STUDY ON ANTI CANCER ACTIVITY OF 2-PYRIDINEFORMAMIDE THIOSEMICARBAZONES AND THEIR COPPER (II) COMPLEXES ON *HeLa* CELLS

A Dissertation
In partial fulfillment of the requirement of Master's Degree in Biotechnology
(M.Sc. 2080)

Submitted to:
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Nepal

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Recommendation

This is to certify that Mr. Rabindra Khatiwada conducted the research work titled '**STUDY ON ANTI-CANCER ACTIVITY OF 2-PYRIDINEFORMAMIDE THIOSEMICARBAZONES AND THEIR COPPER(II) COMPLEXES ON *HeLa* CELLS**' under our supervision. This thesis work was undertaken as partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The results presented herein are his original findings. We hereby recommend this thesis for final evaluation.

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Certificate of Evaluation

This certification acknowledges that the thesis titled **'STUDY ON ANTI-CANCER ACTIVITY OF 2-PYRIDINEFORMAMIDE THIOSEMICARBAZONES AND THEIR COPPER(II) COMPLEXES ON *HeLa* CELLS**,' presented to the evaluation committee by Mr. Rabindra Khatiwada, is deemed satisfactory for the partial fulfillment of the Master of Science in Biotechnology.

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ABSTRACT

The research presented in this thesis aimed to study, identify, and compare the anti-cancer activity of different 2-pyridineformamide thiosemicarbazones and their Cu(II) complexes (HL_1, HL_3, HL_4, HL_5, HL_6, [CuL_1Cl], [CuL_3Cl], [CuL_4Cl]) on *HeLa* cell lines. The specific objectives were to evaluate the Half maximal inhibitory concentration (IC_{50}) of these compounds, perform cell viability assays using MTT reagent, and compare the IC_{50} values among the compounds. The experimental methodology involved culturing *HeLa* cells and treating them with different concentrations (1-50 micro molar) of the compounds in DMEM media. Cell viability was assessed after a 72-hour incubation using MTT reagent, and absorbance was measured at 551nm. Statistical analysis, including non-parametric one-way ANOVA followed by post hoc Tukey's multiple comparison, revealed significant differences in mean viability across the concentrations of all compounds, indicating compound-specific effects. The dose-response curve analysis showed a concentration-dependent reduction in cell viability, with calculated IC_{50} values ranging from 0.08406 µM to 8. 687 µM. It had been observed that the HL ligand had shown less IC_{50} values as compared to its corresponding copper(II) complex Nevertheless, Copper(II) complexes exhibited more potent anticancer activity compared to their respective ligands for maximal cell death, as shown by bar graph and data analysis. For example, [CuL_1Cl] exhibited 96.59% inhibition at 10 µM, while its ligand, HL_1, showed a lower 68.61% inhibition at 10 µM. Similarly, [CuL_3Cl] displayed notably higher 99.79% inhibition at 25 µM compared to HL_3's slightly lower 87.29% inhibition at the 20 µM concentration. [CuL_4Cl]'s ligand counterpart HL_4 showed 92.41% inhibition at 20 µM concentration, [CuL_4Cl] exhibited substantial inhibition of 97.93% at just 5 µM. Furthermore, a comparison among the concentrations of compounds highlighted distinct effects on cell viability, with some concentrations showing expansion in viability at lower concentrations. These findings emphasize the compound-specific and concentration-dependent effects of 2-pyridineformamide thiosemicarbazone its copper(II) complexes on *HeLa* cell viability. The implications of this research may have far-reaching effects in the field of cancer therapeutics and warrant further investigation.

Keywords: Anti-cancer activity, cell viability, concentration-dependent response, compound-specific effects, Copper(II) complexes, *HeLa* cell lines, IC_{50}, ligands

CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	
HL_1	N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide
HL_3	N'-(4-(pyridin-2-yl)piperazine-1-carbonothioyl)picolinohydrazonamide
HL_4	6-methyl-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide
HL_5	6-Methyl-N'-(morpholine-4-carbonothioyl)picolinohydrazonamide
HL_6	6-Methyl-N'-(4-(pyridin-2-yl)piperazine-1-carbonothioyl)picolinohydrazonamide
$[CuL_1Cl]$	((((amino(pyridin-2-yl)methylene)hydrazono)(pyrrolidin-1-yl)methyl)thio)copper(II) chloride
$[CuL_3Cl]$	((((amino(pyridin-2-yl)methylene)hydrazono)(4-(pyridin-2-yl)piperazin-1-yl)methyl)thio)copper(II) chloride
$[CuL_4Cl]$	((((amino(6-methylpyridin-2-yl)methylene)hydrazono)(pyrrolidin-1-yl)methyl)thio)copper(II) chloride

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CHAPTER 1

INTRODUCTION

1.1 Background

A class of organosulfur compounds known as thiosemicarbazones is distinguished by their distinct chemical structure, $H_2NC(S)NHN=CR_2$, where R can be any of several organic groups. These substances have attracted a lot of attention from scientists because of their wide range of biological characteristics and flexible uses in medicinal chemistry. Thiosemicarbazones were first identified by Domagk in the middle of the 20th century for their antibacterial properties. Since then, their potential to fight a variety of diseases, including bacteria, viruses, and malaria, has been the subject of intense investigation. In addition to their antibacterial properties, thiosemicarbazones have been shown to have promise antiviral and anticancer properties, which makes them important research targets for medicinal drugs (Domagk et al., 1946; Shakya, 2016).

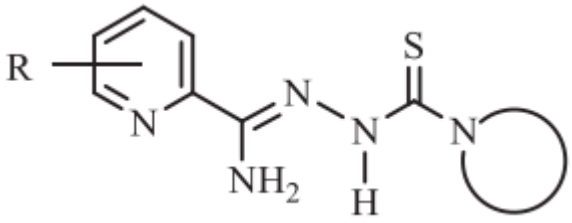


Figure 1 2-Pyridineformamide Thiosemicarbazone

In the study by (Shakya et al., 2016) a set of thiosemicarbazone and its copper(II) complexes were investigated, each characterized by unique properties. The compounds, denoted as HL_1, HL_3, HL_4, HL_5, and HL_6, displayed diverse molecular formulas, molecular masses, colors, yields, and melting points. For instance, HL_1 had a molecular formula of $C_{11}H_{15}N_5S$ with a molecular mass of 249.34 g/mol. It exhibited a yellow color, a yield of 51%, and a melting point range of 159-160°C. Similarly, HL_3 had a molecular formula of $C_{16}H_{19}N_7S$, a molecular mass of 341.43 g/mol, a yellow color, a yield of 48%, and a melting point range of 151-152 °C. HL_4, with a molecular formula of $C_{12}H_{17}N_5S$ and a molecular mass of 263.36 g/mol, showed a yellow color, a yield of 34%, and a melting point range of 152-154 °C.

HL_5, characterized by a molecular formula of $C_{12}H_{17}ON_5S$ and a molecular mass of 279.36 g/mol, presented a yellow color, a yield of 56%, and a melting point range of 163-166 °C. Lastly, HL_6, with a molecular formula of $C_{17}H_{21}N_7S$ and a molecular mass of 355.46 g/mol, displayed a yellow color, a yield of 40%, and a melting point range of 148-150°C.Furthermore, copper complexes, labeled as $[CuL_1Cl]$, $[CuL_3Cl]$, and $[CuL_4Cl]$, were characterized in the study. These complexes exhibited distinct molecular compositions and colors. For example, $[CuL_1Cl]$ had a molecular formula of $C_{11}H_{14}ClCuN_5S$, a molecular mass of 347.32 g/mol, and displayed an emerald color, boasting a high yield of 95% and a melting point range of 263-266°C. Similarly, $[CuL_3Cl]$, with a molecular formula of $C_{16}H_{18}ClCuN_7S$ and a molecular mass of 439.43 g/mol, presented a mint green color, a yield of 90%, and a melting point range of 247-250°C. Lastly, $[CuL_4Cl]$ exhibited a moss green color, had a molecular formula of $C_{12}H_{16}ClCuN_5S$ with a molecular mass of 361.35 g/mol, a high yield of 93%, and a melting point range of 242-244°C. These comprehensive characterizations provide detailed insights into the diverse properties of these thiosemicarbazones and its copper(II) complexes and their copper complexes as documented in Shakya et al.'s study.

1.2 Thiosemicarbazone chemistry

Thiosemicarbazones (TSCs) are synthetic chemicals with a variety of structures and significant biological potential that arise from the reaction of thiosemicarbazide with aldehydes or ketones. To create them, take an aldehyde or ketone used in the synthesis and add "thiosemicarbazone" to the compound's name. The R^1, R^2, R^3, and R^4 as shown in figure 2, substituents that make up TSCs' overall structure enable the creation of diverse derivatives with heterocyclic characteristics as well as their integration into cyclic systems. Because of electron dispersion, TSCs usually take on a flat structure in their solid-state form, which encourages internal hydrogen bonding between particular nitrogen and amine atoms. The electron distribution is mostly affected by aromatic attachments on the azomethine carbon. (Shakya B., 2016). Additionally, during dissolution, TSCs exhibit tautomerism between thione and thiol forms, with the thiol form promoting effective electron distribution across the thiosemicarbazone structure.

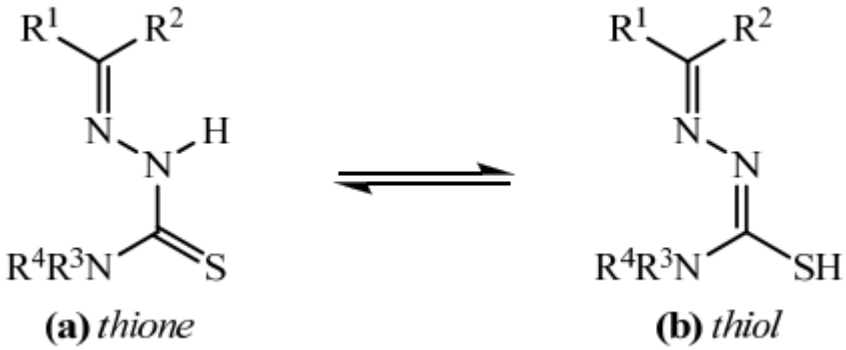


Figure 2 Tautomeric forms of thiosemicarbazone

When heteroaromatic components are added, electron dispersion is enhanced and possible coordination sites are created. Because of its varied structures, biological significance, and ion-sensing capabilities, TSCs are an all-around adaptable chemical class that presents promising opportunities in areas such as pharmaceutical and coordination chemistry (Chattopadhyay et al., 1988).

1.3 Cancer Biology

Individual cell growth, specialization (differentiation), and survival in multicellular organisms are carefully regulated to meet the needs of the organism as a whole. Because they proliferate and divide uncontrollably, cancer cells undermine this strict regulation. When cancer cells proliferate unchecked, they eventually spread throughout the body, interfering with tissues' and organs' ability to function normally (Cooper., 2019). A number of characteristics contribute to the transformation of a normal cell into a cancerous one, each of which accelerates the growth of the cell and eventually results in the formation of a tumor. During this process, genetic alterations modify important components of the cell, enabling them to ignore growth restrictions and display all the characteristics of cancer. Cancer cells develop an innate will to proliferate regardless of outside stimuli, disregard signals that prevent cell division, and thwart the process of natural cell death. Additionally, they could separate from their initial tissue and alter how they interact with nearby cells (De Bock et al., 2011). Because they frequently lack oxygen, tumors require blood flow to enlarge. They accomplish this by encouraging the development of fresh blood arteries that pierce the tumor. Tumors can travel to far-off parts of the body and become more adept at penetrating surrounding tissues as cancer progresses. This transforms the tumor into an aberrant organ that is ready

for rapid development and invasive activity, which may cause it to spread to other areas of the body (Lodish, et.al., 2016). Exposure to carcinogens and errors in DNA replication can result in alterations to the sequence of DNA. All mutagens, including carcinogens, change DNA by modifying nucleotides. Indirect-acting carcinogens are the primary class of carcinogens; they must first be activated in order to damage DNA. The cytochrome P-450 system aids in the removal of hazardous foreign compounds in animals through metabolic activation. However, direct-acting carcinogens, such as DMS and EMS, can harm DNA without requiring alterations to the cells (Heyer et al., 2010).

1.3.1 Cell Division

The four main phases of the cell cycle—G_1, S, G_2, and M—allow for precise cell division and replication. Cell development, monitoring of internal and external environments, and preparation for critical events like DNA synthesis (S phase) and cell division (M phase) are all part of interphase, which consists of the G_1, S, and G_2 phases. Mammalian cells spend a significant amount of time replicating chromosomes and creating sister chromatids during the S phase. These condensed sister chromatids align, split, and finally produce two daughter nuclei during M phase, also known as mitosis. The G_1 and G_2 gap phases serve as checkpoints and promote cell growth by enabling the cell to assess its surroundings prior to committing to DNA replication and division. progression through the cell cycle, particularly at the decision-making stage in the G_1 phase (Alberts et al., 2015).

Ribonucleotide reductase (RNR) is vital for DNA synthesis during cell division. It converts ribonucleotides into deoxyribonucleotides, essential for DNA creation (Torrents et al., 2002). The RNR mechanism involves radical generation at C-3', facilitating deoxyribonucleotide production (Kolberg et al., 2004).

In humans, RNR comprises RRM1 (alpha subunit), RRM2, and RRM2B (beta subunits), needing a di-iron cofactor for ribonucleotide conversion (Larsson et al., 2004; Uppsten et al., 2004). Thiosemicarbazones, like α(N)-heterocyclic carboxaldehyde derivatives, inhibit RNR by disrupting its active-site radical (Kalinowski et al., 2009; Fairman et al., 2011). Notably, FeII-3-AP(Triapine) complex exhibits potent inhibition, deactivating the enzyme and inducing protein

damage via ROS mediated suppression of tyrosyl radical within R2 subunit of Ribonucleotide Reductase (Shao et al., 2006).

These compounds, while hindering DNA synthesis, also induce DNA breaks, although the exact link to RNR inhibition remains incompletely understood These substances can induce chromatid and single -strand break in DNA (Sartorelli et al., 1971). Elevated RNR levels in cancer cells make it a therapeutic target, and agents targeting RNR, like thiosemicarbazones, show promise as anti-cancer treatments (Herrick et.al., 2007).

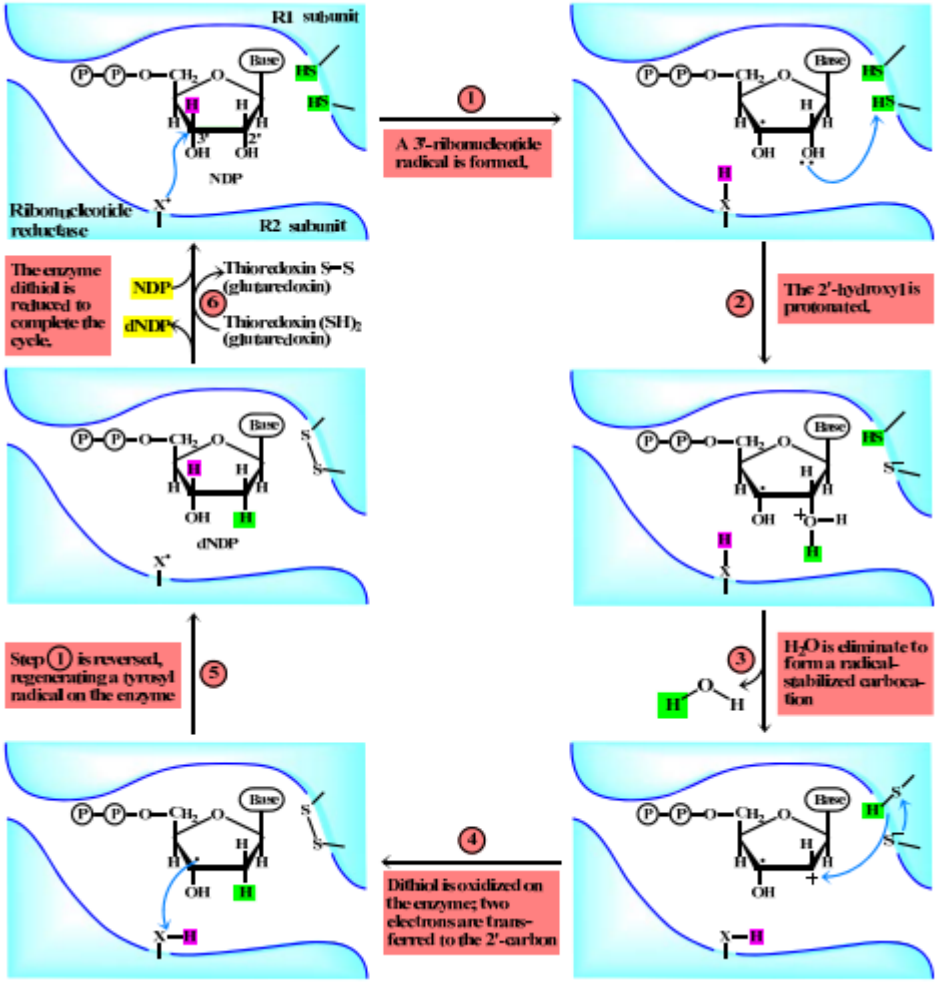


Figure 3 Catalytic mechanism of ribonucleotide reductase

1.3.2 Mechanism of Action of Ribonucleotide Reductase

The initial step involves the generation of a substrate radical at the C-3' position via a thiyl radical abstracting a hydrogen atom—this radical subsequently induces the departure of OH- from the C-2' carbon. The resulting 2'-ketyl radical (state 3) arises after the protonation and release of OH- as water, facilitated by another cysteine residue. To complete the reduction at C-2', a third cysteine residue transfers a hydride ion, reforming a C3 radical and generating a disulfide radical anion (state 5). The release of the deoxyribonucleotide from the enzyme occurs when the C-3' radical recaptures the initial hydrogen atom removed earlier, aided by an electron from R2 to reduce the thiyl radical. Restoring the enzyme's activity involves a disulfide-containing protein, like thioredoxin, which reduces the formed disulfide bond in the enzyme's active site. Subsequently, thioredoxin's oxidized form is reduced by NADH through mediation by thioredoxin reductase, thereby concluding the entire reaction sequence (Kolberg et al., 2004).

