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*Original Article*

# Eugenol's Molecular Warfare against Human Leukemia K562 cells: In Vitro Insights to Chemotherapeutic Potentials

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## ABSTRACT

**Background**: The search for effective anticancer agents has led to an increased focus on natural compounds, given their role as the foundation for many chemotherapeutic drugs. Eugenol (EUG) and Bis-Eugenol (Bis-EUG), derived from clove oil, have shown promise due to their antioxidative, anti-inflammatory, pro-apoptotic, and anti-proliferative properties across various cancer cell lines. This study aims to elucidate the chemotherapeutic potential of EUG and Bis-EUG on human leukemia K562 cells, exploring their ability to modulate apoptosis and cell proliferation.

**Objective**: To assess the in vitro anticancer effects of EUG and Bis-EUG on K562 cell lines through various assays measuring cell viability, apoptosis, gene expression, and nitric oxide release, with a focus on elucidating the mechanisms underlying their anticancer activity.

**Methods**: The study employed an inter-collaborative approach, utilizing MTT assays to determine the IC50 values of EUG and Bis-EUG on K562 cells. Gene expression analysis of pro-apoptotic genes Caspase-3 and Caspase-9 was performed using RT-qPCR. Apoptotic changes were further analyzed through flow cytometry and Hoechst 33258 staining to observe morphological alterations in treated cells. The release of nitric oxide (NO) as an indicator of anti-proliferative activity was measured using a Griess assay. Statistical analysis was conducted using GraphPad Prism Software, Version 9, and Microsoft Excel, with a significance threshold of $p \leq 0.05$.

**Results**: The IC50 values for EUG and Bis-EUG were determined to be 16.7 µM and 0.14 mM, respectively. Gene expression profiling revealed a significant increase in Caspase-3 and Caspase-9 expression, indicating the activation of apoptotic pathways. Flow cytometry and Hoechst 33258 staining confirmed the induction of apoptosis, with noticeable changes in cell morphology and nuclear fragmentation. The NO release assay demonstrated a dose-dependent increase in NO levels, highlighting the compounds' anti-proliferative effects.

**Conclusion**: EUG and Bis-EUG exhibit potent anticancer effects on leukemia K562 cells by inducing apoptosis and inhibiting cell proliferation. The significant increase in NO levels and the activation of caspases suggest that these compounds may serve as effective chemotherapeutic agents. Further in vivo studies are warranted to explore their potential in cancer treatment regimens.

**Keywords**: Eugenol, Bis-Eugenol, K562 cells, Apoptosis, Chemotherapeutic potential, Nitric oxide, Caspase activation, Anticancer activity.

## INTRODUCTION

Leukemia represents a significant global health challenge, contributing substantially to cancer-related morbidity and mortality. In 2021, it was reported that approximately 100,920 individuals were diagnosed with leukemia, leading to 27,021 deaths, highlighting the urgent need for effective treatments (1). In this context, Eugenol (EUG; C10H12O2) and its natural dimer Bis-Eugenol (Bis-EUG;

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C20H22O4), phenylpropanoid compounds derived primarily from clove oil (Syzygium aromaticum), have garnered attention due to their extensive pharmacological potential. These substances, characterized by their colorless to pale yellow oily appearance, exhibit a broad spectrum of biological activities, including antibacterial, antiviral, antifungal, anticancer, anti-inflammatory, and antioxidant effects, making them valuable in medicine and pharmacology (2).

Research has consistently demonstrated the anticancer properties of essential oils, particularly those rich in phenylpropanoids, against a variety of tumor cell lines (3). EUG and Bis-EUG, specifically, have been shown to induce apoptosis in promyelocytic leukemia cells (HJ-60) through mechanisms dependent on reactive oxygen species (ROS) and the modulation of signal transduction pathways, thereby exerting a cytotoxic effect on cancer cells (7). Furthermore, these compounds have been observed to enhance the cytotoxic and pro-apoptotic activity of cisplatin, a chemotherapeutic agent, in both in vivo and in vitro studies focusing on triple-negative breast tumors (8). They also appear to increase the sensitivity of the human immortal cell line from cervical cancer (HeLa cells) to cisplatin, suggesting a potential synergistic effect when combined with conventional chemotherapy (9).

