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Antimutagenic effects of 2,6-Dimethylpyridine-N-oxide using fluctuation ames test

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ABSTRACT

The mutagenic potential of chemical agents is a significant concern in the field of genetic toxicology. The 2,6-Dimethylpyridine-N-oxide is believed to have antimutagenic properties, which could be beneficial for applications in pharmaceutical and environmental sciences. This study aims to evaluate the antigenotoxic potential of 2,6-Dimethylpyridine-N-oxide using the fluctuation Ames test, a reliable method for detecting mutagenic effect. Specifically, the Ames assay was conducted with preincubation in a suspension of reversion Salmonella bacteria. The experimental approach utilized *Salmonella typhimurium* tester strains - TA98, which are characterized by frameshift mutations in the hisD3052 gene, and another strain TA100 featuring base-pair substitution mutations in the hisG46 gene to assess the genotoxic potential of the test compounds. The aim was to evaluate the antigenotoxic potential, these strains were concurrently exposed to positive mutagens: 4-Nitroquinoline-N-oxide (4-NQO) for the strain TA100 and 2-Nitrofluorene (2-NF) for TA98, without the presence of rat liver S9 microsomal fractions. Additionally, 2-Aminoanthracene (2-AA) was used for both strains with S9 activation alongside 2,6-Dimethylpyridine-N-oxide with the same dose level as positive controls by volume. The results indicate that 2,6-Dimethylpyridine-N-oxide exhibits significant antimutagenic effect, as shown by a notable reduction in mutation rates detected by the fluctuation Ames test. These findings support further investigation into the role of 2,6-Dimethylpyridine-N-oxide in preventing mutagenesis and its potential utility in drug development and environmental protection. This research provides essential insights into anti-mutagenesis and highlights the potential of 2,6-Dimethylpyridine-N-oxide in mitigating genotoxic risks.

1. Introduction

The intensification of industrial chemicals production leads to global anthropogenic environmental pollution, which aspects pose risks to human health. It is imperative to note that some of these chemicals may possess the potential for deleterious long-term effects, including mutagenic effect. The mutagenic potential of chemical agents is a profound concern in genetic toxicology, with implications for both human health and environmental safety. Mutagens can lead to genetic mutations (DNA damage, carcinogenesis with biological processes, and chromosomal abnormalities), which are often precursors to cancer and other genetic disorders [1–3]. Therefore, finding agents with antimutagenic properties—those that can reduce or prevent mutations is of great interest in the fields of pharmaceutical development, cancer prevention, and environmental protection [4–9]. The pyridine derivatives hold considerable promise in this regard, given that the heterocyclic pyridine system constitutes the foundation of a broad spectrum of pharmacological agents with diverse therapeutic benefits. A substantial number of effective substances with antioxidant, membrane- and genome-protective, and antimutagenic properties have been identified in these groups of substances [10,11].

A representative of pyridine-N-oxide derivatives, Ivin (2,6-Dimethylpyridine-N-oxide), which is a synthetic compound that mimics the structure and function of naturally occurring plant hormones with demonstrated high biological activity and is recommended as a plant growth regulator (PGR). Studies have demonstrated that Ivin treatment in plants results in increased membrane permeability, altered gene regulation, and changes to the nuclear matrix, ultimately leading to enhanced RNA and protein synthesis [12].

Research conducted on laboratory animals has demonstrated that Ivin reduces lipid peroxidation processes, exhibits a membrane-stabilizing effect, slightly elevates the levels of RNA and DNA, as well

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as the mitotic index of hepatocytes. Additionally, it enhances protein synthesis in liver tissue and does not promote carcinogenesis [13].

Our previous studies have shown that plant growth regulators (PGRs) derived from pyridine-N-oxide, specifically Ivin and Poteytin, when used in conjunction with pesticides, can significantly decrease the acute and subchronic toxicity of these pesticides in mammals. Furthermore, these PGRs demonstrate antioxidant, hepatoprotective, antihypoxic, and adaptogenic effects, while also reducing mutagenic effect [14–17]. This is especially noticeable with cytostatics (positive controls) such as Cyclophosphamide and Dioxidine [18,19].