1.3.3 Anti-Cancer Activities

Phase I clinical trials were initiated in 1956 and the first medicine derived from heterocyclic thiosemicarbazones (HCT) was presented. This progression transpired subsequent to the identification of its formidable capacity to impede the operation of the Ribonucleotide reductase (RR) enzyme. The first use of HCT's anti-cancer capabilities was demonstrated at this milestone when pyridine-2-carboxaldehyde thiosemicarbazone showed promising results against leukemia L1210 in mice (Brockman et al., 1956).

Subsequent studies revealed that an α-(N)-heterocycle plays a critical function in augmenting its biological effectiveness. Interestingly, hydroxyurea, the first RR inhibitor utilized in clinical settings, is noticeably less potent than these particular α-(N)-heterocyclic thiosemicarbazones (Liu et al., 1995). Of these, 3-Hydroxypyridine-2-Carboxaldehyde Thiosemicarbazone (3-HP) has proven to be particularly effective against a number of cancer forms, such as lymphoma L5178Y, Lewis's lung cancer, Ehrlich ascites carcinoma, and L1210 (Sartorelli, 1967).

1.4 Laboratory test of Anti-Cancer Agent

1.4.1 *In vivo* method

The *in vivo* approach is essential to the assessment of possible anticancer drugs. *In vivo* experiments, as opposed to controlled cell culture settings, examine the impact of these drugs on living things, especially animal tumor models. This method takes into account a number of variables, including vascularization, tumor heterogeneity, and interactions between distinct cell types, to provide a more thorough knowledge of how the medications interact with the intricacies of a three-dimensional tumor environment. Researchers may see directly how these drugs affect tumor development, metastasis, and overall survival in a real system through in-vivo investigations, which offer priceless insights that are not entirely attainable in artificial in-vitro conditions. Therefore, before contemplating the therapeutic uses of anticancer medicines, the in- vivo approach is crucial for evaluating their prospective efficacy and safety, even though in-vitro tests serve as an initial screening tool (Zips et al., 2005).

1.4.2 *In vitro* Method

Under carefully monitored lab settings, prospective anticancer drugs are thoroughly assessed through the *in vitro* procedure. First, a variety of tumor cell lines with human or murine origins are chosen to represent various cancer types. Using specialized cell culture media such as Eagles Minimum Essential Medium (EMEM), Dulbecco Modified Eagles Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, and others, these cell lines are exposed to candidate medications in either non-clonogenic or clonogenic experiments. While clonogenic assays evaluate the survival and capacity to form colonies of clonogenic cells—which are frequently started from a single cell—non-clonogenic assays, such as cell counting or dye-based methods like MTT assays, estimate cell survival. Drug concentrations are frequently varied in these tests in order to find the IC_{50} value, which indicates the concentration required to lower the number of cells by 50%. Before moving on to more complicated *in vivo* models for additional assessment, these *in vitro* investigations offer insightful information about possible therapeutic uses by analyzing the impact of these medicines on various tumor cell lines (Zips et al., 2005).

1.5 Rationale of study

This thesis uses *HeLa* cells, a well-known cellular model for cancer research, to examine the effects of various 2-pyridineformamide thiosemicarbazones its copper(II) complexes on cellular activity. The principal aim was to appraise the cytotoxic properties of these compounds and appraise their potential as agents against cancer. This investigation is motivated by the known inhibitory actions of some thiosemicarbazones on ribonucleotide reductase, an enzyme that is essential for DNA synthesis, inhibition of Topoisomerase 2, disruption of mitochondria (Yuan et al.,1998), production of reactive oxygen species (ROS) (Shao et al., 2006), and inhibition of Multi Drug resistance protein (MDR1). Thus, it is expected that this inhibition will prevent the growth of cancer cells, making these compounds promising candidates for anticancer treatments (Shakya et al., 2019).

By investigating the cytotoxicity of various 2-pyridineformamide thiosemicarbazones its copper(II) complexes on *HeLa* cells, this study aimed to shed light on their potential as selective cytotoxic agents against cancer cells. Understanding the impact of these compounds on cell viability could contribute to the development of novel and effective strategies for cancer treatment. Through this exploration, the thesis seeks to elucidate the therapeutic prospects of these derivatives and their plausible role in advancing cancer therapeutics by targeting fundamental cellular processes essential for cancer cell survival and proliferation.

1.6 Research Hypotheses

In this study, we aim to investigate the effects of 2-pyridineformamide thiosemicarbazones and their copper(II) complexes on *HeLa* cell line across eight treatment dose 0μM, 1μM, 5μM, 10μM, 15μM, 20μM, 25μM and 50μM. We will utilize non-parametric one-way ANOVA to determine whether there are statistically significant differences in dose dependent reduction of *HeLa* cell line proliferation on each compound of 2-pyridineformamide thiosemicarbazone.

H_0(Null Hypothesis): There is no effect of the compounds on cell viability across different concentrations. Any observed differences in cell viability between the control and different concentrations are due to random chance or experimental variability among compounds ($\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 = \mu_8$).

H_1(Alternative Hypothesis): There is an effect of compounds concentrations on cell viability. The differences observed in cell viability between the control and different concentrations are not solely due to random chance or variability but rather indicate a genuine impact of the compounds on cell viability ($\mu_1 \neq \mu_2 \neq \mu_3 \neq \mu_4 \neq \mu_5 \neq \mu_6 \neq \mu_7 \neq \mu_8$).

1.7 Objectives

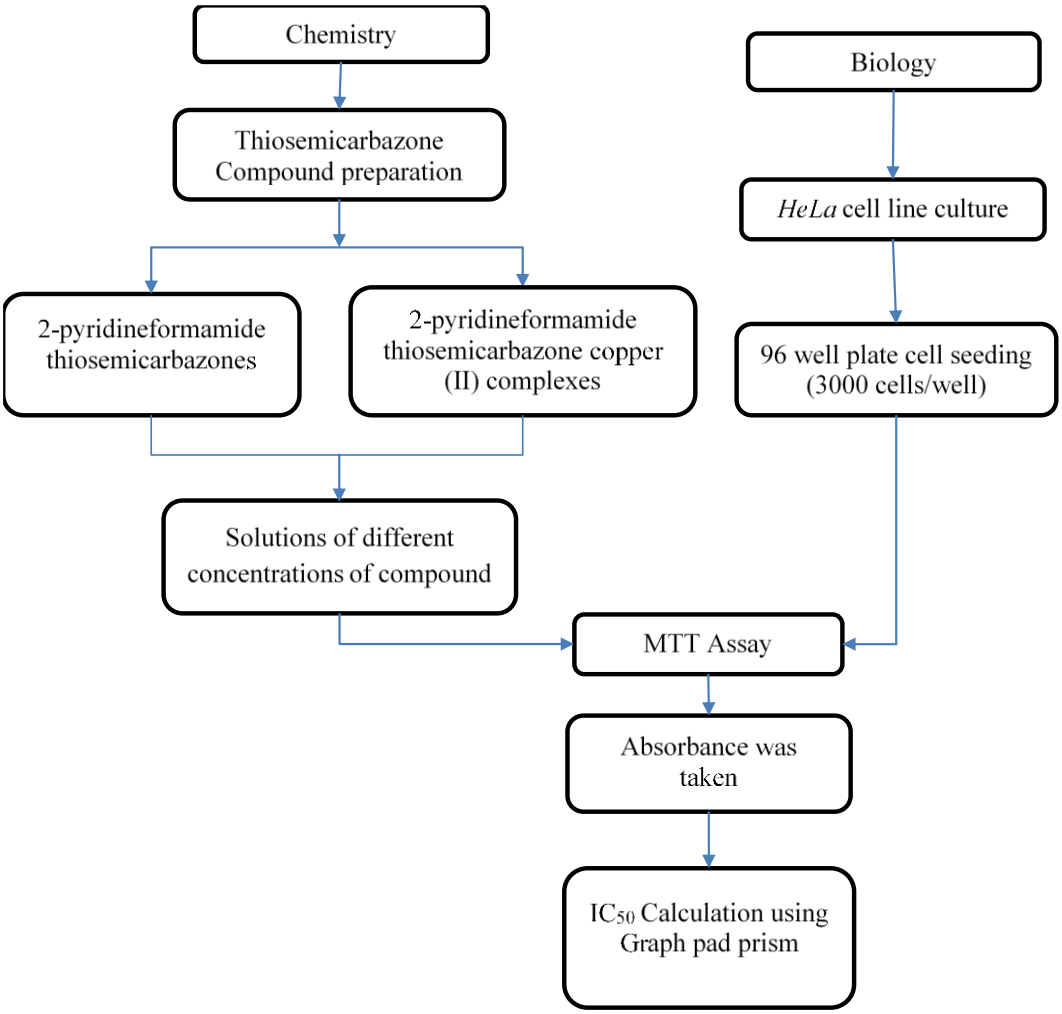
1.7.1 General objective

- To study and compare anti-cancer activity of 2-pyridineformamide thiosemicarbazones and their copper(II) complexes on *HeLa* cell line

1.7.2 Specific objective

- Understanding the in vitro cytotoxicity and cell death effect of 2-pyridineformamide thiosemicarbazones to the *HeLa* cell line.
- Re-examining efficacy of 2-pyridineformamide thiosemicarbazones on *HeLa* cell line.
- Efficacy study of 2-pyridineformamide thiosemicarbazone copper(II) complexes on *HeLa* cell line.
- Comparative IC_{50} efficacy analysis of the 2-pyridineformamide thiosemicarbazones versus their corresponding copper(II) complexes in inducing cytotoxicity and cell death at various concentrations.
- Correlate the obtained viability data from the MTT assay with the compounds' structural characteristics and known anti-cancer activities, establishing potential correlations between compound structure and cytotoxicity.
- Investigate the dose-response relationship by analyzing the impact of different compound concentrations on *HeLa* cell viability, identifying concentration-dependent effects.

1.8 Research Design



CHAPTER 2

LITERATURE REVIEW

2.1 Background

2.1.1 Thiosemicarbazones and its copper(II) complexes

An organosulfur molecule with the chemical formula $H_2NC(S)NHN=CR_2$ is called a thiosemicarbazone. There are other variations of this molecule, such as those where organic groups have been added to one or more of the NH sites (Wattanakanjana et al., 2012).

This investigation targeted potent inhibitors of α-glucosidase for diabetes management, focusing on compounds 3c, 3g, and 3i. These compounds, 3c=(E)-2-((4- chloro-2- oxo-2- H-chromen-3- yl) methylene)- N- (2,4,5 -trichlorophenyl) hydrazine-1- carbothioamide, 3g = (E)-2- ((4- chloro-2- oxo-2H- chromen-3-yl) methylene)-N-phenethylhydrazine-1-carbothioamide, and 3i=(E)-2-((4-chloro-2-oxo-2H-chromen-3-yl)methylene)-N-(4-nitrophenyl)hydrazine-1-carbothioamide, exhibited potent inhibitory activity against α-glucosidase. These compounds demonstrated impressive IC_{50} values ranging from 2.33 to 22.11 µM, surpassing the standard drug acarbose (IC_{50} = 873.34 ± 1.67 µM). Detailed examinations revealed compound 3c's concentration-dependent inhibition and emphasized the pivotal role of the thiosemicarbazide moiety in interacting with α-glucosidase residues. Molecular dynamics simulations highlighted the influence of these specific compounds on protein dynamics within the enzyme's active site. These findings position compounds 3c, 3g, and 3i as promising candidates for further exploration in novel antidiabetic medications (Zahra et.al.,2023)

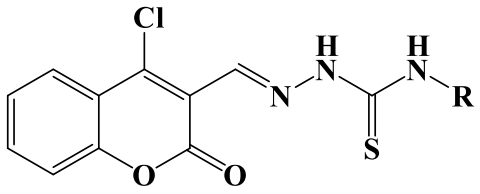


Figure 4 Coumarin-derived thiosemicarbazones

Here when R=2,4,5-Trichlorophenyl i.e(3c), R=β-Phenyl i.e (3g), R=4-Nitrophenyl i.e(3i)

Another study investigated the efficacy of three compounds—Diethyl 2-amino-5-[(E)-[(perfluorophenyl)methylidene]amino]thiophene-3,4-dicarboxylate (3j), Diethyl 2-amino-5-[(E)-[(5-nitrothiophen-2-yl)methylidene]amino]thiophene-3,4-dicarboxylate (3n), and Diethyl 2-amino-5-(6-chloronicotinamido)thiophene-3,4-dicarboxylate (5c)—on cell proliferation. Cancer cells in logarithmic growth phase were seeded at 200ml in 96-well plates at a density of $5 \times 10^{\wedge 3}$ cells per well and treated with these compounds. The assessment of cell proliferation inhibition was conducted by measuring absorbance at 540 nm after 48 hours of treatment.Results indicated the IC_{50} values for normal human embryonic kidney cells (HEK-293) as follows: 31.4 μM (3j), 65.0 μM (3n), and 160.0 μM (5c). Lower IC_{50} values suggested higher toxicity or impact on normal kidney cells at lower compound concentrations. Moreover, the compounds exhibited selectivity towards specific cancer cell lines over normal cells, illustrated by their Selective Index (SI) values. Higher SI values indicated greater selectivity. Compound 5c demonstrated selectivity towards certain cancer cell lines over normal cells (Bozorov et.al 2017).

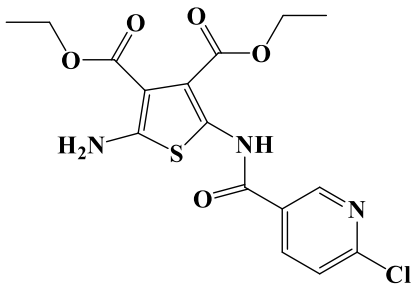


Figure 5 Structure of Diethyl 2-amino-5-(6-chloronicotinamido)thiophene-3,4-dicarboxylate compound

Where A= Pyridine chloride

When interacting with transition metal ions, thiosemicarbazones ($H_2NC(S)NHN=CR_1R_2$) usually act as chelating ligands. They join via the hydrazinic and sulfur nitrogen atoms to create connections. Sometimes, nevertheless, they function as monodentate ligands and create connections only with the sulfur atom (Shakya et.al.,2019)

Not only in cancer, this thiosemicarbazone compounds also inhibit superbugs.The emergence of superbugs, which are bacteria resistant to many drugs, especially

those carrying New Delhi metallo-β-lactamase (NDM-1), is a growing concern. Scientists have been working to create new substances that can stop NDM-1, making these bacteria vulnerable again. They made thirteen different substances, and two of them, called 1a and 1f, were really good at stopping NDM-1. These substances not only blocked NDM-1 but also killed the drug-resistant bacteria in the lab. What's interesting is that one of these substances, 1a, not only stops NDM-1 but also makes other antibiotics work better against these superbugs. This discovery is promising for developing new treatments against these dangerous, drug-resistant bacteria. (Ying et.al.,2021)

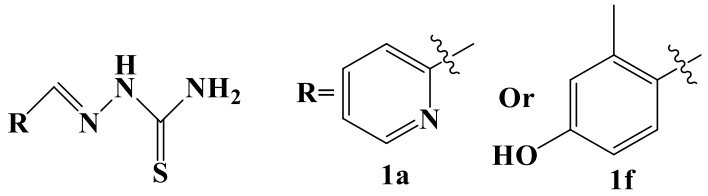


Figure 6 Structure of thiosemicarbazone compound which inhibits superbugs

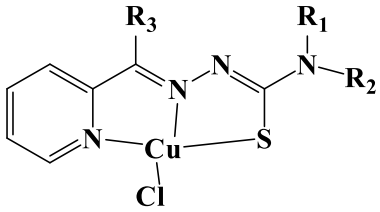
Where R= methyl phenol(1f), and R= pyridine (1a)

2.1.2 Thiosemicarbazones ligands and its copper(II) complex

Thiosemicarbazones, versatile ligands in coordination chemistry, exhibit properties conducive to binding with transition metals. Their structural composition is marked by the presence of pivotal C=N and C=S bonds, enabling effective chelation of metal ions. What sets them apart is their flexible thiosemicarbazone moiety structure, allowing the incorporation of diverse substituents and functional groups. This adaptability enhances their potential to adopt various coordination modes, enriching their coordination ability when engaging with transition metals. Moreover, the addition of heteroatoms, particularly nitrogen and sulfur, further augments their coordination prowess. This enrichment of heteroatoms within the thiosemicarbazone structure refines their coordination mode, rendering them adept at forming stable complexes with transition metals. Leveraging a donor system composed of nitrogen and sulfur atoms (N–N–S), thiosemicarbazones establish stable bonds with metal ions (Stefani et al., 2013).