This study aims to explore the chemotherapeutic potential of EUG and Bis-EUG against the Human Chronic Myeloid Leukemia K562 cell line, positioning these compounds as promising natural anticancer agents with the ability to interfere significantly with solid cancer cell growth through various mechanisms. The investigation into their molecular action as part of a novel cancer treatment strategy seeks to minimize side effects while maximizing efficacy. The antiapoptotic Bcl-2 proteins, in particular, present attractive targets for the development of innovative anticancer therapies, given their role in cell survival pathways (11).

EUG exerts its anticancer effects through diverse yet interconnected mechanisms, including the induction of apoptosis, arrest of the cell cycle, reduction of angiogenesis, and inhibition of inflammation and cellular invasion, alongside its dual roles as an oxidant and pro-oxidant (12). The study also notes the occurrence of autophagy and necroptosis in response to EUG treatment, highlighting its multifaceted impact on cancer cells. The development of nanocarriers for EUG, such as liposomes, microemulsions, and nanoparticles, has been a significant advancement, improving the delivery of phytoconstituents to targeted cancer cells while reducing the adverse effects and resistance often associated with chemotherapy (13).

In conclusion, the pro-apoptotic effects of EUG and Bis-EUG on K562 cells underscore their potential as effective, natural anticancer agents. Their capacity to elicit a broad range of chemotherapeutic properties at low doses offers a promising avenue for the development of cancer treatments with favorable outcomes and minimal side effects, furthering the pursuit of novel strategies in the fight against leukemia.

Table 1 Multidirectional Activity (EUG)

| Mechanism | Effect |
|---|---|
| Inhibition of NF-Kb activation | Reduces cancer cell proliferation and survival |
| Decrease in COX-2 activity | Lowers inflammation, potentially inhibiting tumor growth |
| Cell cycle arrest in S phase | Halts cancer cell division and growth |
| Apoptotic cell death | Induces death of cancer cells by reducing inflammatory cytokines |
| Neuroprotective role | Protects neuronal cells from damage |
| Antioxidant and MAO inhibitor | Prevents oxidative stress and inhibits monoamine oxidase, contributing to its therapeutic effects (4-6) |

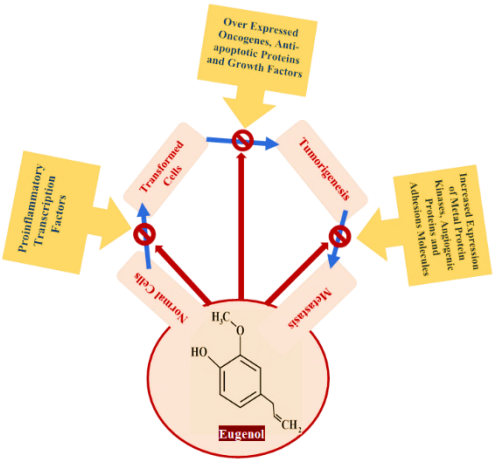


*Figure 1 Flowchart indicating molecular mechanism*

## MATERIAL AND METHODS

In an inter-collaborative effort that spanned one year, this study brought together the expertise and facilities of several distinguished institutions. The research was primarily conducted in the laboratories of Kinnaird College for Women University (KCWU), the Center of Excellence in Molecular Biology (CEMB), and the University of Health Sciences (UHS). The Kauser Abdullah Malik (K.A.M) School of Life Sciences was designated as the nexus for integrating the diverse facets of the study, facilitating a cohesive and synergistic research environment. Following the successful replication of experiments with noteworthy outcomes, an international scholar from Freie University of Berlin, Germany, was tasked with the critical role of analyzing and synthesizing the draft results, thus ensuring a global perspective was incorporated into the study's findings.