It has been hypothesized that 2,6-Dimethylpyridine-N-oxide possesses antimutagenic properties. Due to its unique chemical structure, this compound may interfere with mutagenic processes at the molecular level, potentially lowering the risk of mutations. If its effectiveness is confirmed, 2,6-Dimethylpyridine-N-oxide could serve as a valuable protective agent against mutagens. Its applications could range from pharmaceuticals aimed at minimizing drug-induced mutations to environmental strategies designed to mitigate the effects of pollutants.

This study explores the antimutagenic properties of 2,6-Dimethylpyridine-N-oxide using the fluctuation Ames test, a sensitive and widely recognized method for detecting mutagenic effect. This test employs well-characterized Salmonella strains to identify specific types of mutations [20–23]. The fluctuation Ames test is particularly useful in identifying whether a compound can either induce or inhibit mutations in bacterial cells, serving as a representative system to anticipate potential effects in higher organisms. The results of this study could pave the way for further research into the application of 2,6-Dimethylpyridine-N-oxide in mitigating the effects of mutagens, contributing to advancements in genetic toxicology, and possibly leading to the development of new antimutagenic therapies or environmental interventions.

The current study aims to determine the genotoxic and antigenotoxic potential of 2,6-Dimethylpyridine-N-oxide using a fluctuating version of the Ames assay with preincubation in suspension of reversion *Salmonella* bacteria.

2. Material and methods

The present study used the fluctuating version of the Ames MPF Kit produced by Xenometrix AG, Switzerland. The kit includes bacterial strains, positive controls, and post-mitochondrial supernatant S9 from a lyophilised Aroclor 1254-induced rat liver S9 mix. The bacterial mutant tester strains, *Salmonella typhimurium* TA98 with frameshifts a point mutation in hisD3052 and *Salmonella typhimurium* TA100 with base-pair substitution mutation in hisG46, were used to determine genotoxic and antigenotoxic potentials of the test compounds.

2.1. Chemical reagents

- ***Test compound***: The grow plant regulator Ivin (2,6-Dimethylpyridine-N-oxide, 99.9 %, liquid, clear from colourless to yellowish, miscible with water), was synthesized at the V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine by analogy with the method described by Ochiai [24], which involved the oxidation of 2,6-dimethylpyridine with 30 % perhydrol in acetic acid and acetic anhydride. The chemical structure of 2,6-Dimethylpyridine-N-oxide presented in Fig. 1.
- ***Mutagens*:** The mutagens (Xenometrix AG, Switzerland) used for the antigenotoxicity assays included:
  - o ***4-Nitroquinoline-N-oxide (4-NQO)*** (final test concentration 0.1 μg/ml)**:** Known for inducing base-pair substitutions, particularly in strain TA100 S9–.
  - o ***2-Nitrofluorene (2-NF)*** **(**final test concentration 2 μg/ml)**:** A strong inducer of frameshift mutations that is utilized in experiments with strain TA98 S9–.

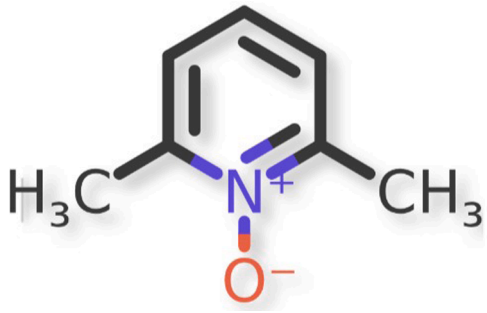

Fig. 1. Chemical structure of 2,6-Dimethylpyridine-N-Oxide.