Furthermore, the inclusion of the copper(II) metal ion refined this ligand. The unique characteristics of copper(II) complexes produced with thiosemicarbazone ligands are essential to their anti-cancer effect, especially against lung cancer cells.

Under the influence of cellular oxidants and reductants, these Copper complexes actually enable Copper to undergo redox cycling between the reduced monovalent and oxidized divalent states (Balsa et al., 2021). Copper complexes exhibit redox activity, cause an excess of reactive oxygen species within cells, and trigger cell death via the mitochondrial apoptotic pathway. Their ability to limit cell proliferation is closely associated with their lipophilicity, which is a crucial property. According to studies, these complexes catalyze hydrogen peroxide, which causes intracellular reactive oxygen species (ROS) and induces death in lung cancer cells. This process acts as a focused method to cause malignant cells to undergo programmed cell death. Additionally, scientists have suggested creating particular copper(II) complexes, by utilizing the His242 residue of the human serum albumin (HSA) carrier's IIA subdomain. These Complexes attach to HSA subdomain IIA by coordination with His242 or hydrophobic interactions. The objective of these designs is to improve these metal preparations' in vivo delivery efficiency, anti-cancer efficacy, and selectivity. With its new approach to enhancing the complexes' specificity and efficacy inside the biological system, this targeted formulation holds potential for cutting-edge cancer therapy techniques (Bai et al., 2022). According to (Bai et al. 2022), Copper complexes exhibited a greater anti-proliferative effect on lung cancer cells (A549 and NCI-H460) than their comparable ligands.



13.R_1=Me, R_2=H,R_3=H
14.R_1=Me,R_2=Me,R_3=ph

Figure 7 Complexes of 2- pyridineformamide thiosemicarbazones

2.1.3 Synthesis of 2-pyridineformamide thiosemicarbazones (Shakya et al., 2019)

The process starts by combining CS_2 and N-methylaniline with NaOH, followed by the addition of sodium chloroacetate and subsequent acidification with HCl. This sequence yields carboxymethyl N-methyl-N-phenyldithiocarbamate, presenting as a pale buff-colored substance with a melting point around 197–198°C. Upon further treatment of carboxymethyl N-methyl-N-phenyldithiocarbamate with hydrazine

hydrate and water, it generates colorless rod-shaped crystals with a melting point of 124–125 °C, forming 4-Methyl-4-phenyl-3-thiosemicarbazide(I).Moving forward, the reaction of 4-methyl-4-phenyl-3-thiosemicarbazide with pyrrolidine in MeCN results in the production of pyrrolidine-1-thiocarboxylic acid hydrazide (II). This product manifests as colorless needle-shaped crystals, melting at 172-174 °C. 2-Cyanopyridine, Sodium and dried methanol was added in (II) to prepare (III) 2-pyridineformamide N(4)-ring incorporated Thiosemicarbazones. Finally (III) is treated with $CuCl_2$ to form copper(II) complex of respected ligand i.e. (IV). The diagram below illustrates the overall procedure for generating the precursor involved in thiosemicarbazones and its Copper(II) complexes synthesis.

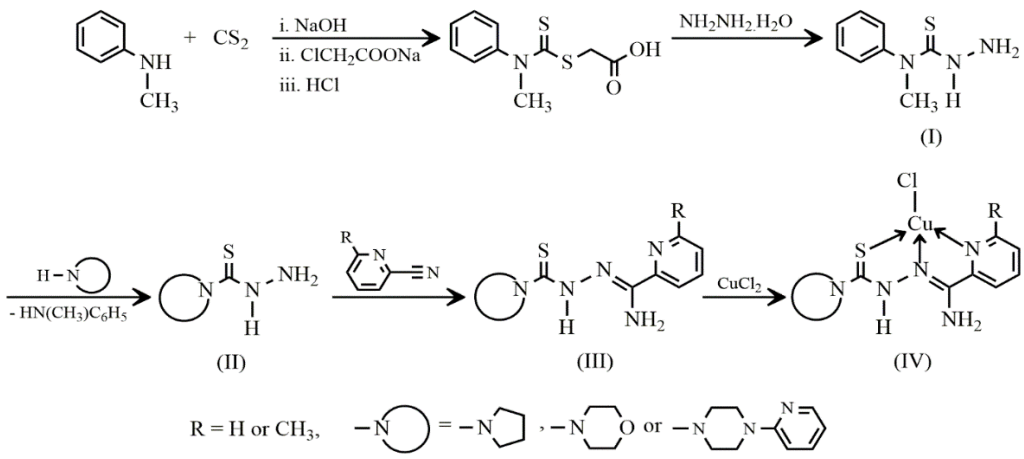


Figure 8 Synthetic route of 2-pyridineformamide thiosemicarbazones and their Copper(II) complexes

2.1.4 Cancer immunology

Highly responsive to detecting and defending against pathogens, the innate immune system relies on inflammation as a primary defense. Paradoxically, persistent inflammation can accelerate tumor growth, mainly through pathways involving cytokines such as VEGF, IL-10, and IL-6, along with gene alterations like BRAF, impacting both the tumor environment and immune responses. This persistent inflammation prompts immune suppression and aids tumor progression by stimulating proteins like STAT3 and fostering suppressor cell formation, notably in places like the spleen. Serving as a crucial connection between the innate and adaptive immune systems, dendritic cells (DCs) detect danger signals and present antigens to T cells. Insights into DC biology have inspired novel immunotherapies,

where ex vivo techniques create mature DCs from monocytes or CD34+ stem cells, loading them with specific tumor antigens before reintroduction into patients. Alternatively, in vivo strategies target DCs by combining tumor-specific antigens with adjuvants and specific antibodies. Despite notable successes, like sipuleucel-T's use in treating metastatic prostate cancer, achieving significant clinical responses in DC-based immunotherapies remains challenging. Current research focuses on refining these approaches, tapping into DCs' potential to develop more effective cancer treatments (Harris et al.,2013).

In the meantime, the field of cancer immunotherapy is developing quickly, with monoclonal antibodies being a key tool in the treatment of different kinds of tumors. For example, rituximab targets CD20 in lymphomas, trastuzumab targets HER-2 in breast cancer, and the recently approved T-DM1 combines trastuzumab with the powerful chemotherapeutic drug emtansine (DM1) to provide targeted delivery while limiting systemic damage. Through mechanisms such as complement-dependent cytotoxicity (CDCC) and antigen-dependent cytotoxicity (ADCC) via natural killer (NK) cells, monoclonal antibodies help destroy tumor cells by obstructing pro-survival signals. Notable therapeutic responses have been demonstrated by clinical developments including T cell engineering employing chimeric antigen receptors (CARs) targeting antigens like CD19 and antibodies coupled with cytotoxic drugs like brentuximab vedotin (Jiang et.al.,2011).

The complexities of the tumor microenvironment, which include immune suppressive factors, barriers to T cell activation, and inhibitory pathways, make it difficult to build a successful anti-tumor response. Comprehending the biology of T cells, encompassing antigen identification through MHC molecules and co-stimulatory signaling pathways, is essential for their activation and proper operation. The Immuno score in colorectal cancer serves as an example of how memory T cells, which are important for long-lasting responses after re-exposure to antigens, can be found in a variety of subsets and are important in cancer immunotherapy. Tumor antigenicity can be determined in a number of ways, which aopperffects the activation of an immune response. Research into the ability of cancer vaccines to activate particular T cells is also ongoing. Enhancing anti-tumor immunity involves adoptive T cell therapies (TILs, CAR-T cells), understanding T cell fatigue mechanisms, and developing strategies to bypass immune inhibitory

pathways such as immunological checkpoints (PD-1, CTLA-4). These diverse methods represent the changing face of cancer immunotherapy, offering better patient matching and creative ways to boost responses in patient groups that have historically had lower response rates (Porter et al., 2011).

2.1.5 Role of thiosemicarbazones on cancer cells

According to research by Shakya et al. (2019), compound 5FAmPyrr (5-Fluoro-N'-(pyrrolidine-1-carbonothioyl)picolinohydrazonamide) had the strongest impact on MCF-7 breast cancer cells. The compound's ability to block pro-caspase-3 and PARP-1 suggests that it induces cell death intrinsically. Furthermore, it was discovered that Compound 5FAmPyrr inhibited JNK (Jun N-terminal kinase), an essential member of the MAPK superfamily, at dosages of 1 and 3 μM. This indicates that the JNK pathway is the mechanism via which the chemical causes MCF-7 cells to undergo apoptosis. Therefore, by blocking the JNK pathway, Compound 5FAmPyrr, a derivative of 2-pyridineformamide thiosemicarbazone, has strong anti-MCF-7 breast cancer cell activities. This substance may be used to treat breast cancer as an anti-cancer contender.

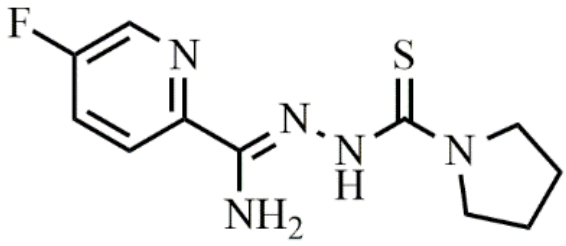


Figure 9 Structure of 5FAmPyrr

The study conducted by (Sartorelli and Booth in 1967) explored the anti-tumor properties of Copper(II)KTS, (copper(II) chelate of 3-ethoxy-2- oxobutyraldehyde bis(thiosemicarbazone),), and its interactions with various compounds in inhibiting the growth of Sarcoma 180 ascites cells. Initially, Copper(II)KTS showed significant efficacy by notably extending the survival of mice with tumors derived from these cells. In contrast, other compounds like cupric chloride, copper stearate, and kethoxal bis(thiosemicarbazone) alone did not have substantial effects on the lifespan of similar tumor-bearing animals. However, combining Copper(II)KTS with specific compounds, such as 6-thioguanine or Ar-isopropyl-a-(2-methylhydrazino)-ptoluamide, resulted in synergistic inhibition of tumor growth.

In particular, the combination of 6-thioguanine and Copper (II)KTS emerged as highly potent in impeding tumor development. Additionally, while mixtures of Copper(II)KTS with 5-fluorouracil showed additive effects in slowing down the progression of Sarcoma 180, combinations involving uracil mustard did not demonstrate significant therapeutic benefits. It focused on emphasizing the potential of these compounds to act through complementary pathways, leading to enhanced inhibition of DNA synthesis and consequently restraining tumor growth. Ultimately, the research suggests promising possibilities for utilizing Copper(II)KTS and specific compound combinations as potential strategies to combat tumor progression, particularly through their interactions affecting DNA biosynthesis (Sartorelli & Booth, 1967).

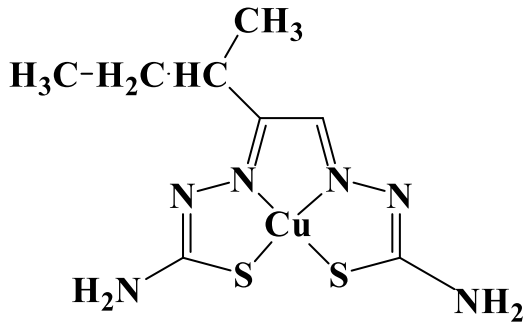


Figure 10 Structure of Cu(KTS)

The di-2-pyridylketone thiosemicarbazone, 2-benzoylpyridine thiosemicarbazone, and 2-acetylpyridine thiosemicarbazone series are examples of new-wave thiosemicarbazones that have been created and show strong anticancer activity in vitro. Furthermore, research conducted *in vivo* using Dp44mT (Di-2-pyridylketone-4,4-dimethyl 3-thiosemicarbazone), one of these ligands, demonstrates notable effectiveness in specifically impeding the growth of certain cancers.DNA topoisomerase IIa has been demonstrated to be specifically inhibited by Dp44mT, causing cytotoxicity in breast cancer cells. Dp44mT, which has an IC_{50} value of 0.01 µM showed anti-cancer activity against SK-N-MC neuroepithelioma cells (Kalinowski et al., 2009).

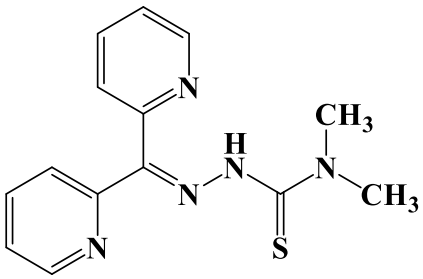


Figure 11 Structure of Dp44mT

In 2009 Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) emerged as the most potent chelator (Meyboom, 2009). Dp44mT works by inhibiting the uptake of iron from transferrin and promoting the release of cellular iron. In a study involving mice, after a duration of 7 weeks, the melanoma xenografts in mice treated with Dp44mT exhibited only 8% of the growth observed in mice treated with the vehicle alone. Interestingly, there wasn't a significant decrease in systemic iron levels. Additionally, Dp44mT increased the expression of the iron-responsive tumor growth and metastasis suppressor gene Ndrg1 specifically within the tumor, without impacting its expression in the liver.

According to (Mrozek W et al., 2019) the compound (2c) 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide had a particularly strong apoptotic effect on the different cell line. In various assays and analyses, MCF-7 cells (breast cancer cell line) showed a significant response to compound 2c treatment in his study (Mrozek W et al., 2019).

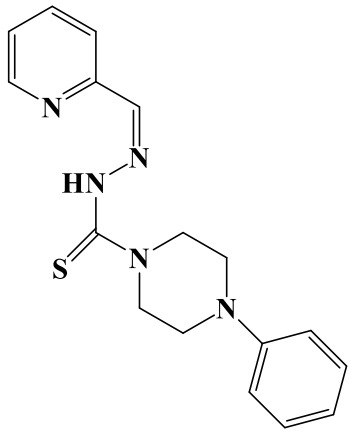


Figure 12 The Structure of (2c) 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide

Where R = 4-No_2

1. **Annexin V Binding Assay:** The apoptotic effect of compound 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide was more pronounced in MCF-7 cells, where a high percentage of apoptotic cells was observed.
2. **Immunoblotting Analysis:** In the immunoblotting analysis of protein expression, MCF-7 cells exhibited substantial changes in the levels of key proteins involved in apoptosis and cell cycle regulation upon treatment with compounds.
3. **Cytochrome c Release:** MCF-7 cells showed a marked decrease in mitochondrial cytochrome c levels upon treatment with compound 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide, indicating a disruption of mitochondrial integrity and activation of apoptotic pathways.
4. **DNA Intercalation:** Compound 4- (4-nitrophenyl)- N'-[(pyridin-2 -yl) methylidene] piperazine-1 -carbothiohydrazide had a strong intercalation effect with DNA, with MCF-7 cells and others showing significant hypochromism and redshift in absorption maxima.
5. **ROS Generation:** Compound 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide induced a significant increase in the generation of reactive oxygen species (ROS) in cell lines, further suggesting its potent apoptotic effect.

Taken together, the data suggest that compound 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide had a more pronounced apoptotic effect on the MCF-7 cell line compared to other cell lines such as HCT 116(colon cancer cell line). This indicates that MCF-7 cells were more susceptible to the cytotoxic and apoptotic effects of compound 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide, making it a promising candidate for targeting breast cancer cells.

1 ((2 (4-Methoxyphenyl) 1H indol -3-yl) methylene) 4 -methylthiosemicarbazide, (6n), showed the most anti-cancer activity among the chemicals examined in one of the experiments carried out by (Bakherad et al., 2019). Because compound 6n was at least three times more powerful against A-549 cells than the commercially

available reference medication etoposide, according to the cytotoxicity experiments, it was shown to have high potency. Moreover, chemical 1 ((2 (4-Methoxyphenyl) 1H indol -3-yl) methylene) 4 -methylthiosemicarbazide,, caused apoptosis in the A-549 cells, as demonstrated by further investigations using flow cytometry and acridine orange/ethidium bromide double labeling. To further validate these experimental findings, molecular studies were conducted to explore the potential binding interactions of the most potent compound 1 ((2 (4-Methoxyphenyl) 1H indol -3-yl) methylene) 4 -methylthiosemicarbazide, with tubulin, as well as colchicine, and with the ATPase domain of topoisomerase IIα active sites. Furthermore, the final derivatives of the compounds showed good radical scavenging potential in the 0.015–0.630 μM range, which is similar to the 0.655 μM range of conventional ascorbic acid. (Bakherad et al., 2019).

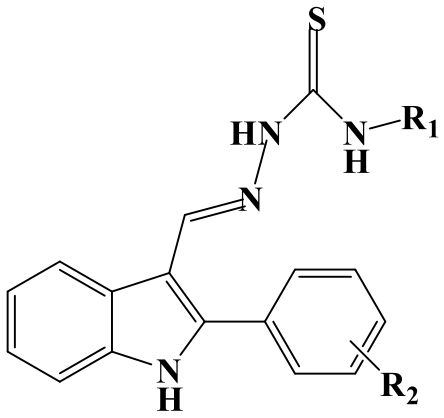


Figure 13 Structure of 1 ((2 (4-Methoxyphenyl) 1H indol -3-yl) methylene) 4 -methylthiosemicarbazide

Where R_1=Methyl (Me) and R_2= Methoxy (OMe)

In a separate scholarly work, researchers focused on synthesizing novel thiosemicarbazone derivatives containing 5-methoxy isatin, specifically modifying them at the N(4) position. The synthesized compounds underwent thorough characterization. Notably, among these derivatives, (MeOIstPyrd) 5-Methoxyisatin N(4)-Pyrrolidinyl Thiosemicarbazone exhibited remarkable efficacy against A431 skin cancer cells, demonstrating an IC_{50} of 0.9 μM. Importantly, it demonstrated low toxicity towards normal human fibroblast cells (HLF-1) and human embryonic kidney cells (HEK293).The compound MeOIstPyrd exerted its effects by impeding cell proliferation, migration, and spheroid formation. Its mechanism of action involved activating the mitochondrial intrinsic apoptotic pathway. Additionally, it

induced DNA damage, triggered p53 activation, and disrupted the interaction between p53 and MDM2. These findings underscore the compound's potential in targeting mutant p53 and suggest its promising role in cancer therapeutics as an effective strategy (Shahi et.al.,2023).

