due to low cell seeding density in this study, the viability is lower than the general criterion (75%) for genotoxicity tests.[32,33] It seems that the cytotoxic effect of six N-nitroso compounds is different at the same concentration, suggesting that individual NOCs react differently with cellular macromolecules.[27] The divergence in cell fates is partially mediated by repair pathway-specific responses to cytotoxicity and mutagenicity induced by NOCs, which have been demonstrated in tobacco-specific nitrosamines.[29] The final concentration of

individual NOCs needs to be further optimized based on these pilot tests.

HPV16 E5 is the smallest oncoprotein with many functions, such as regulating cell proliferation and apoptosis.[19,34] It inhibits the degradation of epidermal growth factor receptors and downregulates the level of tumor suppressors p21 and p27, resulting in enhanced growth factor signaling.[19,20] E5 oncoprotein hijacks the host DNA replication machinery and inhibits the apoptosis of cells.[34,35] BALB/c 3T3-E5 cells exhibited higher viability than BALB/c 3T3 cells treated with NPYR or NMOR. The biological activity of E5 perhaps partly counteracts the NOCs-induced cytotoxicity, resulting in relatively high viability of BALB/c 3T3-E5 cells. On the contrary, the BALB/c-E5 cells have lower viability than BALB/c cells treated with NDMA, NDEA, and NPIP. The hypothesis is that the extended E5 expression may harm the cells above.[36] The impaired viability caused by E5 has also been reported in trophoblastic and cervical cell lines.[37] The cells expressing E5 exhibit high sensitivity to apoptosis induced by osmotic stress.[38] Based on these findings, E5 appears to exert different influences on cell fate.

E5 is suggested to function in the early stages of normal progression into malignant cells.[39] Previous studies reported that E5 alone has a weak transforming activity, and robust activity is observed when E5 acts synergistically with the E6 and E7 oncoproteins.[21] Likewise, E5 has been found to have an enhanced transforming potency combined with NOCs, especially NDPA, in this study. However, the molecular networks of interaction between NOCs and HPV E5 have yet to be elucidated. NOCs may play a tumor initiator,[40,41] and the E5 oncoprotein acts as a tumor promoter[42,43] in carcinogenesis. A study demonstrated that exposure to tobacco-specific nitrosamines modulates the expression of transformation-associated genes in HPV16-immortalized human cervical epithelial cells.[44] Another study also confirmed that cigarette smoke components activate the expression of E6 and E7 oncogenes by activating the HPV Long Regulatory Elements (LCR) in cervical cancer cells.[45] After all, the evidence about the intervention of NOCs on HPV is limited. More convincing evidence is required to substantiate the above explanations.

The BALB/c 3T3 two-stage cell transformation assay demonstrates multiple advantages, such as a standardized protocol, high sensitivity to a wide spectrum of carcinogens, a clear transformation endpoint, and good stability of data. The BALB/c 3T3-E5 cell is an ideal model for rapidly screening the synergetic effect of NOCs. The keratinocyte cell line (HaCaT) and the esophageal epithelial cell line (SHEE and Het-1A) are suitable for investigating the synergistic carcinogenesis mechanism between NOCs and HPVs. These models are expected to investigate the effects of intrinsic molecules on cellular behavior and identify target genes and signaling pathways, similar to GAB2 in HPV and MNNG-induced malignant Het-1A cells, as previously reported.[18] The limitation of this study is the lack of some assays of cellular behaviors such as proliferation, migration, and invasion, as well as *in vivo* tumorigenicity. These will be the focus of our subsequent work.