  - o **2-*Aminoanthracene (2-AA):*** Employed in assays with metabolic activation (S9 mix) for both strains TA98 (final test concentration 1 μg/ml) and TA100 (final test concentration 2.5 μg/ml) to determine the influence of 2,6-Dimethylpyridine N-oxide on mutagenesis under simulated metabolic conditions.

2.2. Bacterial strains

- ***Salmonella typhimurium TA98*:** This strain carries a frameshift mutation in the hisD3052 gene, which makes it highly sensitive to mutagens that cause frameshift mutations.
- ***Salmonella typhimurium TA100*:** This strain has a base-pair substitution mutation in the hisG46 gene, making it suitable for detecting mutagens that induce base-pair substitutions.

2.3. Ames fluctuation assay

The genotoxicity test was conducted according to the established protocol. The test compound's genotoxic potential was determined according to OECD TG 471 [25]. Each bacterial strain was treated with the substance at concentrations of 0.0016, 0.008, 0.04, 0.2, 1 and 5 μL/ml (the maximum test concentration for soluble non-cytotoxic substances recommended by OECD 471).

Dimethyl sulfoxide (DMSO) was used as a solvent and negative control. The mutagens 4-Nitroquinoline-N-oxide (4-NQO) for *Salmonella typhimurium* TA100, 2-Nitrofluorene (2-NF) for *Salmonella typhimurium* TA98 without microsomal fractions of rat liver (-S9), and 2-Aminoanthracene (2-AA) for both strains with metabolic activation (+S9) were used as positive controls to verify bacterial susceptibility to established genotoxins.

- ***Test Procedure*:** The Ames fluctuation assay was chosen for its sensitivity in detecting mutagenic and antimutagenic activities. This variant of the Ames test is particularly useful in detecting low levels of mutagenic effect in a liquid culture environment.
- ***Preincubation*:** To facilitate better chemical interaction, the bacterial strains were pre-incubated with the test compound in a nutrient-rich medium for 90 min in a shaking incubator at 37 °C. This step is crucial for assessing the compound's ability to interact with bacterial DNA or mutagens.
- ***Reversion Assay*:** Following preincubation, the bacterial cultures were exposed to either the test compound alone (*for genotoxicity testing*) or in combination with a known mutagen (*for antigenotoxicity testing*). The cultures were then transferred from 24-well to a 384-well plate, and the number of wells showing bacterial growth (indicating reversion to a histidine-independent state) was recorded.
- **Controls:** The assay included various controls:

  - ***Negative Control*:** To establish baseline mutation rates, bacteria were treated with a vehicle control (solvent DMSO) without the test compound or mutagen.

- ***Positive Control*:** To verify the assay's responsiveness, bacteria were exposed to known mutagens (4-NQO for TA100, 2-NF for TA98, and 2-AA for both strains) without the test compound.

2.4. Metabolic activation

- ***S9 Mix Preparation*:** The Aroclor 1254-induced rat liver lyophilized homogenate S9 fraction produced by Xenometrix AG, Switzerland as part of reagents of Ames MPF kit. The S9 mix was prepared with cofactors by Sigma-Aldrich (Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, potassium chloride, magnesium chloride, phosphate buffer) to simulate the metabolic activation of test compounds in a mammalian liver. This component was crucial for assessing the effects of 2,6-Dimethylpyridine-N-oxide in both metabolically active (with S9 mix) and non-active (without S9 mix) conditions.
- ***Assays with S9 Mix*:** Experiments were conducted with and without the S9 mix to determine whether metabolic activation affects the genotoxic or antigenotoxic potential of 2,6-dimethylpyridine N-oxide. The presence of S9 mix allows for the evaluation of the compound's interaction with activated metabolites, providing a more comprehensive understanding of its potential effects in a living organism.