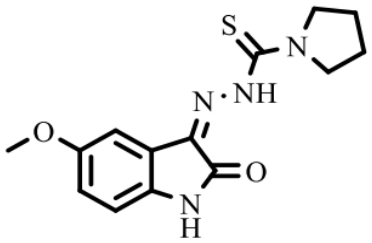


Figure 14 Structure of MeOIstPyrd

The novel (Pt(TSC)Cl complex) platinum complex of N(4)-phenyl-2-formylpyridine thiosemicarbazone (HTSC) displayed robust anticancer activity against A549, MCF-7, and Caco-2 cell lines, exhibiting low micromolar IC_{50} values (200–1.75 μM), as 10,000 cells/well were seeded, Particularly noteworthy was its heightened selectivity in Caco-2 cells ($IC_{50} = 2.3$ μM) compared to cisplatin ($IC_{50} = 107$ μM) after 48 hours of treatment. Significantly, this complex showed reduced nephrotoxicity on normal Hek293 cells compared to cisplatin. Mechanistic insights revealed its efficacy in inhibiting cancer cell proliferation, predominantly inducing apoptosis through the intrinsic pathway via modulation of the Bax/Bcl-2-Casp9-Casp3/Casp7 axis. The compound's ability to induce apoptosis in Caco-2 cells while demonstrating low nephrotoxicity underscores its potential as a valuable candidate for colorectal cancer treatment, warranting further optimization and development (Salehi et.al., 2022).

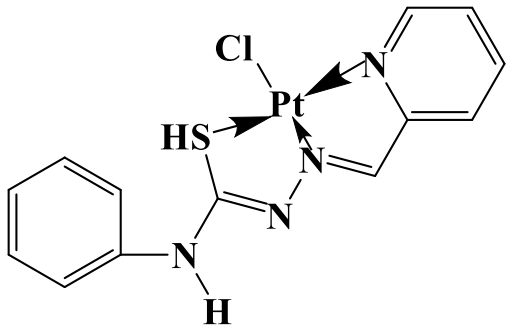


Figure 15 Structure of platinum complex of N(4)-phenyl-2-formylpyridine thiosemicarbazone (HTSC)

2.1.6 Mammalian cell culture

Mammalian cell cultures serve as indispensable tools in the study of anti-cancer activities due to their ability to mimic human cellular responses. There exists a diverse array of mammalian cell culture types employed in anti-cancer research, each presenting distinct features and advantages. Adherent cell cultures, exemplified by *HeLa* cells, MCF-7 cells, and HepG2 cells, exhibit attachment to culture surfaces, resembling the *in vivo* cellular environment and facilitating vital cell-cell and cell-matrix interactions. In contrast, suspension cell cultures like HL-60 cells, Jurkat cells, and K562 cells grow freely in suspension without surface attachment, necessitating specific culture conditions for growth, such as shaking or stirring. Primary cell cultures, derived directly from tissue without immortalization, like primary human fibroblasts and hepatocytes, retain characteristics akin to the original tissue but have a limited lifespan in culture. Immortalized cell lines such as HEK-293, NIH/3T3, and IMR-90 cells bypass senescence, allowing prolonged culture while retaining specific cellular traits, though they may differ from primary cells. Additionally, three-dimensional (3D) cell cultures, including spheroids, organoids, and tumor explants, provide a more intricate microenvironment that mimics in vivo conditions, allowing for natural cell-cell interactions resembling tumor architecture. Co-culture systems involving various cell types, like cancer cells with fibroblasts, immune cells, or endothelial cells, model complex tumor-stromal interactions and the intricate tumor microenvironment. Lastly, patient-derived cell cultures (PDCs) or patient-derived xenografts (PDX), derived directly from patient tumors, maintain tumor heterogeneity and offer greater clinical relevance in studying patient-specific responses to treatments. These various cell culture models cater to diverse research objectives, be it exploring drug efficacy, elucidating molecular mechanisms, or evaluating tumor responses within a clinically relevant context, contributing uniquely to anti-cancer activity studies (Lovitt et al., 2014).

2.1.7 The MTT Assay (3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a type of single tetrazolium salt. Its structure comprises a central quaternary tetrazole ring with a positive charge, encircled by three aromatic rings –

two phenyl groups and one thiazolyl ring. When MTT is reduced, the core tetrazole ring is altered, giving rise to a violet-blue molecule called formazan that doesn't dissolve in water (Berridge et al., 2005).MTT's unique properties allow it to pass through both the cell membrane and the inner mitochondrial membrane of viable cells. This is due to its positive charge and its lipophilic (fat-attracting) structure. Metabolically active cells reduce MTT to formazan (Stockert et al., 2018).This reduction reaction is chromogenic, meaning it produces a color change. This property forms the basis for the MTT assay. The assay is mainly used to assess cell metabolic activity. However, it has also been extended to infer other cellular states or processes, like viability, although such inferences can sometimes lack solid validation (Mosmann, 1983).In the MTT assay, cells are typically exposed to MTT for a few hours, causing the formation of water-insoluble formazan. This formazan is then dissolved using a solvent like Dimethyl sulfoxide (DMSO). Next, a microplate reader measures the decrease in light transmission due to absorbance and other factors in the MTT-formazan solution. This measurement is taken at a wavelength where MTT-derived formazan absorbs the most light (around 570 nm). The optical density (OD) values obtained are used to estimate formazan concentration and, consequently, the level of MTT reduction within the cells (Ghasemi et al., 2021).

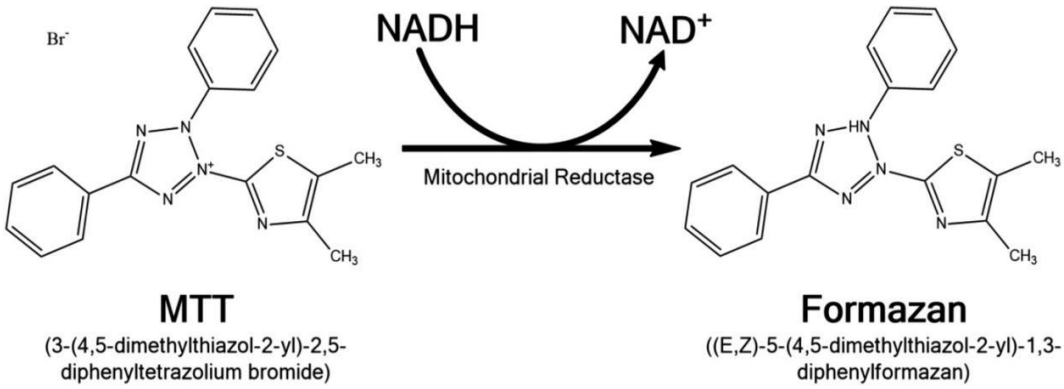


Figure 16 Reduction of MTT to formazan crystal

CHAPTER 3

MATERIALS AND METHODS

The experimental study, conducted at the Central Department of Biotechnology in Kritipur, Kathmandu, Nepal, aimed to assess the impact of five different ligands and three Copper(II) complexes of 2-pyridineformamide thiosemicarbazones its copper(II) complexes labelled as HL_1, HL_3, HL_4, HL_5, HL_6, $[CuL_1Cl]$, $[CuL_3Cl]$, and $[CuL_4Cl]$. These compounds had been selected from a variety of samples provided by (Shakya et.al., 2019). The study began by preparing the materials, equipment, and reagents required for the experiments, all detailed in **Appendix A**. One day before the experimentation, the *HeLa* cell line passaging and culture protocol, also listed in **Appendix A**, were executed to ensure the cells were in the appropriate state for the experiment.

The process of seeding *HeLa* cells involved meticulous steps to ensure the proper culture setup. The culture flask containing the *HeLa* cells had its old media removed and was washed twice with a 2 ml Phosphate buffer saline (PBS) solution. Subsequently, 2 ml of trypsin was added to detach the cells, which were then incubated at 37 ℃ for two minutes. Once the cells were detached, they were collected in 1 ml PBS solution, transferred to an Eppendorf tube, and centrifuged at 3000rpm for 1 minute. After discarding the supernatant, the cells were homogenized in 1 ml of complete Dulbecco modified eagle's medium (DMEM) media to ensure cell viability. The preparation of complete DMEM is outlined in **Appendix A.**

The seeding density was crucial and calculated to be 3000 cells per well. This calculation was performed using a hemocytometer, where 10 µL of homogenized cells mixed with trypan blue dye were counted under a microscope. Post-cell count, the seeded cells were treated with various concentrations of serially diluted compounds (1, 5, 10, 15, 20, 25, and 50 µM) in DMEM media and incubated for 72 hours at 37 °C. After this incubation period, the cells were treated with MTT reagent (5mg/ml in PBS) and further incubated for 3 hours. Later, the supernatant was removed, and 150 µL of DMSO was added to each well, mixed thoroughly, and the

absorbance was measured at 551nm using an ELISA reader machine, following the protocol outlined by (Shakya et.al.,2019). Additionally, the protocols for the *HeLa* cell line and the preparation of trypsin solution were precisely detailed, ensuring the accuracy and reproducibility of the experiment.

3.1 Quality Control

3.1.1 Quality monitoring of the laboratory equipment, reagent and media

Laboratory equipment like incubator, autoclave, hot air oven, refrigerator, biosafety cabinet etc. were regularly monitored for their performance and immediately repaired if any deviation occurred. Reagent and biochemical media were checked for their manufacture and expiry date before using them. Each reagent and media were labeled with preparation date, expiry date and stored in a proper condition.

3.1.2 Quality control during MTT assay duration

The DMEM media, FBS (Fetal bovine serum), MTT reagent, *HeLa* cell line were checked for lot number, manufacture date, expiry date and storage.

3.2 Data analysis

Data were statistically analyzed with non-parametric analysis of variance (ANOVA) followed by post hoc test for group-wise comparison. A value of $p \leq 0.05$was assumed. Where even applicable and 95% confidence interval along with the exact p value were presented. All statistical procedures were performed using Graph pad prism version 9.5.1.

CHAPTER 4

RESULTS

4.1 Comparison of cell viability effects of five 2-pyridineformamide thiosemicarbazone ligands and three Copper(II) complexes

The impact on cell viability was assessed in *HeLa* cell lines using a cell viability assay to study five distinct ligands and three Copper(II) complexes derived from 2-pyridineformamide thiosemicarbazone compounds. The viability results are expressed as mean percentages ± standard deviation compared to the control group.

Table 1 Mean of % Cell viability and its standard deviation (SD) of compounds

% (mean)cell viability ±Standard Deviation									
Compounds									
Concentrations(µM)		HL_1	HL_3	HL_4	HL_5	HL_6	$[CuL_1Cl]$	$[CuL_3Cl]$	$[CuL_4Cl]$
	0	100 ±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0
	1	53.1289 ±11.20579	92.45994± 4.796445	50.50049± 3.34398	60.21262± 0.556808	49.26491± 5.435576	78.40181± 14.76861	88.14185± 0.965344	8.590138± 4.151664
	5	39.35824± 8.060482	45.20591± 2.062746	25.82669± 1.122842	43.19538± 5.033483	36.10842± 6.750071	51.60125± 3.901276	63.4942±2. 487803	2.068884± 1.561844
	10	31.39901± 4.704302	31.62488± 2.062102	17.68493± 2.263877	36.00234± 3.618197	19.97446± 5.845596	3.414642± 1.164108	55.15715± 1.80974	1.235621± 0.378374
	15	24.25364± 2.267662	23.7427±3. 605885	12.47646± 0.595175	31.05842± 3.474472	12.84435± 4.088431	1.161725± 2.174163	39.90837± 3.89102	0.676427± 0.607431
	20	19.17968± 1.188827	12.7077± 1.8847	7.585686± 1.201839	25.96802± 4.168946	9.646337± 3.976662	1.574766± 1.863176	7.059405± 2.658674	0.368973± 0.819385
	25	17.40808± 1.795792	9.400417± 2.022946	7.440548± 1.604755	19.9969±1 .953031	8.073328± 2.142464	1.329053± 1.461288	0.204582± 2.125715	- 0.0322± 0.81337
	50	10.93615± 4.719084	4.247012± 1.265448	6.972803± 1.344497	11.38039± 0.702306	5.304994± 2.431548	2.190189± 4.020694	-1.03611± 2.125947	0.503052± 1.137744

The study investigated the impact of different compounds (HL_1, HL_3, HL_4, HL_5, HL_6, $[CuL_1Cl]$, $[CuL_3Cl]$, $[CuL_4Cl]$) on cell viability at various concentrations, measured as percentages with standard deviations. Each compound demonstrated a dose-dependent decrease in cell viability as concentrations increased.For HL_1, HL_3, HL_4, HL_5, and HL_6, escalating concentrations led to reduced cell viability, highlighting their cytotoxic effects. $[CuL_1Cl]$ showed viability declines from 1 µM to 10 µM, with a substantial decrease at 15 µM and beyond, indicating significant cytotoxicity. Similarly, $[CuL_3Cl]$ exhibited reduced viability from 15 µM, reaching

critically low levels at 25 µM and 50 µM. [CuL_4Cl] showcased declining viability from 1 µM to extremely low levels at 20 µM and beyond.

Notably, statistical analysis indicated no significant differences in cell viability mean values within certain concentration ranges for some compounds ([CuL_1Cl] at 20 µM, 25 µM, 50 µM; [CuL_3Cl] at 25 µM, 50 µM; [CuL_4Cl] at 5 µM, 10 µM, 15 µM, 20 µM, 25 µM, 50 µM). This suggests consistent impacts on cell viability within these concentration ranges without discernible differences.

In summary, these compounds demonstrated dose-dependent cytotoxicity on cell viability, emphasizing their potential as agents affecting cellular function, particularly at higher concentrations for certain compounds.

Note: The negative value for cell viability at 50 µM of [CuL_3Cl] and at 25 µM of [CuL_4Cl] might indicate experimental artifacts and should be further investigated for clarity.

Table 2 Compound Analysis

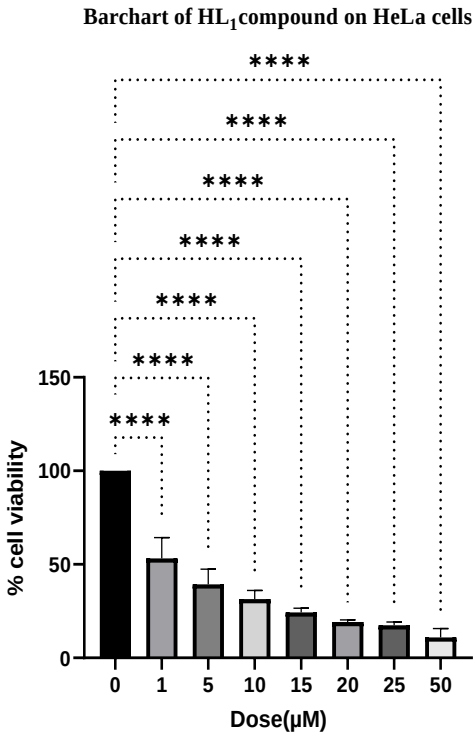
Compound	Max Inhibition	Concentration (µM)	Compound	Max Inhibition	Concentration (µM)
HL_1	68.61%	10	[CuL_1Cl]	96.59%	10
HL_3	87.29%	20	[CuL_3Cl]	99.79%	25
HL_4	92.41%	20	[CuL_4Cl]	97.93%	5
HL_5	88.61%	50		-	-
HL_6	80.03%	10		-	-

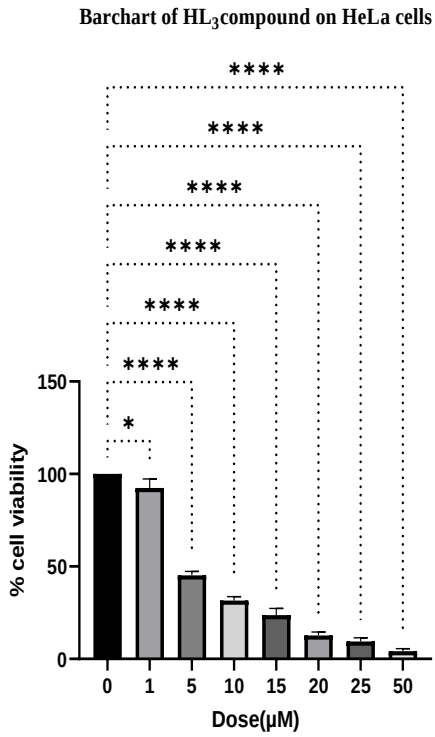
The table illustrates the maximum inhibitory percentages and their respective concentrations measured in micromoles (µM) for various compounds categorized within two groups: HL and [CuLCl]. Among the HL group, HL1 showed a maximum inhibition of 68.61% at a concentration of 10 µM, while HL_3 displayed a higher inhibition rate of 87.29% at the 20µM concentration. HL_4 exhibited a notable inhibition of 92.41% at a concentration of 20 µM, while HL5 and HL6 showed inhibitory effects of 88.61% and 80.03%, respectively, both observed at a concentration of $_{50}$ µM and 10µM. In the [CuLCl] group, [CuL_1Cl] demonstrated a significant inhibition of 96.59% at a concentration of 10 µM, and [CuL_3Cl]

showcased an impressive inhibition of 99.79% at a concentration of 25 µM. [CuL_4Cl] also exhibited substantial inhibition, recording 97.93% at a concentration of 5 µM.from above table it can be noticed that Copper(II) complexes([CuLCl]s)showed efficient inhibition at lower compound concentration as compared to the ligands (HLs).