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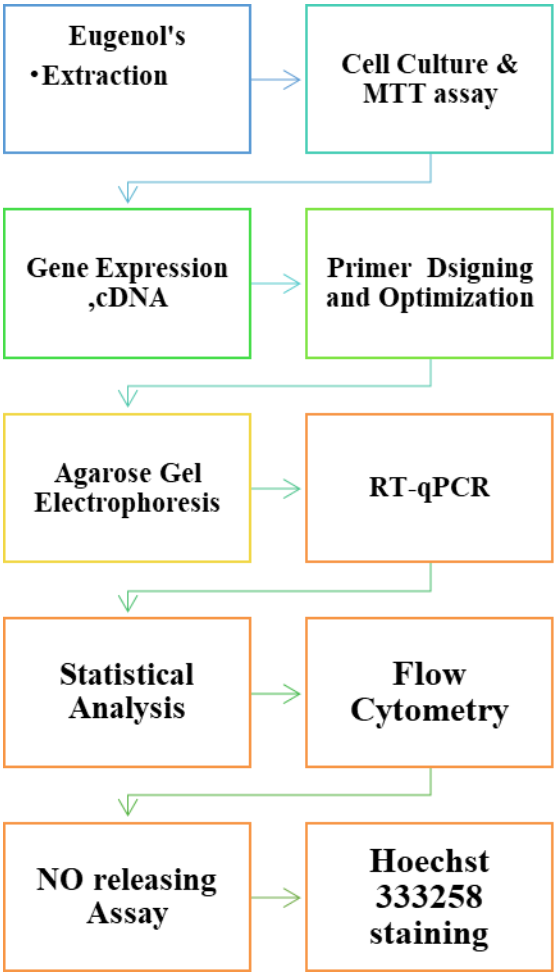


*Figure 2 Schematic representation of the experimental plan (In vitro study)*

The chemical cornerstone of this research was Eugenol (EUG), a compound primarily extracted from clove oil, which is known for its high EUG content ranging between 72–90%. Both EUG and its derivative, Bis-Eugenol (Bis-EU), demonstrated commendable solubility in organic solvents, a property that significantly contributed to the ease of conducting the experiments. The Human Leukemia K562 cell line served as the biological model for the study, with cells being exposed to various concentrations of EUG and Bis-EUG to assess cytotoxic effects via MTT assays conducted at intervals of 24, 48, and 72 hours. This meticulous approach allowed for the determination of IC50 values, reflecting the concentration at which a 50% reduction in cell viability was observed, with a particular focus on the 24-hour treatment period for enhanced reliability of results.

Gene expression analysis was a critical component of the study, employing RT-qPCR techniques to scrutinize the impact of treatments on specific gene activities. The selection of primers for Caspase-3 and Caspase-9 was executed with precision, following comprehensive design and optimization processes to ensure the validity of the expression data. The employment of conventional PCR methods and agarose gel electrophoresis further solidified the reliability of the primer sequences used. The CFX 96 qPCR system from Bio-Rad, complemented by reagents sourced from Thermo Fisher Scientific, USA, facilitated a rigorous assessment of gene expression, with β-Actin serving as a stable internal control to normalize the data.

Statistical analyses were conducted using advanced software tools, including GraphPad Prism, Version 9, and Microsoft Excel, with a defined significance threshold of $p \leq 0.05$. This analytical framework was instrumental in evaluating relative gene expression levels and other critical parameters, ensuring a robust statistical foundation for the study's conclusions. The evaluation of cell apoptosis and nitric oxide release were integral to understanding the cellular mechanisms influenced by EUG and Bis-EU treatments, with flow cytometry and colorimetric assays providing quantitative insights into these processes. Furthermore, the application of Hoechst 33258 staining offered a qualitative assessment of nuclear changes, augmenting the study's comprehensive analysis of treatment effects.

Statistical analysis played a pivotal role in the evaluation and interpretation of the data collected during this study. The research team employed GraphPad Prism Software, Version 9, and Microsoft Excel for this purpose. These tools were selected for their robust statistical capabilities and user-friendly interfaces, facilitating a comprehensive analysis of the study's findings. The primary focus of the statistical evaluation was on the relative gene fold changes among other variables, which were scrutinized to ascertain the effects of Eugenol (EUG) and Bis-Eugenol (Bis-EU) on the Human Leukemia K562 cells.