2.5. Antigenotoxicity testing was conducted as follows

In order to ascertain the antigenotoxic potential of the test compound, the same strains were also used in combination with the same mutagens and 2,6-Dimethylpyridine-N-oxide. The study was conducted in two variations: in the first version, the test compound was added to the mutagen-treated strains before pre-incubation (bacterial culture + Ivin + PC + pre-incubation for 90 min). In the second protocol, the test compound was introduced after pre-incubation (bacterial culture + PC + pre-incubation for 90 min + Ivin). Following pre-incubation, the wells were exposed in incubator 37°C for 48 h before calculations were made. In both versions, the native test compound and mutagens were added in the same quantity by volume (10 μL each). The antigenotoxic effects were evaluated by measuring the reduction in the number of revertant bacterial colonies, comparing samples treated with mutagens alone to those with test compounds added either before or after the pre-incubation period [26]. To evaluate our data, we used a grading scale where 25–40 % inhibition was classified as moderate antimutagenicity, 40 % or higher inhibition as strong antimutagenicity, and less than 25 % inhibition as no antimutagenicity [27].

2.6. Data analysis

- ***Reversion Rate Calculation*:** The number of revertant colonies (indicative of mutations) was counted in each well. The mutation frequency was calculated by comparing the number of revertant colonies in treated samples to those in the control samples.
- ***Statistical Analysis*:** The validated Xenometrix software "Ames MPF dilutions calculator" was used for statistical data processing. The following values were calculated: Mean Number of Positive Wells per Concentration, Standard Deviation (SD) of the Number of Positive Wells per Concentration. The Baseline is calculated by adding the Mean Number of positive wells in the negative control and the SD-value. A Fold increase relative to the baseline, which is determined by dividing the mean number of positive wells at each dose by that of the negative (solvent) control baseline. Student's *t*-test (unpaired one-tailed) was used to determine the significance of differences at a significance level set at $p \leq 0.05$.

A fold increase in the number of revertants less than two times the baseline level is not considered a positive effect since the differences are not significant. A test sample is classified mutagenic when the dose-dependent effect or a fold increase is greater than two times the baseline is recorded.

The antigenotoxicity data were statistically analysed using GraphPad Prism. The data were analyzed using one-way ANOVA, and the significance of inter-group differences was analyzed using Tukey's test. A p-value of less than 0.05 was considered statistically significant.

2.7. Reproducibility and validation

***Replicates*:** To ensure reproducibility, all experiments were conducted in triplicate. Results were reported as mean ± standard deviation.

***Inter-Laboratory Comparison*:** In 2013–2015, we conducted an inter-laboratory comparison involving the results of positive and negative controls of studies on 58 pesticides, comparing our findings with common historical controls from 18 laboratories from seven countries. The analysis included positive and negative controls using strains TA 98 and TA 100, with and without metabolic activation. The results demonstrated that the figures correlated within the standard deviation limits, indicating our methodology's robustness and reproducibility. This supports the quality of our studies on the potential mutagenic activity of pesticides, as assessed by the Ames fluctuation test at L.I. Medved's Research Center of Toxicology, MH, Ukraine [28].

3. Results and discussion

3.1. Genotoxicity assessment

A range of concentrations of 2,6-Dimethylpyridine-N-oxide (ranging from 0.0016 to 5 μL/ml) was investigated to evaluate the genotoxic effects on *Salmonella typhimurium* strains TA98 and TA100 (according to OECD 471 guidelines) [25]. These concentrations were selected based on preliminary toxicity assessments to ensure they were within the recommended guidelines while remaining within a range capable of revealing potential genotoxic or antigenotoxic effects.

The results of the genotoxicity study indicated that 2,6-Dimethylpyridine-N-oxide did not exhibit genotoxic effect in either strain (TA98 or TA100) at any of the tested concentrations (not published yet). This suggests that the compound does not induce mutations through frameshift (strain TA98) or base-pair substitution mechanisms (strain TA100), making it relatively safe from a genotoxic perspective under the conditions tested.