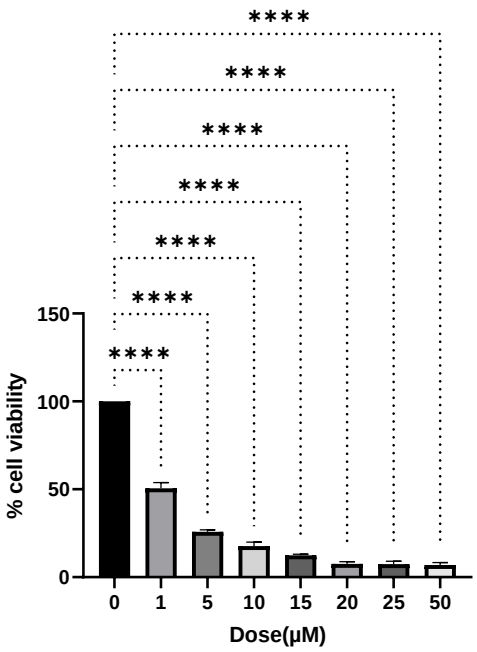
4.1.1 Dose-Response Curve Analysis

Dose-response curves were fitted for each compound's viability data, showing distinct concentration-dependent trends. Detail data of dose response curve is provided in APPENDIX B

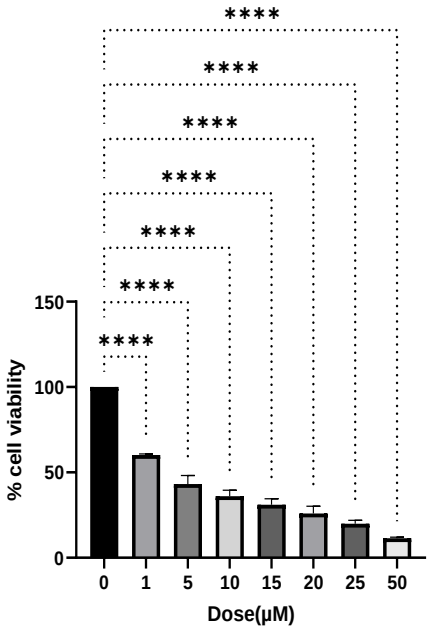




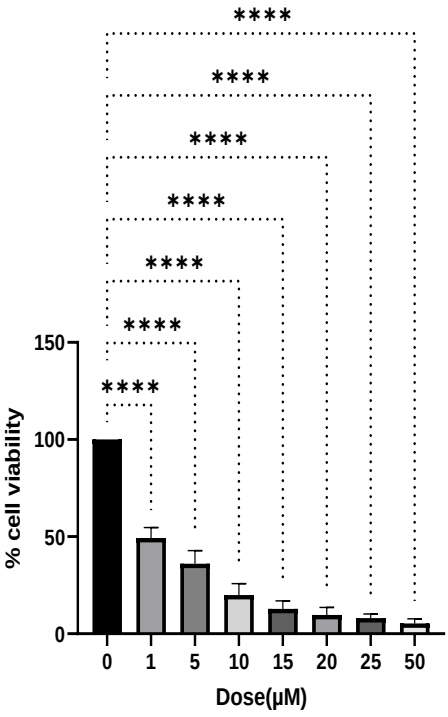
Barchart of HL_4compound on HeLa cells



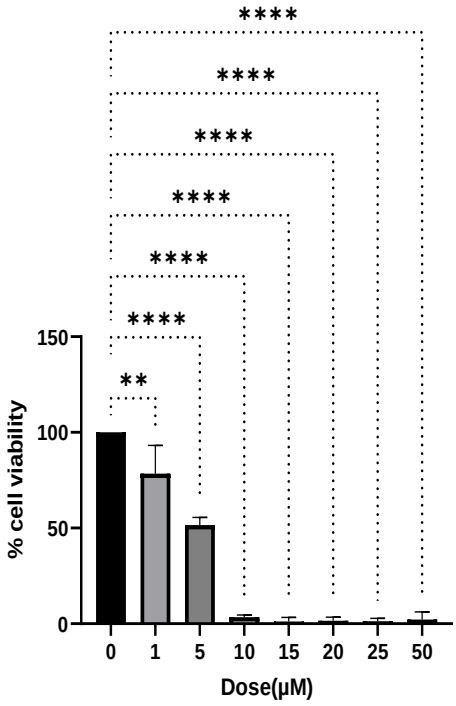
Barchart of HL_5 compound on HeLa cells



Barchart of HL_6 compound on HeLa cells



Barchart of $[CuL_1Cl]$ compound on HeLa cells



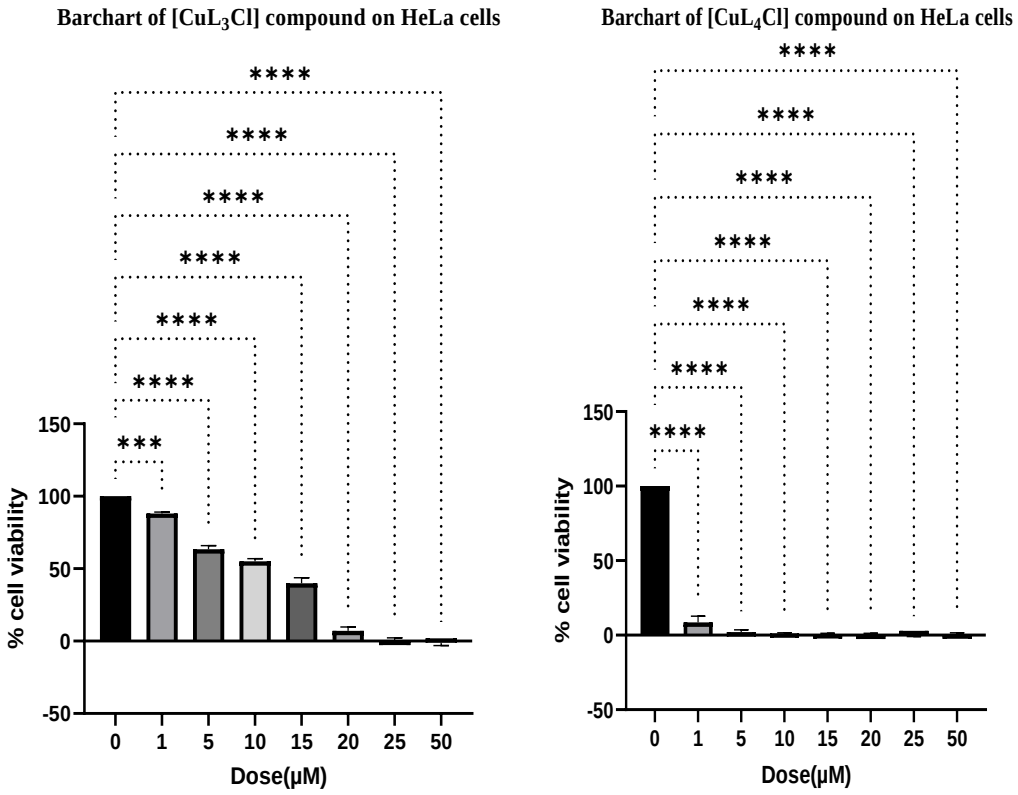


Figure 17 Bar chart of HL Compounds and its copper(II)complexs

The analysis of various compounds' dose-response curves on the *HeLa* cell line reveals significant inhibitory effects on the biological response. HL_1 exhibited a strong impact, with an IC_{50} of 1.598 (CI: 1.014 - 2.247) and a Hill slope of -0.5225, showcasing a well-defined inhibition pattern. HL_3 showed substantial inhibition, with an IC_{50} of 5.002 (CI: 4.525 - 5.498) and a steep Hill slope of -1.297, indicating a pronounced drop in response. HL_4 displayed a notable effect, presenting an IC_{50} of 1.065 (CI: 0.9293 - 1.204) and a moderate Hill slope of -0.7343. HL_5 demonstrated a discernible impact, with an IC_{50} of 2.675 (CI: 2.091 - 3.300) and a moderate Hill slope of -0.5467. HL_6 exhibited inhibition with an IC_{50} of 1.181 (CI: 0.7922 - 1.609) and a Hill slope of -0.6819. Additionally, $[CuL_1Cl]$ revealed a substantial effect, displaying an IC_{50} of 5.062 (CI: 3.092 - 5.622) and a steep Hill slope of -4.478, indicating a profound response reduction with a Hill slope interval of ??? to -1.457. The appearance of "???" in the confidence interval of the Hill slope for the $[CuL_1Cl]$ compound in Graph pad prism 9.1.5 indicates a failure or challenge in the computation process during asymmetric (profile likelihood) calculations for nonlinear regression parameters. This issue arises when Prism encounters difficulties in determining one or both confidence limits for the Hill slope

parameter. The specific reasons for this may include the complexity of the data, limitations in the dataset, or other inherent factors in the nonlinear regression analysis of the [CuL_1Cl] compound. The "???" notation signifies that the confidence limits for the Hill slope parameter could not be reliably determined due to these encountered challenges or failures in the calculation process. As a result, the confidence limit denoted by "???" lacks a defined value, indicating uncertainty about the lower range of the Hill slope parameter. [CuL_3Cl] showcased a notable impact, with an IC_{50} of 8.687 (CI: 6.812 - 10.55) and a Hill slope of -1.807. [CuL_4Cl] demonstrated a potent effect, revealing an IC_{50} of 0.08406 (CI: 0.02466 to 0.2314) with a Hill slope of -0.9542, indicating a steep decline in response. In all cases, the goodness of fit (R^2) values were notably high, ranging from 0.9249 to 0.9982, indicating robust correlations between predicted and observed values. These analyses collectively emphasize the diverse inhibitory impacts of the compounds on the biological response, supported by well-defined IC_{50} values and strong goodness of fit metrics.

4.1.2 Significant Difference Among Concentrations, Statistical Analysis:

Non-parametric one-way ANOVA followed by Tukey's multiple comparisons showed significant differences in mean viability across various concentrations of compounds: [CuL_1Cl], [CuL_3Cl], [CuL_4Cl], HL_1, HL_3, HL_4, HL_5, and HL_6 ($p < 0.001$ for all). Specifically, post hoc Tukey's test indicated significant differences between concentrations (1μM, 5μM, 10μM, 15μM, 20μM, 25μM, 50μM) of each compound ($p < 0.001$ for all comparisons). The detailed ANOVA results are available in **Appendix B**. The conducted analysis, encompassing ANOVA, Brown-Forsythe test, and Bartlett's test, reveals non-significant differences in means and variances among the eight tested compounds: HL_1, HL_3, HL_4, HL_5, HL_6, [CuL_1Cl], [CuL_3Cl], and [CuL_4Cl]. Specifically, the F-value of 0.6173 and P-value of 0.7393 in ANOVA indicate no statistically significant variation in anti-cancer activity means across these compounds. Additionally, the non-significant results from the Brown-Forsythe ($P = 0.9045$) and Bartlett's tests ($P = 0.9719$) imply that the variances in anti-cancer activity are not significantly different among the groups. The non-significant findings suggest that observed differences in anti-cancer activity among the compounds are likely due to random variability rather than inherent distinctions.

CHAPTER 5

DISCUSSION

This study, aimed to investigate the effects of 2-pyridineformamide thiosemicarbazones and it's copper(II) complexes on the proliferation of *HeLa* cell lines across a range of concentrations. The primary objective was to determine whether these compounds exhibit anti-cancer properties and if there are significant differences in their effectiveness. Our hypotheses were formulated to assess the potential variability among the concentration of compounds' effects on cell viability.

Effect of 2-pyridineformamide thiosemicarbazone Concentration on Cell Viability:

Our experimental findings demonstrate a clear concentration-dependent effect of 2-pyridineformamide thiosemicarbazones on cell viability on *HeLa* cell line. As 2-pyridineformamide thiosemicarbazones and it's copper(II) complexes concentration increased, we observed a significant reduction in cell viability, indicating its potent cytotoxic properties.

Variation in mean of % Cell viability and its standard deviation (SD) of compound

The cell viability percentages and their corresponding standard deviations (SD) for compounds HL_1, HL_3, HL_4, HL_5, HL_6, $[CuL_1Cl]$, $[CuL_3Cl]$, and $[CuL_4Cl]$ reveal a wide range of effects on cellular viability across different concentrations. However, some intriguing patterns have emerged. For instance, at lower concentrations of most compound treatments, there appears to be a higher Standard Deviation among replicates. This discrepancy might stem from inconsistencies in cell seeding density, suggesting a need for further study in this area. Another unexpected observation is seen in certain compounds like $[CuL_1Cl]$ and $[CuL_4Cl]$, where cell viability initially decreases as the compound concentration increases. However, at a certain point, there is a reversal, indicating an increase in cell viability as the compound concentration rises. This suggests the presence of a specific dosage

where the compound maximally inhibits cell density but then allows for increased viability beyond that threshold. These diverse ranges in viability percentages and standard deviations underscore the varying degrees of impact on cellular health and highlight the concentration-dependent effects of these compounds on cell viability. Some datasets approach or fall below 0% viability, indicating potential cytotoxic effects at higher concentrations. This underscores the crucial necessity for optimizing concentrations in these cellular assays.

IC_{50} Determination and Therapeutic Implications:

The calculated IC_{50} values provided valuable insights into the concentration of 2-pyridineformamide thiosemicarbazones and it's copper(II) complexes required to achieve a 50% reduction in cell viability for *HeLa* cell line. These values serve as crucial indicators of drug potency and can guide dosing strategies in clinical applications. The lower IC_{50} values obtained for $[CuL_4Cl]$ (0.08406µM) cells suggest that this cell line is particularly strong cytotoxic effects for *HeLa* cell line. Conversely, the higher IC_{50} values for $[CuL_3Cl]$ (8.687µM), $[CuL_1Cl]$ (5.062µM) and HL_3(5.002 µM) indicate a relatively lower cytotoxicity, possibly implying a need for higher concentrations of these drugs to achieve similar therapeutic effects. We have also noticed that the structure of some compound in HL family containing methyl group at sixth carbon position of pyridine ring reduces the IC_{50} value such as IC_{50} value of HL_3 =5.002µM without methyl group and IC_{50} value of HL_6=1.81µM Containing methyl group at fifth carbon position of pyridine ring as shown in figure below;(Shakya et.al.,2016) also suggest that, the essential condition for biological effectiveness is the presence of a nitrogen atom within a heterocyclic structure positioned adjacent to the thiosemicarbazide side chain, which gives improved water solubility. The addition of methyl or other hydrophobic groups to the 3, 4, or 5 carbon atoms of the pyridine ring, or the presence of the benzene ring in isoquinoline, enhanced enzyme suppressive action (Sartorelli et al., 1971). On the contrary HL_1 and HL_4 IC_{50} values are not so significantly different HL_1 IC_{50}=1.598µM and HL_4 IC_{50}=1.065µM; despite being similar in other structure. This might be due to presence of pyrrolidine ring as we can observe. it in HL_3, IC_{50}=5.002µM and HL_1, IC_{50}=1.598µM. This suggest that comprehensive study and research is needed in this sector.