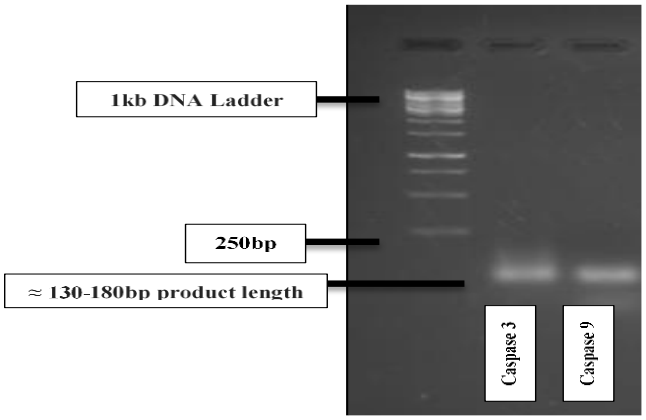


*Figure 3 Primer optimization results on 1.8% agarose gel, run at 120V for 45 min*

The analysis began with the calculation of IC50 values, determining the concentrations at which EUG and Bis-EU induced a 50% reduction in cell viability. This step was crucial for establishing the effective dose ranges of the compounds under investigation. Subsequently, the expression levels of targeted genes, specifically Caspase-3 and Caspase-9, were analyzed through RT-qPCR, with β-Actin serving as an internal control to normalize the data. The differential expression of these genes provided insights into the apoptotic mechanisms triggered by the treatments.

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Table 2 Primer sequences of required genes with their melting temperature

| GENE NAME | PRIMER SEQUENCES |
|---|---|
| Caspase-3 | Forward: 5'-GCGAATCAATGGACTCTGG-3' |
| | Reverse: 5'-GACATCTGTACCAGACCGAG-3' |
| Caspase-9 | Forward: 5'-CAGGCCCCATATGATCGAG-3' |
| | Reverse: 5'-CTGTGTCCTCTAAGCAGGAGA3' |

GraphPad Prism Software was instrumental in performing various statistical tests, including one-way ANOVA and post-hoc analyses, to compare the gene expression levels and cytotoxic effects across different treatment groups. This software's graphical capabilities also allowed for the visual representation of the data, enhancing the clarity and interpretability of the results. Microsoft Excel complemented these analyses by facilitating data organization, preliminary calculations, and the preparation of charts for initial data exploration.

A p-value of ≤ 0.05 was established as the threshold for statistical significance, ensuring that the findings were rigorously vetted for reliability. This criterion was applied across all statistical tests to discern meaningful differences between treatment groups and control conditions. The adoption of this stringent standard underscores the study's commitment to scientific integrity and the generation of credible, actionable insights.

## RESULTS

In the study exploring the chemo-preventive potential of Eugenol (EU) and Bis-Eugenol (Bis-EU) on the Human K562 cell line, a series of in vitro experiments were conducted to elucidate their anticancer properties. MTT assays were performed on K562 cells treated with serial concentrations of EU (5, 10, 15, 20, 25, and 30 µM) and Bis-EU (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mM), with the assays conducted over 24, 48, and 72 hours. The 24-hour assay was specifically chosen for further analysis due to its optimal determination of IC50 values for EU and Bis-EU, which were found to be 16.7 µM and 0.14 mM, respectively. The cell viability analysis, represented through a graph with a dotted black line indicating the IC50 of both compounds, showed a comparison with Cisplatin used as a control, illustrating the compounds' efficacy in reducing cell viability at these concentrations.

Further analysis focused on the expression of pro-apoptotic genes, with significant attention given to Caspase 3 and Caspase 9. The expression profiling revealed a marked increase in the relative gene fold for both biomarkers, indicating the potent pro-apoptotic capability of EU and Bis-EU. Notably, the expression of Caspase-9 was significantly higher than that of Caspase-3, suggesting a more pronounced effect of Bis-EU on the apoptotic pathway within K562 cells.

The cell apoptosis assay provided additional insights into the mechanisms underlying cell death induced by EU and Bis-EU. Following treatment with IC50 concentrations of both compounds, K562 cells were stained with Annexin V-FITC and propidium iodide (PI) and analyzed via flow cytometry. The apoptotic rates, as determined by fluorescence measurements, were positively correlated with the IC50 values, confirming the pro-apoptotic potential of EU and Bis-EU.