3.2. Antigenotoxicity assessment

Despite the lack of intrinsic genotoxicity, 2,6-Dimethylpyridine-N-oxide demonstrated some degree of antigenotoxic effect when co-administered with known mutagens, particularly 2-Aminoanthracene (2-AA), 4-Nitroquinoline-N-oxide (4-NQO), and 2-Nitrofluorene (2-NF) in equal volume doses (10 μL) as PCs. The antigenotoxic effects were evaluated by measuring the reduction in the number of revertant bacterial colonies, comparing samples treated with mutagens alone to those with test compounds added either before or after the preincubation period.

- **2,6-Dimethylpyridine-N-oxide +4-NQO:** The inhibition rates of mutagenesis induced by 4-NQO at the *Salmonella typhimurium* TA100 without metabolic activation (-S9) were low (or insignificant) in both application variants of 2,6-dimethylpyridine N-oxide: ranging from 5.56 % before preincubation to 1.40 % after preincubation (Fig. 2. A). While these figures suggest some level of interference with 4-NQO's mutagenic effects, the observed impact was negligible, indicating that 2,6-dimethylpyridine N-oxide may have limited effectiveness against this type of mutagen.
- **2,6-Dimethylpyridine-N-oxide +2-NF:** Similarly, in experimental procedures incorporating 2-NF, a known inducer of frameshift

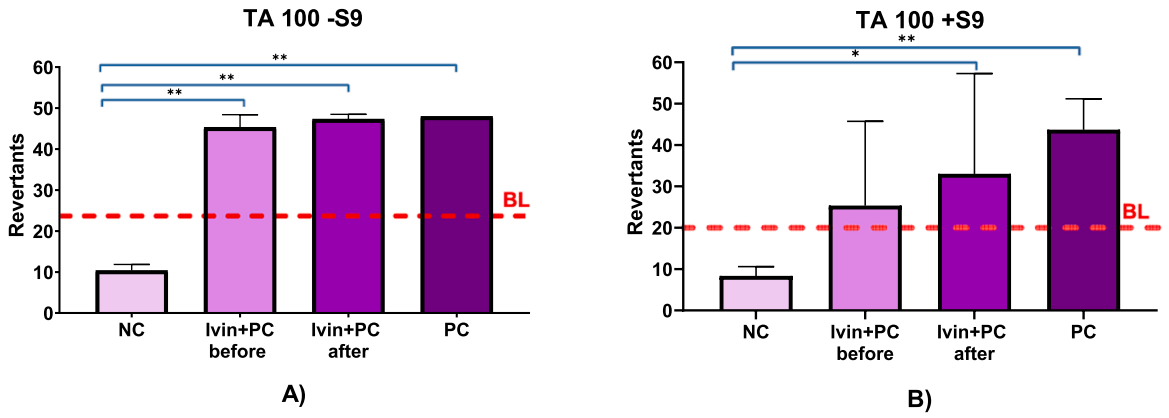

Fig. 2. The number of revertant colonies of Salmonella typhimurium TA100 following combined action of the 2,6-Dimethylpyridine N-oxide (Ivin) (10 μl) and mutagens 4-NQO without metabolic activation (A) and 2-AA with metabolic activation (B) under conditions before preincubation and after preincubation. NC – negative control, Ivin+PC before – Ivin + positive control before preincubation, Ivin+PC after - Ivin + positive control before preincubation, PC – positive control, BL - Basic line (zero line); Statistically significant: *- $p \leq 0.05$, **- $p \leq 0.001$.

mutations in *Salmonella typhimurium* TA98 strain, 2,6-Dimethylpyridine-N-oxide generated notably increased antigenotoxic effect. The inhibition rates of mutagenesis for *Salmonella typhimurium* TA98 strain ranged from 12.28 % added before preincubation to 59.52 % after preincubation (Fig. 3.A). The results suggest that the 2,6-Dimethylpyridine-N-oxide can mitigate the mutagenic effects of 2-NF in both experimental protocols. The data demonstrated that administration of 2,6-Dimethylpyridine-N-oxide to bacterial cultures previously exposed to the mutagen exhibited enhanced efficacy, suggesting this compound's potential role in ameliorating mutagen-induced cellular damage. Referenced studies [5,27] indicate that mutagenesis inhibition exceeding 40 % is indicative of potent antimutagenic properties, which was observed with 2,6-Dimethylpyridine-N-oxide in this investigation.