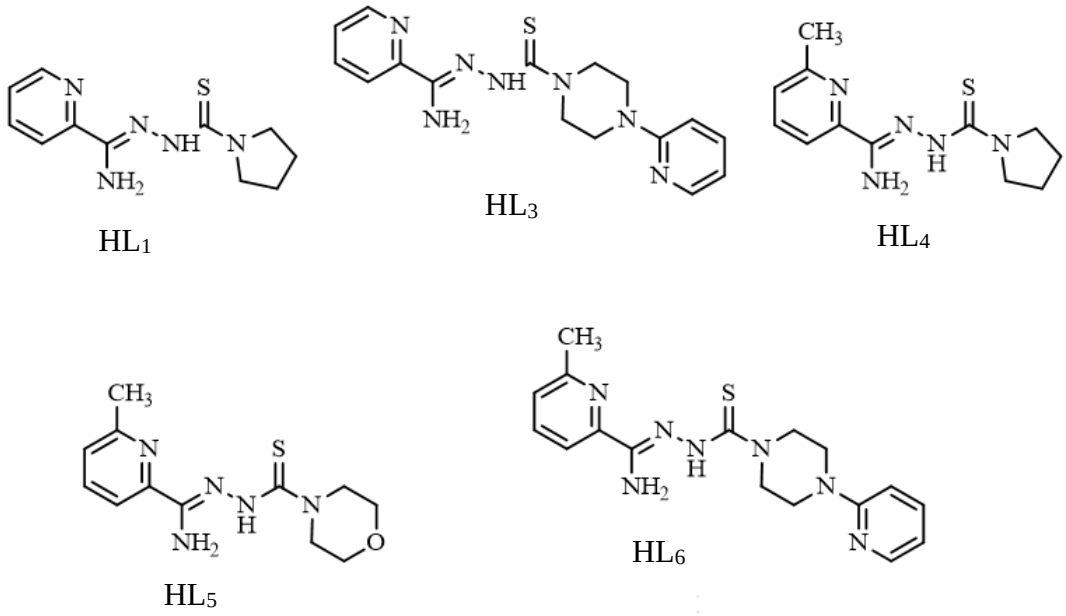


Figure 18 Structure of 2-pyridineformamide thiosemicarbazone ligands

The compounds of [CuLCl] series are the copper(II) complexes of the corresponding ligands of HL series(Shakya, 2016).It has also shown similar effects as the compound which contain 6- methyl pyridine ring or pyrrolidine ring attached has less IC_{50} values as compared to other members of the same family One of the surprising observation done was that the compound containing both 6- methyl pyridine ring and pyrrolidine ring attached to 2-pyridineformamide thiosemicarbazone has more or less significant drop of IC_{50} value, example of it are HL_4=1.065µM and $[CuL_4Cl]$=0.08406µM as shown in figure below

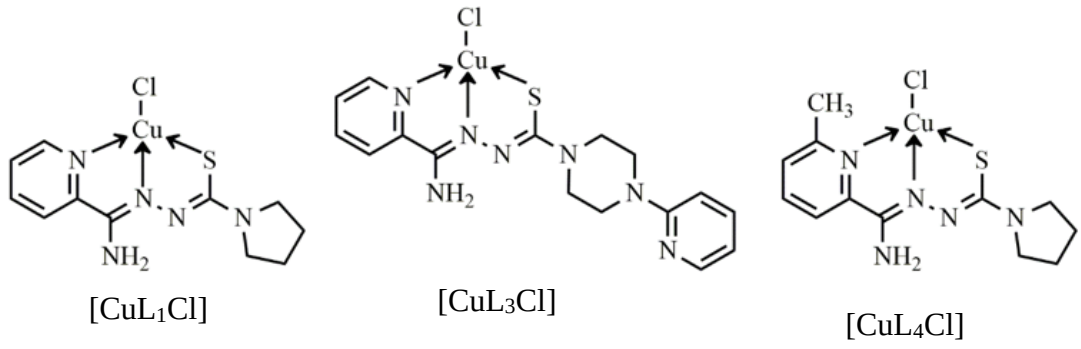


Figure 19 Structure of Copper(II) complexes of 2-pyridineformamide thiosemicarbazones

The comparison between [CuLCl] (Copper(II) complexes) and HL (ligands) in inhibiting cancer growth, considering concentration data and inhibition percentages, highlights the unique advantages of [CuLCl] compounds. Notably,

[CuL_4Cl] displayed an exceptionally low IC_{50} of 0.08406, indicating its efficacy in inhibiting cancer cell growth at remarkably low concentrations compared to the ligands. However, some, including [CuL_1Cl]'s IC_{50} of 5.062 and [CuL_3Cl]'s IC_{50} of 8.687, are higher than those of HL_1 and HL_3, respectively. This may be the case because these compounds have a particular dosage response that can kill 50% of the cell; nevertheless, [CuLCl] drugs have demonstrated robust and considerable activity against the *HeLa* cell line at considerably lower concentrations that required for maximal cell death. For example, [CuL_1Cl] exhibited 96.59% inhibition at 10 µM, while its ligand, HL_1, showed a lower 68.61% inhibition at 10 µM. Similarly, [CuL_3Cl] displayed notably higher 99.79% inhibition at 25 µM compared to HL_3's slightly lower 87.29% inhibition at 20µM concentration. [CuL_4Cl]'s ligand counterpart HL_4 showed 92.41% inhibition at 20µM concentration, [CuL_4Cl] exhibited substantial inhibition of 97.93% at just 5 µM. This heightened inhibitory effect at lower concentrations indicates the superior potency of [CuLCl] compounds against cancer cells compared to their ligands.

The efficacy of [CuLCl] compounds stem from various factors, including their unique interactions with cellular components. [CuLCl] compounds, like [CuL_4Cl], potentially engage in hydrophobic or coordinate binding mechanisms with human serum albumin (HSA), utilizing the His242 residue of HSA's IIA subdomain as suggested by (Bai et al.,2022). This interaction with HSA holds significance as it could enhance the delivery efficiency and selectivity of these copper complexes within the biological system. Through forming hydrophobic or coordinated bonds with specific regions on HSA, [CuLCl] compounds might extend circulation times, improve stability, and enable targeted delivery to cancerous sites. This selective binding to HSA could also enhance the complexes' bioavailability and distribution within the body, thereby augmenting their anti-cancer efficacy. Additionally, the inherent fluctuating redox and oxidation activity of [CuLCl] compounds especially copper(II) metal ion of [CuLCl] within the cell triggers an increase in reactive oxygen species (ROS) production within cells, disrupting cancer cell defense mechanisms and promoting pathways that induce cell death—a feature notably absent in ligand forms. HL ligands are characterized by their structural elements containing crucial C=N and C=S bonds. These bonds involve nitrogen and sulfur atoms that possess lone pairs of electrons in their hybrid orbitals, allowing effective

binding to metal ions and essential cellular components. In the iron (II) chelates found within copper(II) complexes, they can easily change to the ferrous form in blood or upon exposure to thiols. This ferrous state has the ability to eliminate the tyrosyl radical within the enzyme by undergoing a single-electron reduction reaction, which depends on the presence of oxygen (Preidecker et al., 1980). This targeted action, combined with hydrophobic or coordinated binding to proteins like HSA, and their lipophilicity, which is a crucial property to enter inside cell, increases the efficacy of [CuLCl] compounds in inhibiting cancer growth, positioning them as promising candidates for advanced anti-cancer therapies (Bai et al.,2022).

Significant Differences Among Concentrations (one and one compound comparison)

Non-parametric one-way ANOVA tests indicated statistically significant differences in cell viability among the treatment groups for all compounds. The p-values were highly significant ($p < 0.001$) for all compounds, emphasizing that different concentrations of each compound had distinct effects on *HeLa* cell viability. Also, the homogeneity of variances among the compared groups is also not violated i.e., one-way ANOVA tests can reliably be used. Post hoc Tukey's multiple comparison tests revealed significant differences among most concentrations for each compound**.** [CuL_1Cl] exhibited a noteworthy trend by significantly reducing cell viability across various concentrations, which is indicative of its potential as an effective anti-cancer agent. However, a surprising observation was made at the 1 µM concentration, where a slight expansion compared to the control was observed in Post hoc Tukey's multiple comparison tests. This expansion, although statistically significant with a (p-value of 0.0052 ***), suggests that [CuL_1Cl]'s efficacy may have a concentration-dependent aspect, warranting further investigation. Similarly, compound [CuL_3Cl] showed a pattern of significantly lower cell viability at most concentrations, indicating its strong potential for cancer inhibition. However, like [CuL_1Cl], it displayed a unique behavior at the 1 µM concentration, where an expansion compared to the control was observed. This expansion, despite its statistical significance (p-value of 0.002 ***), suggests that there might be a specific concentration range where [CuL_3Cl]'s

anti-cancer effects are less pronounced. In contrast, compound [CuL_4Cl] consistently exhibited highly significant reductions in cell viability at all tested concentrations. The (p-value of <0.001 ****) underscores its consistent and robust anti-cancer activity. Compounds HL_1, HL_4, HL_5, and HL_6 displayed a consistent pattern of significantly lower cell viability at all concentrations tested, emphasizing their potent and reliable anti-cancer potential. The p-values for these compounds were all <0.001 (****), signifying their strong inhibitory effects on *HeLa* cells. Compound HL_3, on the other hand, exhibited lower cell viability at most concentrations, but what sets it apart is the slight expansion compared to the control observed at the 1 µM concentration. This expansion, despite being statistically significant (p-value of 0.0414 *), remained greater than $p < 0.001$. Thus, while HL_3 generally displayed effective inhibition of *HeLa* cell growth, its specific concentration range might have a slightly reduced efficacy.

These findings underscore the distinct and complex nature of each compound's effects on *HeLa* cell viability. While some compounds demonstrate consistent and potent anti-cancer activity, others show unique dose-dependent behaviors. These observations are crucial for optimizing the therapeutic potential of these compounds in cancer treatment, offering valuable insights for further research and drug development.

Findings from Different Literature

We compared our study results with those of (Shakya et al.,2019), who found that certain compounds exhibited inhibitory effects on the *HeLa* cell line, with IC_{50} values ranging between 0.9-8.6 µM across different cell culture conditions (HL_1 to HL_6). They noted that the lowest IC_{50} was observed in HL_6 and the highest in HL_3, using 3000 cells/100µL/well and measuring absorbance at 570 nm.

Contrastingly, our investigation identified IC_{50} values ranging from 1.065-5.002 µM for the *HeLa* cell line across HL_1 to HL_6. Interestingly, we observed the lowest IC_{50} in HL_4 and the highest in HL_3, employing the same cell seeding density and measuring absorbance at 551 nm. Notably, our study found a close correlation with (Shakya et al.,2019).

One of the astonishing finding is that the IC_{50} value of HL_1 of (shakya,et,al.,2019) is 6.2µM ; however, we found out that its value is 1.598µM, This might be because the compound treatment concentration in our experiment was greater as compared to (shakya et.al 2019), if it is so, than why, anti-cancer activity of HL_5 was comparatively low as in both study IC_{50} value was similar i.e 2.2µM on (shakya.et.al 2019) and 2.675µM on our study or might have different absorption characteristics at 570nm and 551nm due to its molecular structure. If the compound absorbs light strongly at one wavelength and less at another, this difference in absorption can affect the accuracy of the assay or measurement, or some compounds or impurities might absorb light at specific wavelengths, leading to interference or background signals in the assay. This interference can affect the accuracy of the IC_{50} determination, especially if the compound's absorption overlaps with the wavelength used for measurement.

Shakya et al., (2019) highlighted Compound 5FAmPyrr's efficacy against MCF-7 breast cancer cells through JNK pathway inhibition. These findings suggest the potential utility of our compounds in the treatment of breast cancer, indicating a promising direction for further exploration.

Moreover, other studies indicate the potential of thiosemicarbazone derivatives in anti-cancer activity (Sartorelli et.al.,1967; Kalinowski et al., 2009). The structural modifications and interaction with target proteins observed in these studies support our findings of compound-specific effects.

In a study by Mrozek-Wilczkiewicz et al., (2019), compound the pyridine derivative 4-(4-nitrophenyl) piperazine-1-carbothiohydrazide; precursor of 2c exhibited the highest potency among the newly synthesized compounds, with an IC_{50} value of 0.010 µM against HCT 116 p53-/- cells (colon cancer cell line). However, its effectiveness significantly decreased (30-fold) when tested on the wild-type HCT 116 cells. Compound (2c) 4-(4-nitrophenyl)-N'-[(pyridin-2-yl) methylidene] piperazine-1-carbothiohydrazide primarily suppressed the proliferation of MCF-7(breast cancer cell line) cells (IC_{50} = 0.021 µM) and U-251(gliomas) cells (IC_{50} = 0.036 µM) but demonstrated resistance in Hs 683(gliomas) cells (IC_{50} = 4.68 µM) The cells were seeded in 96-well plates (Nunc) at a density of 5,000 cells/well and measured at 490nm

(E)-1-((2-(4-methoxyphenyl)-1H-indol-3-yl)methylene)-4-methylthiosemicarbazide (6n), as shown by Bakherad et al. (2019), exhibited strong anti-cancer activity against various cell lines, with low IC_{50} values. Compound 6n, characterized by a methyl group substitution on the thiosemicarbazone and a 4-methoxyphenyl substitution at the C-2 position of the indole ring, displayed the most potent inhibitory activity against the A-549 cell line(lung), showcasing an IC_{50} of 12.5 µm. using s (1×10^4 cells/well) in RPMI-1640 medium at 492nm observance. These findings suggest that the presence of the methoxy group at the para position of the phenyl ring on the C-2 position of the indole and the methyl group substitution on the thiosemicarbazone segment significantly contribute to the inhibitory efficacy of compound 6n against the A-594 cell line. These studies reinforce the potential of 2-pyridineformamide thiosemicarbazone derivatives in cancer therapy.

French et al. (1974) conducted a comparative analysis of the inhibitory effects in vitro on ribonucleotide reductase and the tumor-inhibitory effects on three murine neoplasms using 61 pyridine compounds and 36 other related α-(N)-heterocyclic carboxaldehyde thiosemicarbazones. Their findings did not reveal a clear and direct quantitative relationship between inhibitory activity against partially purified human ribonucleotide reductase and sensitivity to the three tumors they examined. Nevertheless, among the compounds that exhibited significant inhibitory effects on the enzyme (with IC_{50} values below 10^{-6} M), 25 out of 27 demonstrated anticancer activity in at least one of the tumor systems employed. In contrast, among the compounds with intermediate or low activity against the enzyme, only 33 out of 51 and 2 out of 19, respectively, displayed antitumor properties. Interestingly, a substantial portion of the compounds that could impede tumor growth were comprised of the esters and ethers of 3-hydroxy- and 5-hydroxy-2-formyl pyridine thiosemicarbazone. Notably, these compounds possessed only intermediate inhibitory activity (with IC_{50} values falling between 10^{-4} and $5 * 10^{-5}$ M) against ribonucleotide reductase.

Structure and its Anti-cancer activity relationship of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes on *HeLa* cells

In the investigation of 2-pyridineformamide thiosemicarbazones and their copper(II) complexes on *HeLa* cells it has been found from literature that Electron-

withdrawing groups, like the 2-benzoylpyridine present in HL_4, identified as (2-benzoylpyridine N(4)-methylthiosemicarbazone), conferred exceptional anti-tumor efficacy against K562 leucocythemia cells (IC_{50}= 0.002 µm) and BEL7402 liver cancer cells (IC_{50}= 0.138 µm) (Li et al., 2009). Similarly, meta-fluorinated substituents in compound 4g, N-(Diethylcarbamothioyl)- 2-(2-(diethylcarbamothioyl) hydrazono)-2- (3-fluorophenyl) -acetamide, exhibited superior potency (IC_{50}= 9.0 µM, CC_{50} > 200 µM) with a higher selectivity index compared to non-substituted compounds for anti-parasitic property (Salsi et al., 2018). These electron-withdrawing groups are known to augment bioactivity by reducing electron density in aromatic rings, potentially heightening reactivity toward biological targets. Conversely, electron-donating groups, like thymol in compound (4b) thymol substituted thiosemicarbazone, demonstrated significant cytotoxicity against HT-1080 and A-549 cancer cells, with IC_{50} values of 7.10 ± 0.32 and 14.40 ± 0.36 µM, respectively. This compound's capability to induce caspase-dependent apoptosis and G2/M phase cell cycle arrest underscores its potential for targeted anti-cancer therapies (Laamari et al., 2023). Additionally, in our study, 6-methyl pyridine substituents within thiosemicarbazones showcased significant effects, potentially heightening interaction due to stabilized electron density within the molecule. The impact of these substituents on thiosemicarbazone activities remains contextual, influenced by compound structure, target specificity, and mechanisms of action. While electron-withdrawing groups generally enhance bioactivity, their effects on anticancer and anti-parasitic efficacy can vary based on compound structure and biological targets.

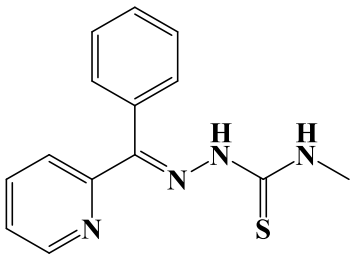


Figure 20 Structure of (HL4) 2-benzoylpyridine N(4)-methylthiosemicarbazone

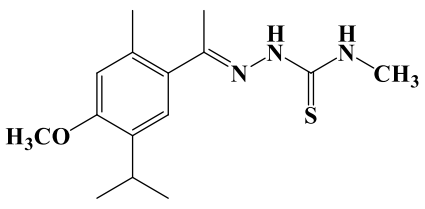


Figure 21 Structure of (4b) thiosemicarbazone containing thymol group

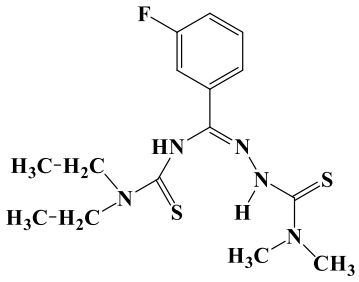


Figure 22 Structure of (4g) N-(Diethylcarbamothioyl)-2-(2-(diethylcarbamothioyl)hydrazono)-2-(3-fluorophenyl)- acetamide

Comparison of % yield of all compounds with their IC_{50} values

Comparing the % yield of compounds with their respective IC_{50} values reveals interesting insights into their potential efficacy. Compounds HL_1, HL_4, and $[CuL_4Cl]$, despite varying yields (HL_1: 51%, HL_4: 34%, $[CuL_4Cl]$: 93%), exhibited notably low IC_{50} values (HL_1: 1.598, HL_4: 1.065, $[CuL_4Cl]$: 0.08406). This suggests that compounds with lower yields might possess higher potency, indicating a potential solely based on yield.inverse relationship between yield and biological activity. However, this correlation isn't uniform across all compounds. For instance, HL_5 displayed a higher yield of 56% but had an IC_{50} value of 2.675, indicating moderate inhibitory activity. Conversely, $[CuL_1Cl]$ showcased a high yield of 95% and an IC_{50} value of 5.062, denoting substantial inhibition. Such inconsistencies might stem from varied compound structures or interactions, emphasizing the need for a nuanced understanding of structure-activity relationships to predict biological efficacy

Our study highlights the significance of compound-specific effects on cell viability and emphasizes the importance of concentration-dependent responses. These findings provide valuable insights into the potential anti-cancer properties of 2-pyridineformamide thiosemicarbazones and their copper(II) complexes and their suitability for further investigation as anti-cancer agents. The differences in IC_{50} values and the unique responses observed for each compound warrant further exploration to identify the mechanisms underlying these varying effects.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

In conclusion, our study provides a comprehensive analysis of the effects of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes on *HeLa* cell line. The non-parametric one-way ANOVA gave that there is statically significance difference between the mean of each dose from (0µM to 50µM) of compound 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. The concentration-dependent response underscores the importance of personalized approaches in drug development. The calculated IC_{50} values and insights gained from our analysis pave the way for more targeted therapeutic interventions and contribute to our understanding of the potential clinical applications of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes.