Table 3 In vitro anticancer studies of Eugenol

| Cancer Type | Tested Compound | Cell Line Used | Effect and Mechanism | IC50 Value |
|---|---|---|---|---|
| Leukemia | EUG | K562 | - Increase in ROS-generated apoptosis<br>- Increase in mitochondrial permeability transition<br>- Decrease in Bcl-2<br>- Increase in cytochrome c release (14) | 16.7 µM |
| Leukemia | EUG and Bis-EUG<br>(3, 3'-dimethoxy-5,5'-di-2-propenyl-2,2'-diol) | K562 | - Increase in cytotoxicity<br>- Increase in apoptosis<br>- Decrease in COX-2 gene expression (15) | 0.14 mM (Bis-EUG) |

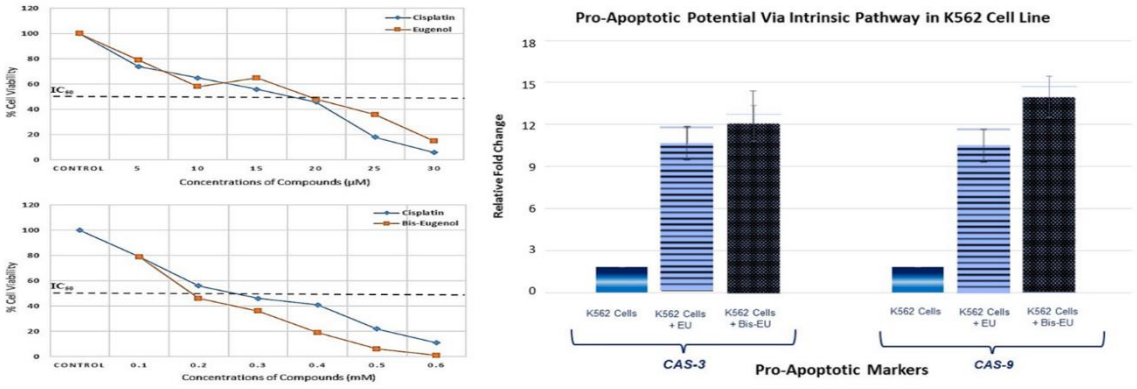


*Figure 4 MTT assay of K562 cells showing IC50 for EU (16.7μM) and Bis-EU (0.14mM) versus Cisplatin; Expression of Caspase-3 and -9 in K562 cells, highlighting Bis-EU's enhanced pro-apoptotic effect*

K562 cells exposed to 16.7uM of EUG and 0.14mM of Bis-EUG showed brightly blue fluorescence along with apoptotic morphology after treatment, including rupture of cell membranes and Nuclear Fragmentations.

Morphological changes induced by the treatments, observed through Hoechst 33258 staining, highlighted distinctive features such as cell shrinkage, chromatin condensation, and apoptotic body formation, with the nuclear alterations seen as bright blue nuclei indicative of apoptosis, contrasting with the control cells, which exhibited no significant morphological changes.

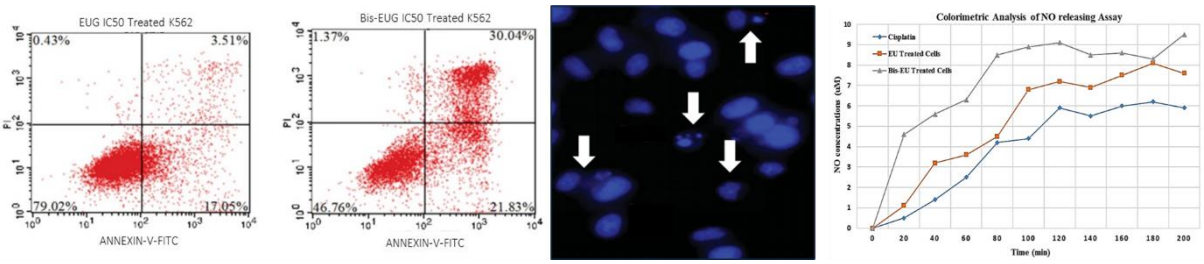


Figure 5 Flow cytometry of K562 cells after 24h with EUG (16.7μM) and Bis-EU (0.14mM), using annexin V-FITC/PI; Hoechst 33258 staining; NO assay: Bis-EUG outperforms EUG and Cisplatin in growth inhibition at 100-120 min

The nitric oxide (NO) releasing assay further corroborated the anti-proliferative effects of EU and Bis-EU. Following treatment with IC50 concentrations of the compounds, a continuous rise in NO levels was observed, underscoring the compounds' ability to enhance anti-proliferative activity in a dose-dependent manner. The elevation in NO levels served as a significant marker of the compounds' efficacy in inhibiting cell proliferation, aligning with the observed pro-apoptotic and cytotoxic effects.