- **2,6-Dimethylpyridine-N-oxide +2-AA:** More substantial antigenotoxic effects were observed with mutagen 2-AA and metabolic activation (+S9 mix). The inhibition of mutagenesis by 2-AA for *Salmonella typhimurium* TA100 ranged from 47.23 % before preincubation to 31.25 % after preincubation, Indicating a significant reduction in mutation rates (Fig. 2.B). Similarly, for *Salmonella typhimurium* TA98, the inhibition of mutagenesis by 2-AA was 17.56 % before preincubation and 46.59 % after preincubation (Fig. 3.B).

The reduction in the number of revertant bacterial colonies was particularly notable in assays conducted with the S9 mix, suggesting that 2,6-Dimethylpyridine-N-oxide may interact with metabolically activated mutagens or their metabolites to reduce their mutagenic potential.

Notably, the study also investigated the impact of 90 min preincubation in Salmonella suspension on the antigenotoxic effect of 2,6-Dimethylpyridine-N-oxide. The results suggest that preincubation with 2,6-Dimethylpyridine-N-oxide may decrease the compound's ability to inhibit mutagenesis for *Salmonella typhimurium* TA100. Conversely, in *Salmonella typhimurium* TA98, a more pronounced antimutagenic effect was observed when 2,6-Dimethylpyridine-N-oxide was added after preincubation of the culture with the mutagen. This could be attributed to various factors, such as changes in the chemical stability of the compound during preincubation, alterations in its interaction with mutagenic agents over time, or the nature of the mutations (such as frameshift mutations or base pair substitutions in the gene).