Comparison with Existing Literature: MTT Assay Results

In the present study, we conducted MTT assay to assess the cytotoxic effects of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes on the viability of *HeLa* cancer cells. Our findings revealed a concentration-dependent decrease in cell viability following 2-pyridineformamide thiosemicarbazones and its copper(II) complexes treatment. To provide context to our results, we compared our findings with existing literature in the field.

Consistency with Previous Studies:

Our results are in line with several studies that have investigated the cytotoxic potential of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes in *HeLa* cancer cell line. For instance, Shakya et al., (2019) demonstrated a similar dose-response relationship, reporting a significant reduction in cell viability in response to 2-pyridineformamide thiosemicarbazones and its copper(II) complexes treatment across *HeLa* cell line. Such as, he found that compounds showed to be inhibitory against *HeLa* cell line with IC_{50} between 0.9-8.6µM fromHL_1, HL_3, HL_4, HL_5, HL_6, $[CuL_1Cl]$, $[CuL_3Cl]$, $[CuL_4Cl]$ which is similar with our findings 1.065-5.002µM. These consistent findings suggest that Drug 2-pyridineformamide

thiosemicarbazones and its copper(II) complexes 's cytotoxic effects might be a common trait among *HeLa* cell line.

Observing at the IC_{50} values provided for both [CuLCl] and HL compounds, the $[CuL_4Cl]$ compound stands out as the most potent anti-cancer agent among all the compounds. Its exceptionally low IC_{50} of 0.08406 indicates the lowest concentration needed to inhibit the biological response by 50%. followed closely by $HL_4$and HL_6. The descending order of potency based on their IC_{50} values is evident: $[CuL_4Cl] > HL_4 > HL_6 > HL_1 > HL_5 > HL_3 > [CuL_1Cl] > [CuL_3Cl]$. This suggests that $[CuL_4Cl]$ possesses the highest potency in inhibiting cancer cell growth or activity compared to all other HL and [CuLCl] compounds mentioned.

While observing at compound analysis table the comparison between the HL (ligands) and [CuLCl] (Cu(II) complexes) compounds in terms of their anticancer activity shows that the [CuLCl] compounds generally display higher inhibitory effects at lower concentrations compared to their respective ligands. $[CuL_1Cl]$ exhibited a remarkable inhibition of 96.59% at a concentration of 10 µM, whereas its corresponding ligand, HL_1, showed a lower inhibition of 68.61% at a concentration of 10 µM. $[CuL_3Cl]$ demonstrated an even higher inhibition of 99.79% at a concentration of 25 µM, while its ligand, HL_3, displayed a slightly lower inhibition of 87.29% at 20 µM concentration. $[CuL_4Cl]$ showed substantial inhibition of 97.93% at a concentration of 5 µM, whereas its ligand counterpart showed 92.41 at 20µM.This pattern across the compounds indicates that the Copper(II) complexes ([CuLCl]s) tend to exhibit more potent inhibitory effects against cancer cells at lower concentrations compared to their ligands (HLs). The [CuLCl] compounds generally achieve higher maximum inhibitions at lower micromolar concentrations compared to their respective ligands, showcasing their potential as more efficient anticancer agents. Therefore, among the listed [CuLCl] and HL compounds, $[CuL_4Cl]$ exhibits the highest anti-cancer activity, considering its remarkably low IC_{50} value.

Limitations and Future Directions:

It is important to note that while our study provides valuable insights, our comparison with existing literature also reveals gaps in our understanding of 2-pyridineformamide thiosemicarbazones its its copper(II) complexes' effects.

Further studies could elucidate the differential sensitivity in cell lines, exact mechanisms behind drug resistance and the specific signaling pathways modulated by 2-pyridineformamide thiosemicarbazones and its copper(II) complexes in *HeLa* cells line.

In conclusion, our MTT assay results align with previous research on 2-pyridineformamide thiosemicarbazones and its copper(II) complexes' cytotoxic effects, showcasing its potential as an anticancer agent.

Interpretation of Biological Significance: MTT Assay Results

The MTT assay results obtained in this study provide valuable insights into the biological significance of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes' effects on *HeLa* cervical cancer cells. The concentration-dependent reduction in cell viability signifies the potential cytotoxicity of the drug and its implications for cancer treatment. The following interpretations elucidate the biological significance of our findings.

The observed decrease in cell viability upon 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. Treatment supports the notion that this compound exerts a targeted cytotoxic effect on *HeLa* cells. The significant reduction in viability indicates that 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. interferes with vital cellular processes, potentially inducing cell death through apoptosis or other mechanisms. This targeted approach aligns with the growing trend in precision medicine, where therapies are tailored to specific cellular vulnerabilities.

The cytotoxic effects of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. hold promising implications for cancer therapy. With *HeLa* cells commonly associated with cervical cancer, our findings suggest that 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. could be explored as a potential candidate for cervical cancer treatment. By selectively targeting cancer cells while minimizing harm to normal cells,2-pyridineformamide thiosemicarbazones and its copper(II) complexes may offer a more effective and tolerable therapeutic option compared to conventional treatments with broader toxicities.

The biological significance of our findings extends beyond the laboratory setting. The robust cytotoxic effects of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. warrant further investigation and preclinical and clinical studies. The potential integration of 2-pyridineformamide thiosemicarbazones and its copper(II) complexes. into chemotherapy regimens or targeted therapies holds promise for improving patient outcomes. However, rigorous evaluations of safety, pharmacokinetics, pharmacodynamics and long-term effects are essential before considering its translation to clinical practice.

Limitations and Further Exploration:

While our results provide crucial insights, the study has certain limitations. It focuses solely on in vitro cytotoxicity and does not account for normal cell treatment and its side effects, the complex tumor microenvironment. Future studies could delve into drug 2-pyridineformamide thiosemicarbazones and its copper(II) complexes' effects on 3D cell cultures or animal models to assess its behavior in a more physiologically relevant context.

In summary, from discussion section we can probably say that 2-pyridineformamide thiosemicarbazone family containing either 6-methyl pyridine ring or pyrrolidine ring showed drop in IC_{50} values as compared to other members of the family. If both the 6-methyl pyridine ring as well as pyrrolidine ring is present in the compound, then it showed significant drop in IC_{50} values of a compound.in comparison to other members example HL_4 and $[CuL_4Cl]$. MTT assay results underscore the biological significance of drug 2-pyridineformamide thiosemicarbazones and its copper(II) complexes' potential as a selective cytotoxic agent for *HeLa* cervical cancer cells. The observed cytotoxicity aligns with the paradigm shift towards personalized medicine and targeted therapies as shown by the literature. The findings open avenues for further exploration, emphasizing the need for comprehensive evaluations to harness drug 2-pyridineformamide thiosemicarbazones and its copper(II) complexes' therapeutic potential while addressing challenges associated with drug resistance and translational application

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CHAPTER 7

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APPENDIX-A

EQUIPMENTS, CHEMICALS AND PREPARATION OF REAGENTS

1. EQUIPMENTS, GLASSWARES AND OTHERS

Equipments

- 5% Carbondioxide Incubator
- T25 animal cell culture flask
- laminar air flow wood
- centrifuge
- Cryo storage container
- Liquid nitrogen container
- Inverted microscope
- Simple microscope
- Hemocytometer

Glassware and plastic ware

- Beaker
- Pipette
- Measuring cylinder
- Horizontal and vertical flask
  T25
  T75
  T100
- Petri plate
- Microscope slide
- Cover slips
- Eppendorfs tube

2. Protocol of *HeLa* cell line seeding:

1. Take the old media containing *HeLa* cell line and remove the culture media from culture flask.
2. Wash the cell containing flask with 2 to 3 ml 1x PBS either 2 to 3 times observing the confluency of the cell.
3. Add 2ml of trypsin and incubate for 2 minutes at 37℃.After that remove trypsin and collect cells on required volume
4. Transfer required volume of solution to new T25 animal cell culture flask and incubate it at incubator until the confluency of cells is acquired (Shakya et al., 2019).

3. Protocol for preparation of trypsin solution

- Dextrose:0.05% of 50 ml PBS
- EDTA:0.02% of 50ml PBS
- Trypsin:0.1% of 50ml PBS
- PH Must be maintained at 7- 7.5
- MTT reagent was prepared in PBS of 5mg/ml (Shakya et al., 2019)

Preparation of Dulbecco's Modified Eagle Medium (DMEM)

The preparation of standard DMEM (Dulbecco's Modified Eagle Medium) involved several sequential steps conducted under sterile conditions. Initially, the basal DMEM powder was combined with distilled water in accordance with the manufacturer's instructions to achieve the desired concentration. Upon complete dissolution of the powder, adjustments were made to the pH, typically around 7.2 - 7.4, if required. To facilitate cell growth and prevent contamination, 10% fetal bovine serum (FBS) and antibiotics, typically 100 IU/ml of penicillin and 100 µg/ml of streptomycin, were introduced into the solution. Following a thorough mixing process to ensure even distribution of components, the medium underwent sterile filtration to remove potential contaminants. The resulting final sterile DMEM

solution underwent careful storage in labeled, sterile containers at recommended temperatures until its utilization for cell culture experiments, ensuring the maintenance of sterility and quality conducive to optimal cell growth(Morton H., 1970)

Determination of Seeding Density:

The calculation of 3000 cells per well using a hemocytometer and trypan blue dye under a microscope was completed. The total number of live cells was determined in the hemocytometer using the formula (number of live cells counted / number of squares in the hemocytometer * Dilution factor * 10000).

Subsequently, the total number of cells required per 3000 cells per well was calculated, along with the total volume of DMEM needed in µl per 100µl of DMEM per well

Total cell required =3000*no of wells*no of compound (in number)

Total DMEM required=100*no of wells*no of compound (in µl)

The calculation for the total number of cells required per 3000 cells per well involved using the formula (total cells required (for 3000cells/ well) / total cells in the hemocytometer * 1000). This determined the volume of cells required in µl for a certain µl of DMEM medium(Palla et al., 2022)

Compound preparation

One Molar compound solution of 1 ml in DMSO (Dimethyl sulfoxide) was prepared using a specific formula by weighing.

$$\therefore \text{ Molarity (M)} = \frac{w \times 1000}{(\text{Mol. wt of solute}) \times V_{\text{in ml}}}$$

One millimolar compound solution of 1ml in DMSO was prepared using the formula

M1V1=M2V2.

Furthermore, 300µl (for triplicates) of compound dilutions in DMEM with varying concentrations, specifically 0, 1, 5, 10, 15, 20, 25, 50 µM, was prepared using the formula M1V1=M2V2(Shakya et al., 2019).

APPENDIX-B

Data of 2- pyridineformamide thiosemicarbazones and its copper(II) complexes including dose response, ANOVA

Table 3 Data of dose response curve for HL_1

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	1.598
HillSlope	-0.5225
$logIC_{50}$	0.2035
95% CI (profile likelihood)	
IC_{50}	1.014 to 2.247
HillSlope	-0.6290 to -0.4214
$logIC_{50}$	0.006164 to 0.3517
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9635
Sum of Squares	653.7
Sy.x	5.451
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 4 Data of Non-parametric one-way ANOVA of HL_1

Table Analyzed	Data 12				
Data sets analyzed	0-50µM(con)				
ANOVA summary					
F	81.39				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9727				
Brown-Forsythe test					
F (DFn, DFd)	0.8276 (7, 16)				
P value	0.5793				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				

Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	17428	7	2490	F (7, 16) = 81.39	P<0.0001
Residual (within columns)	489.4	16	30.59		
Total	17917	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 5 Data of Tukey comparison of HL_1

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	46.87	31.24 to 62.51	Yes	****	<0.0001	A-B		
0 vs. 5	60.64	45.01 to 76.28	Yes	****	<0.0001	A-C		
0 vs. 10	68.60	52.97 to 84.24	Yes	****	<0.0001	A-D		
0 vs. 15	75.75	60.11 to 91.38	Yes	****	<0.0001	A-E		
0 vs. 20	80.82	65.19 to 96.46	Yes	****	<0.0001	A-F		
0 vs. 25	82.59	66.96 to 98.23	Yes	****	<0.0001	A-G		
0 vs. 50	89.06	73.43 to 104.7	Yes	****	<0.0001	A-H		
1 vs. 5	13.77	-1.864 to 29.41	No	Ns	0.1061	B-C		
1 vs. 10	21.73	6.095 to 37.36	Yes	**	0.0037	B-D		
1 vs. 15	28.88	13.24 to 44.51	Yes	***	0.0002	B-E		
1 vs. 20	33.95	18.31 to 49.58	Yes	****	<0.0001	B-F		
1 vs. 25	35.72	20.09 to 51.36	Yes	****	<0.0001	B-G		
1 vs. 50	42.19	26.56 to 57.83	Yes	****	<0.0001	B-H		
5 vs. 10	7.959	-7.676 to 23.59	No	Ns	0.6510	C-D		
5 vs. 15	15.10	-0.5302 to 30.74	No	Ns	0.0622	C-E		
5 vs. 20	20.18	4.544 to 35.81	Yes	**	0.0072	C-F		
5 vs. 25	21.95	6.315 to 37.58	Yes	**	0.0033	C-G		
5 vs. 50	28.42	12.79 to 44.06	Yes	***	0.0002	C-H		
10 vs. 15	7.145	-8.489 to 22.78	No	Ns	0.7538	D-E		
10 vs. 20	12.22	-3.415 to 27.85	No	Ns	0.1900	D-F		

10 vs. 25	13.99	-1.644 to 29.63	No	Ns	0.0973	D-G		
10 vs. 50	20.46	4.828 to 36.10	Yes	**	0.0064	D-H		
15 vs. 20	5.074	-10.56 to 20.71	No	Ns	0.9419	E-F		
15 vs. 25	6.846	-8.789 to 22.48	No	Ns	0.7889	E-G		
15 vs. 50	13.32	-2.317 to 28.95	No	Ns	0.1264	E-H		
20 vs. 25	1.772	-13.86 to 17.41	No	Ns	0.9999	F-G		
20 vs. 50	8.244	-7.391 to 23.88	No	Ns	0.6136	F-H		
25 vs. 50	6.472	-9.163 to 22.11	No	Ns	0.8295	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	53.13	46.87	4.516	3	3	14.68	16
0 vs. 5	100.0	39.36	60.64	4.516	3	3	18.99	16
0 vs. 10	100.0	31.40	68.60	4.516	3	3	21.48	16
0 vs. 15	100.0	24.25	75.75	4.516	3	3	23.72	16
0 vs. 20	100.0	19.18	80.82	4.516	3	3	25.31	16
0 vs. 25	100.0	17.41	82.59	4.516	3	3	25.86	16
0 vs. 50	100.0	10.94	89.06	4.516	3	3	27.89	16
1 vs. 5	53.13	39.36	13.77	4.516	3	3	4.312	16
1 vs. 10	53.13	31.40	21.73	4.516	3	3	6.805	16
1 vs. 15	53.13	24.25	28.88	4.516	3	3	9.043	16
1 vs. 20	53.13	19.18	33.95	4.516	3	3	10.63	16
1 vs. 25	53.13	17.41	35.72	4.516	3	3	11.19	16
1 vs. 50	53.13	10.94	42.19	4.516	3	3	13.21	16
5 vs. 10	39.36	31.40	7.959	4.516	3	3	2.493	16
5 vs. 15	39.36	24.25	15.10	4.516	3	3	4.730	16
5 vs. 20	39.36	19.18	20.18	4.516	3	3	6.319	16
5 vs. 25	39.36	17.41	21.95	4.516	3	3	6.874	16
5 vs. 50	39.36	10.94	28.42	4.516	3	3	8.901	16
10 vs. 15	31.40	24.25	7.145	4.516	3	3	2.238	16
10 vs. 20	31.40	19.18	12.22	4.516	3	3	3.827	16
10 vs. 25	31.40	17.41	13.99	4.516	3	3	4.381	16
10 vs. 50	31.40	10.94	20.46	4.516	3	3	6.408	16
15 vs. 20	24.25	19.18	5.074	4.516	3	3	1.589	16
15 vs. 25	24.25	17.41	6.846	4.516	3	3	2.144	16
15 vs. 50	24.25	10.94	13.32	4.516	3	3	4.171	16
20 vs. 25	19.18	17.41	1.772	4.516	3	3	0.5548	16
20 vs. 50	19.18	10.94	8.244	4.516	3	3	2.582	16
25 vs. 50	17.41	10.94	6.472	4.516	3	3	2.027	16