Together, these results demonstrate the potent anticancer activities of Eugenol and Bis-Eugenol against K562 cells, highlighting their potential as effective agents in the treatment of leukemia through mechanisms involving apoptosis induction, gene expression modulation, and nitric oxide-mediated anti-proliferative effects.

## DISCUSSION

The exploration of natural compounds as potential antineoplastic agents has become increasingly significant, as the majority of chemotherapeutic drugs currently in clinical use are either natural substances or derivatives thereof (16). In this context, Eugenol (EUG) and Bis-Eugenol (Bis-EUG) have garnered attention for their capacity to modulate various cellular processes associated with cancer initiation and progression. Our study's findings corroborate the antioxidative, anti-inflammatory, pro-apoptotic, anti-proliferative, and anti-tumorigenic properties of these compounds across multiple cancer cell lines, including the human leukemia K562 cells (17). Specifically, the enhanced expression of Caspase-3 and Caspase-9 observed in treated K562 cells underscores the pro-apoptotic potential of EUG and Bis-EUG, contributing significantly to their anticancer efficacy (18). This observation is in line with studies on other compounds, such as piperine, which also induced apoptosis through Caspase activation in cancer cell lines (19).

Previous research has demonstrated EUG's chemotherapeutic properties against lung cancer, highlighting its ability to inhibit cell viability and prevent metastasis at low doses by targeting the PI3K/Akt pathway and MMP activity (20). Similarly, EUG's effect on E2F1 deregulation suggests its potential as a targeted agent for melanoma treatment, given the critical role of E2F transcription factors in melanoma cell proliferation (21). Our study extends these findings, showing that EUG and Bis-EUG not only inhibit leukemia cell growth but also induce nitric oxide (NO) release, a marker of anti-proliferative activity observed in other leukemia and MCF-7 breast cancer cell lines (22).

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The apoptotic effects of EUG and Bis-EUG were further validated through flow cytometric analysis, which revealed changes in cell size and granularity similar to those observed with other anticancer agents like cinnamaldehyde and Thymoquinone (23). Additionally, the morphological alterations detected via Hoechst 33258 staining, including nuclear fragmentation and apoptotic body formation, emphasize the compounds' ability to disrupt cellular integrity and induce apoptosis (26). These findings align with previous studies documenting EUG's membrane permeability and its role in disrupting cellular metabolism (25).

The potential of EUG to enhance the efficacy of cisplatin against breast cancer stem cells, by inhibiting ALDH activity and enhancing NF-κB pathway inhibition, underscores its utility as a complementary therapy for challenging cancer types like triple-negative breast tumors (27). This study's results, indicating the significant anticancer potential of both EUG and Bis-EUG, particularly in enhancing the sensitivity of cancer cells to traditional chemotherapeutic agents, highlight the need for further research into their synergistic effects across various cell lines (29).

## CONCLUSION

In conclusion, our findings affirm the potent anticancer effects of EUG and Bis-EUG on K562 cells, with Bis-EUG exhibiting a higher chemoprotective potential. The differential sensitivity of K562 cells to these compounds suggests that Bis-EUG may have superior anti-proliferative and pro-apoptotic effects. Future studies should focus on in vivo assessments of these compounds' mechanisms of action and explore their synergistic potential with conventional chemotherapeutic agents to mitigate chemotherapy-induced adverse effects. While our study provides valuable insights into the anticancer properties of EUG and Bis-EUG, limitations such as the unanalyzed purity of the compounds post-extraction highlight the need for further validation of our findings. Future research should include comprehensive chemical analysis, such as HPLC, to ascertain the purity and specific contributions of EUG and Bis-EUG to the observed anticancer effects, thereby enhancing the development of novel cancer treatments with minimal side effects.

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