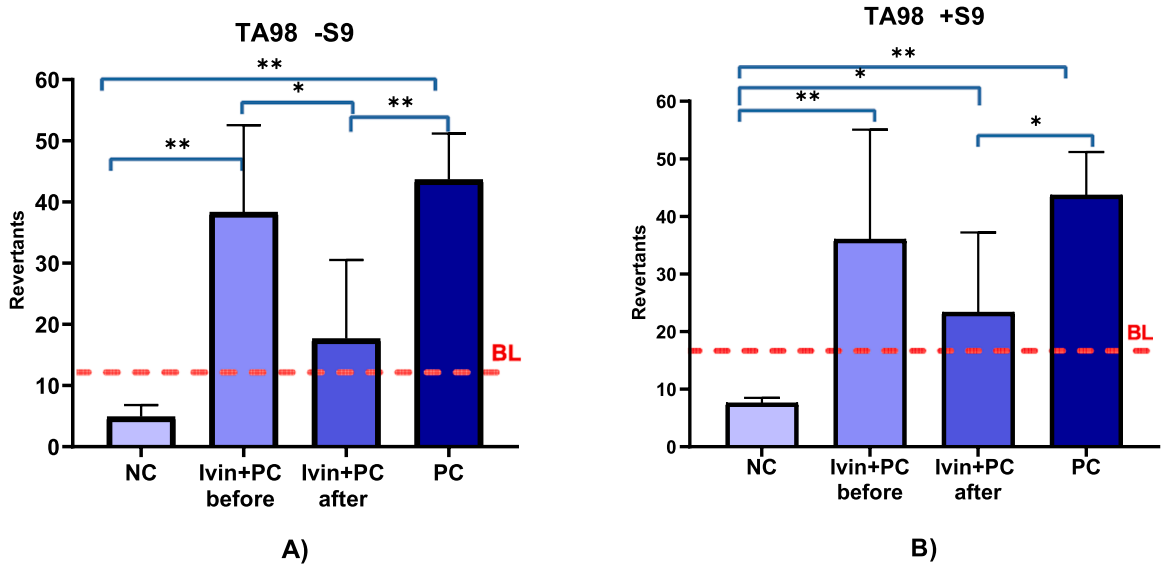

Fig. 3. The number of revertant colonies of Salmonella typhimurium TA98 following combined action of the 2,6-Dimethylpyridine N-oxide (Ivin) (10 μl) and mutagens 2-NF without metabolic activation (A) and 2-AA with metabolic activation (B) under conditions before preincubation and after preincubation. NC – negative control, Ivin+PC before – Ivin + positive control before preincubation, Ivin+PC after - Ivin + positive control before preincubation, PC – positive control, BL - Basic line (zero line); Statistically significant: *- $p \leq 0.05$, **- $p \leq 0.001$.

Based on the experimental findings, the following conclusions can be drawn from the study on the mutagenic and antigenotoxic potentials of 2,6-Dimethylpyridine-N-oxide, the compound was found to have no mutagenic effect across all tested concentrations and bacterial strains in Ames assay. However, it exhibited protective effects against mutagenesis induced by multiple agents, including 2-aminoanthracene (2-AA), 4-nitroquinoline N-oxide (4-NQO), and 2-nitrofluorene (2-NF). This suggests that 2,6-Dimethylpyridine-N-oxide may have potential as a chemopreventive agent against mutagen-induced damage.

Earlier, we conducted and published results [18,19] of an in vivo chromosome aberration assay in mice using 2,6-Dimethylpyridine-N-oxide, which demonstrated its ability to alter the cytogenetic effects in mouse bone marrow cells caused by the known mutagens cyclophosphamide and dioxine. It was found that 2,6-Dimethylpyridine-N-oxide (at doses ranging from 71 to 0.07 mg/kg), when administered to mice together with Cyclophosphamide or Dioxidine, significantly reduced the frequency of metaphases with chromosome aberrations by 55–74 % for both mutagens. This confirms the antigenotoxic potential of 2, 6-Dimethylpyridine-N-oxide.

Further research is warranted to elucidate the precise mechanisms of antimutagenic action, determine structure-activity relationships, explore potential molecular targets, and evaluate the compound's effectiveness in other test systems. This research establishes 2,6-Dimethylpyridine-N-oxide as a promising antimutagenic agent while highlighting the need for mechanistic studies to fully understand its protective properties.

4. Conclusion

The findings of this study provide compelling evidence that 2,6-Dimethylpyridine-N-oxide possesses significant antimutagenic properties. The compound effectively reduces mutation rates, as demonstrated by the fluctuation Ames test, particularly against known mutagens such as 2-aminoanthracene (2-AA). This suggests its potential role in preventing genetic mutations that could lead to serious health issues.

The results warrant further investigation into the underlying mechanisms of action of 2,6-Dimethylpyridine-N-oxide. Future research should focus on elucidating its interactions with DNA, its effects on mutagen activation and detoxification pathways, and its broader implications for genetic integrity.

Moreover, the potential applications of 2,6-Dimethylpyridine-N-oxide are significant in various fields, including drug development, where it could enhance the safety profiles of pharmaceuticals by mitigating genotoxic risks. Its role in environmental protection is also noteworthy, as it may help alleviate the harmful effects of environmental mutagens, thereby promoting public health and safety.

In summary, this research contributes valuable insights to the field of antimutagenesis and positions 2,6-Dimethylpyridine-N-oxide as a promising candidate for addressing genotoxic risks.

Continued exploration of this compound could lead to substantial advancements in genetic toxicology and its applications across multiple industries.

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CRediT authorship contribution statement

Mykola Prodanchuk: Conceptualization, Resources. **Petro Zhminko:** Supervision, Conceptualization. **Tetyana Usenko:** Investigation, Formal analysis. **Volodymyr Bubalo:** Writing – original draft, Methodology, Investigation, Formal analysis. **Olesia Vasetska:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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