CONCLUSION

This study provides a glimpse of the synergistic effect between NOCs and HPV E5 in rodent cell transformation. The synergistic effect is more significant than a single NOC or E5 and increases in a dosage-dependent manner. These models and assays are rapidly designed to elucidate the influence of the interaction of NOCs with macromolecules on cellular fates. Understanding the interaction mechanisms between NOCs and HPV will contribute to providing some experimental evidence for preventing and treating HPV-related tumors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPV: human papillomavirus; **NOCs:** N-nitroso compounds; **NDMA:** N-nitrosodimethylamine; **NDEA:** N-nitrosodiethylamine; **NDPA:** N-nitrosodi-n-propylamine; **NPYR:** N-nitrosopyrrolidine; **NMOR:** N-nitrosomorpholine; **NPIP:** N-nitrosopiperidine; **ADMET:** absorption, distribution, metabolism, excretion and toxicity; **SMILES:** Simplified Molecular Input Line Entry System; **ORF:** Open Reading Frame.

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Cite this article: Liu H, Li S, Li J. A Glimpse of the Interaction between N-Nitrosamines and HPV E5 in Rodent Cell Transformation. Indian J of Pharmaceutical Education and Research. 2026;60(3):1224-32.

Table S1: Toxicity of NDMA in ADMET evaluation models.

(SMILES: CN(C)N=O)		
hERG Blockers	---	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	-	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-). The output value is the probability of being toxic.
DILI	++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	+++	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic.
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	+	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer.
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	--	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	+++	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.

Table S2: Toxicity of NDEA in ADMET evaluation models.

(SMILES: CCN(CC)N=O)		
hERG Blockers	---	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	-	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-) The output value is the probability of being toxic.
DILI	++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	-	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic,
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	++	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer.
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	++	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+++	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	+++	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.

Table S3: Toxicity of NDPA in ADMET evaluation models.

(SMILES: CCCN(CCC)N=O)		
hERG Blockers	--	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	-	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-). The output value is the probability of being toxic.
DILI	++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	+	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic.
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	+	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer..
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	++	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+++	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	++	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.

Table S4: Toxicity of NPYR *in vivo* model in ADMET evaluation models.

(SMILES: C1CCN(C1)N=O)		
hERG Blockers	---	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	+	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-). The output value is the probability of being toxic.
DILI	++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	++	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic.
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	++	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer.
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	++	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+++	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	++	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.

Table S5: Toxicity of NMOR *in vivo* model in ADMET evaluation models.

(SMILES: C1COCCN1N=O)		
hERG Blockers	--	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	-	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-). The output value is the probability of being toxic.
DILI	+++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	-	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic.
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	++	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer.
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	+	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+++	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	--	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.

Table S6: Toxicity of NPIP *in vivo* model in ADMET evaluation models.

(SMILES: C1CCN(CC1)N=O)		
hERG Blockers	---	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
H-HT	-	Human Hepatotoxicity Category 1: H-HT positive (+); Category 0: H-HT negative (-). The output value is the probability of being toxic.
DILI	++	Drug induced Liver injury. Category 1: drugs with a high risk of Dl; Category 0: drugs with no risk of Dl. The output value is the probability of being toxic.
AMES Toxicity	+++	Category 1: Ames positive (+); Category 0: Ames negative (-); The output value is the probability of being toxic.
Rat Oral Acute Toxicity	+	Category 0: low-toxicity; Category 1: high-toxicity. The output value is the probability of being highly toxic.
FDAMDD	---	Maximum Recommended Daily Dose Category 1: FDAMDD (+); Category 0: FDAMDD (-) The output value is the probability of being positive.
Skin Sensitization	++	Category 1: Sensitizer, Category 0: Non-sensitizer. The output value is the probability of being sensitizer.
Carcinogencity	+++	Category 1: carcinogens; Category 0: non-carcinogens, The output value is the probability of being toxic.
Eye Corrosion	++	Category 1: corrosives; Category 0: non-corrosives The output value is the probability of being corrosives.
Eye Irritation	+++	Category 1: irritants; Category 0: non-irritants. The output value is the probability of being irritants.
Respiratory Toxicity	++	Category 1: respiratory toxicants, Category 0: respiratory non-toxicants The output value is the probability of being toxic.