Table 6 Data of dose response curve for HL_3

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	5.002
HillSlope	-1.297
logIC_{50}	0.6991
95% CI (profile likelihood)	
IC_{50}	4.525 to 5.498
HillSlope	-1.439 to -1.171
logIC_{50}	0.6557 to 0.7402
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9894
Sum of Squares	307.5
Sy.x	3.738
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 7 Data of Non-parametric one-way ANOVA of HL_3

Table Analyzed	Data 3				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	616.9				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9963				
Brown-Forsythe test					
F (DFn, DFd)	0.4069 (7, 16)				
P value	0.8842				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Treatment (between columns)	29019	7	4146	F (7, 16) = 616.9	P<0.0001
Residual (within columns)	107.5	16	6.720		
Total	29127	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 8 Data of Tukey multiple comparison of HL_3

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	7.540	0.2120 to 14.87	Yes	*	0.0414	A-B		
0 vs. 5	54.79	47.47 to 62.12	Yes	****	<0.0001	A-C		
0 vs. 10	68.38	61.05 to 75.70	Yes	****	<0.0001	A-D		
0 vs. 15	76.26	68.93 to 83.59	Yes	****	<0.0001	A-E		
0 vs. 20	87.29	79.96 to 94.62	Yes	****	<0.0001	A-F		
0 vs. 25	90.60	83.27 to 97.93	Yes	****	<0.0001	A-G		
0 vs. 50	95.75	88.42 to 103.1	Yes	****	<0.0001	A-H		
1 vs. 5	47.25	39.93 to 54.58	Yes	****	<0.0001	B-C		
1 vs. 10	60.84	53.51 to 68.16	Yes	****	<0.0001	B-D		
1 vs. 15	68.72	61.39 to 76.05	Yes	****	<0.0001	B-E		
1 vs. 20	79.75	72.42 to 87.08	Yes	****	<0.0001	B-F		
1 vs. 25	83.06	75.73 to 90.39	Yes	****	<0.0001	B-G		
1 vs. 50	88.21	80.88 to 95.54	Yes	****	<0.0001	B-H		
5 vs. 10	13.58	6.253 to 20.91	Yes	***	0.0002	C-D		
5 vs. 15	21.46	14.14 to 28.79	Yes	****	<0.0001	C-E		
5 vs. 20	32.50	25.17 to 39.83	Yes	****	<0.0001	C-F		
5 vs. 25	35.81	28.48 to 43.13	Yes	****	<0.0001	C-G		
5 vs. 50	40.96	33.63 to 48.29	Yes	****	<0.0001	C-H		
10 vs. 15	7.882	0.5541 to 15.21	Yes	*	0.0305	D-E		
10 vs. 20	18.92	11.59 to 26.25	Yes	****	<0.0001	D-F		
10 vs. 25	22.22	14.90 to 29.55	Yes	****	<0.0001	D-G		
10 vs. 50	27.38	20.05 to 34.71	Yes	****	<0.0001	D-H		
15 vs. 20	11.03	3.707 to 18.36	Yes	**	0.0017	E-F		
15 vs. 25	14.34	7.014 to 21.67	Yes	****	<0.0001	E-G		
15 vs. 50	19.50	12.17 to 26.82	Yes	****	<0.0001	E-H		
20 vs. 25	3.307	-4.021 to 10.64	No	Ns	0.7644	F-G		

20 vs. 50	8.461	1.133 to 15.79	Yes	*	0.0180	F-H		
25 vs. 50	5.153	-2.175 to 12.48	No	Ns	0.2891	G-H		
Test details	Mean	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	92.46	7.540	2.117	3	3	5.038	16
0 vs. 5	100.0	45.21	54.79	2.117	3	3	36.61	16
0 vs. 10	100.0	31.62	68.38	2.117	3	3	45.68	16
0 vs. 15	100.0	23.74	76.26	2.117	3	3	50.95	16
0 vs. 20	100.0	12.71	87.29	2.117	3	3	58.32	16
0 vs. 25	100.0	9.400	90.60	2.117	3	3	60.53	16
0 vs. 50	100.0	4.247	95.75	2.117	3	3	63.98	16
1 vs. 5	92.46	45.21	47.25	2.117	3	3	31.57	16
1 vs. 10	92.46	31.62	60.84	2.117	3	3	40.65	16
1 vs. 15	92.46	23.74	68.72	2.117	3	3	45.91	16
1 vs. 20	92.46	12.71	79.75	2.117	3	3	53.29	16
1 vs. 25	92.46	9.400	83.06	2.117	3	3	55.50	16
1 vs. 50	92.46	4.247	88.21	2.117	3	3	58.94	16
5 vs. 10	45.21	31.62	13.58	2.117	3	3	9.074	16
5 vs. 15	45.21	23.74	21.46	2.117	3	3	14.34	16
5 vs. 20	45.21	12.71	32.50	2.117	3	3	21.71	16
5 vs. 25	45.21	9.400	35.81	2.117	3	3	23.92	16
5 vs. 50	45.21	4.247	40.96	2.117	3	3	27.37	16
10 vs. 15	31.62	23.74	7.882	2.117	3	3	5.266	16
10 vs. 20	31.62	12.71	18.92	2.117	3	3	12.64	16
10 vs. 25	31.62	9.400	22.22	2.117	3	3	14.85	16
10 vs. 50	31.62	4.247	27.38	2.117	3	3	18.29	16
15 vs. 20	23.74	12.71	11.03	2.117	3	3	7.373	16
15 vs. 25	23.74	9.400	14.34	2.117	3	3	9.583	16
15 vs. 50	23.74	4.247	19.50	2.117	3	3	13.03	16
20 vs. 25	12.71	9.400	3.307	2.117	3	3	2.210	16
20 vs. 50	12.71	4.247	8.461	2.117	3	3	5.653	16
25 vs. 50	9.400	4.247	5.153	2.117	3	3	3.443	16

Table 9 Data of dose response curve for HL_4

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	1.065
HillSlope	-0.7343
$logIC_{50}$	0.02725
95% CI (profile likelihood)	
IC_{50}	0.9293 to 1.204
HillSlope	-0.7891 to -0.6816
$logIC_{50}$	-0.03183 to 0.08059
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9955
Sum of Squares	98.92
Sy.x	2.120
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# X values	24
# Y values analyzed	24

Table 10 Data of Non-parametric one-way ANOVA of HL_4

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	1057				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9978				
Brown-Forsythe test					
F (DFn, DFd)	0.8382 (7, 16)				
P value	0.5719				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					

Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	21964	7	3138	F (7, 16) = 1057	P<0.0001
Residual (within columns)	47.50	16	2.969		
Total	22012	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 11 Data of Tukeys multiple comparison of HL_4

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	49.50	44.63 to 54.37	Yes	****	<0.0001	A-B		
0 vs. 5	74.17	69.30 to 79.04	Yes	****	<0.0001	A-C		
0 vs. 10	82.32	77.44 to 87.19	Yes	****	<0.0001	A-D		
0 vs. 15	87.52	82.65 to 92.39	Yes	****	<0.0001	A-E		
0 vs. 20	92.41	87.54 to 97.28	Yes	****	<0.0001	A-F		
0 vs. 25	92.56	87.69 to 97.43	Yes	****	<0.0001	A-G		
0 vs. 50	93.03	88.16 to 97.90	Yes	****	<0.0001	A-H		
1 vs. 5	24.67	19.80 to 29.54	Yes	****	<0.0001	B-C		
1 vs. 10	32.82	27.94 to 37.69	Yes	****	<0.0001	B-D		
1 vs. 15	38.02	33.15 to 42.89	Yes	****	<0.0001	B-E		
1 vs. 20	42.91	38.04 to 47.79	Yes	****	<0.0001	B-F		
1 vs. 25	43.06	38.19 to 47.93	Yes	****	<0.0001	B-G		
1 vs. 50	43.53	38.66 to 48.40	Yes	****	<0.0001	B-H		
5 vs. 10	8.142	3.271 to 13.01	Yes	***	0.0006	C-D		
5 vs. 15	13.35	8.480 to 18.22	Yes	****	<0.0001	C-E		
5 vs. 20	18.24	13.37 to 23.11	Yes	****	<0.0001	C-F		
5 vs. 25	18.39	13.52 to 23.26	Yes	****	<0.0001	C-G		

5 vs. 50	18.85	13.98 to 23.72	Yes	****	<0.0001	C-H		
10 vs. 15	5.208	0.3378 to 10.08	Yes	*	0.0317	D-E		
10 vs. 20	10.10	5.229 to 14.97	Yes	****	<0.0001	D-F		
10 vs. 25	10.24	5.374 to 15.12	Yes	****	<0.0001	D-G		
10 vs. 50	10.71	5.842 to 15.58	Yes	****	<0.0001	D-H		
15 vs. 20	4.891	0.02015 to 9.761	Yes	*	0.0487	E-F		
15 vs. 25	5.036	0.1653 to 9.907	Yes	*	0.0401	E-G		
15 vs. 50	5.504	0.6330 to 10.37	Yes	*	0.0212	E-H		
20 vs. 25	0.1451	-4.725 to 5.016	No	Ns	>0.9999	F-G		
20 vs. 50	0.6129	-4.258 to 5.484	No	Ns	0.9998	F-H		
25 vs. 50	0.4677	-4.403 to 5.338	No	Ns	>0.9999	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	50.50	49.50	1.407	3	3	49.76	16
0 vs. 5	100.0	25.83	74.17	1.407	3	3	74.56	16
0 vs. 10	100.0	17.68	82.32	1.407	3	3	82.75	16
0 vs. 15	100.0	12.48	87.52	1.407	3	3	87.98	16
0 vs. 20	100.0	7.586	92.41	1.407	3	3	92.90	16
0 vs. 25	100.0	7.441	92.56	1.407	3	3	93.05	16
0 vs. 50	100.0	6.973	93.03	1.407	3	3	93.52	16
1 vs. 5	50.50	25.83	24.67	1.407	3	3	24.80	16
1 vs. 10	50.50	17.68	32.82	1.407	3	3	32.99	16
1 vs. 15	50.50	12.48	38.02	1.407	3	3	38.22	16
1 vs. 20	50.50	7.586	42.91	1.407	3	3	43.14	16
1 vs. 25	50.50	7.441	43.06	1.407	3	3	43.29	16
1 vs. 50	50.50	6.973	43.53	1.407	3	3	43.76	16
5 vs. 10	25.83	17.68	8.142	1.407	3	3	8.185	16
5 vs. 15	25.83	12.48	13.35	1.407	3	3	13.42	16
5 vs. 20	25.83	7.586	18.24	1.407	3	3	18.34	16
5 vs. 25	25.83	7.441	18.39	1.407	3	3	18.48	16
5 vs. 50	25.83	6.973	18.85	1.407	3	3	18.95	16
10 vs. 15	17.68	12.48	5.208	1.407	3	3	5.236	16
10 vs. 20	17.68	7.586	10.10	1.407	3	3	10.15	16
10 vs. 25	17.68	7.441	10.24	1.407	3	3	10.30	16
10 vs. 50	17.68	6.973	10.71	1.407	3	3	10.77	16
15 vs. 20	12.48	7.586	4.891	1.407	3	3	4.916	16
15 vs. 25	12.48	7.441	5.036	1.407	3	3	5.062	16
15 vs. 50	12.48	6.973	5.504	1.407	3	3	5.533	16
20 vs. 25	7.586	7.441	0.1451	1.407	3	3	0.1459	16
20 vs. 50	7.586	6.973	0.6129	1.407	3	3	0.6161	16
25 vs. 50	7.441	6.973	0.4677	1.407	3	3	0.4702	16

Table 12 Data of dose response curve for HL_5

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC50	2.675
HillSlope	-0.5467
$logIC_{50}$	0.4273
95% CI (profile likelihood)	
IC_{50}	2.091 to 3.300
HillSlope	-0.6233 to -0.4736
$logIC_{50}$	0.3203 to 0.5185
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9789
Sum of Squares	352.1
Sy.x	4.001
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 13 Data of Non-parametric one-way ANOVA of HL_5

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	261.2				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9913				
Brown-Forsythe test					
F (DFn, DFd)	0.9315 (7, 16)				
P value	0.5092				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	16569	7	2367	F (7, 16) = 261.2	P<0.0001
Residual (within columns)	145.0	16	9.062		
Total	16714	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 14 Data of Tukeys multiple comparison of HL_5

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	39.79	31.28 to 48.30	Yes	****	<0.0001	A-B		
0 vs. 5	56.80	48.29 to 65.31	Yes	****	<0.0001	A-C		
0 vs. 10	64.00	55.49 to 72.51	Yes	****	<0.0001	A-D		
0 vs. 15	68.94	60.43 to 77.45	Yes	****	<0.0001	A-E		
0 vs. 20	74.03	65.52 to 82.54	Yes	****	<0.0001	A-F		
0 vs. 25	80.00	71.49 to 88.51	Yes	****	<0.0001	A-G		
0 vs. 50	88.62	80.11 to 97.13	Yes	****	<0.0001	A-H		
1 vs. 5	17.02	8.508 to 25.53	Yes	****	<0.0001	B-C		
1 vs. 10	24.21	15.70 to 32.72	Yes	****	<0.0001	B-D		
1 vs. 15	29.15	20.64 to 37.66	Yes	****	<0.0001	B-E		
1 vs. 20	34.24	25.73 to 42.75	Yes	****	<0.0001	B-F		
1 vs. 25	40.22	31.71 to 48.73	Yes	****	<0.0001	B-G		
1 vs. 50	48.83	40.32 to 57.34	Yes	****	<0.0001	B-H		
5 vs. 10	7.193	-1.317 to 15.70	No	Ns	0.1314	C-D		
5 vs. 15	12.14	3.627 to 20.65	Yes	**	0.0029	C-E		
5 vs. 20	17.23	8.718 to 25.74	Yes	****	<0.0001	C-F		
5 vs. 25	23.20	14.69 to 31.71	Yes	****	<0.0001	C-G		
5 vs. 50	31.81	23.31 to 40.32	Yes	****	<0.0001	C-H		
10 vs. 15	4.944	-3.566 to 13.45	No	Ns	0.5041	D-E		
10 vs. 20	10.03	1.525 to 18.54	Yes	*	0.0152	D-F		
10 vs. 25	16.01	7.496 to 24.52	Yes	***	0.0002	D-G		
10 vs. 50	24.62	16.11 to 33.13	Yes	****	<0.0001	D-H		
15 vs. 20	5.090	-3.419 to 13.60	No	Ns	0.4702	E-F		
15 vs. 25	11.06	2.552 to 19.57	Yes	**	0.0067	E-G		
15 vs. 50	19.68	11.17 to 28.19	Yes	****	<0.0001	E-H		

20 vs. 25	5.971	-2.539 to 14.48	No	Ns	0.2914	F-G		
20 vs. 50	14.59	6.078 to 23.10	Yes	***	0.0004	F-H		
25 vs. 50	8.617	0.1068 to 17.13	Yes	*	0.0461	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	60.21	39.79	2.458	3	3	22.89	16
0 vs. 5	100.0	43.20	56.80	2.458	3	3	32.68	16
0 vs. 10	100.0	36.00	64.00	2.458	3	3	36.82	16
0 vs. 15	100.0	31.06	68.94	2.458	3	3	39.67	16
0 vs. 20	100.0	25.97	74.03	2.458	3	3	42.60	16
0 vs. 25	100.0	20.00	80.00	2.458	3	3	46.03	16
0 vs. 50	100.0	11.38	88.62	2.458	3	3	50.99	16
1 vs. 5	60.21	43.20	17.02	2.458	3	3	9.791	16
1 vs. 10	60.21	36.00	24.21	2.458	3	3	13.93	16
1 vs. 15	60.21	31.06	29.15	2.458	3	3	16.77	16
1 vs. 20	60.21	25.97	34.24	2.458	3	3	19.70	16
1 vs. 25	60.21	20.00	40.22	2.458	3	3	23.14	16
1 vs. 50	60.21	11.38	48.83	2.458	3	3	28.10	16
5 vs. 10	43.20	36.00	7.193	2.458	3	3	4.139	16
5 vs. 15	43.20	31.06	12.14	2.458	3	3	6.983	16
5 vs. 20	43.20	25.97	17.23	2.458	3	3	9.912	16
5 vs. 25	43.20	20.00	23.20	2.458	3	3	13.35	16
5 vs. 50	43.20	11.38	31.81	2.458	3	3	18.31	16
10 vs. 15	36.00	31.06	4.944	2.458	3	3	2.845	16
10 vs. 20	36.00	25.97	10.03	2.458	3	3	5.773	16
10 vs. 25	36.00	20.00	16.01	2.458	3	3	9.209	16
10 vs. 50	36.00	11.38	24.62	2.458	3	3	14.17	16
15 vs. 20	31.06	25.97	5.090	2.458	3	3	2.929	16
15 vs. 25	31.06	20.00	11.06	2.458	3	3	6.364	16
15 vs. 50	31.06	11.38	19.68	2.458	3	3	11.32	16
20 vs. 25	25.97	20.00	5.971	2.458	3	3	3.436	16
20 vs. 50	25.97	11.38	14.59	2.458	3	3	8.393	16
25 vs. 50	20.00	11.38	8.617	2.458	3	3	4.958	16

Table 15 Data of dose response curve for HL_6

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	1.181
HillSlope	-0.6819
$logIC_{50}$	0.07219
95% CI (profile likelihood)	
IC_{50}	0.7922 to 1.609
HillSlope	-0.8111 to -0.5617
$logIC_{50}$	-0.1012 to 0.2065
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9697
Sum of Squares	664.0
Sy.x	5.494
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 16 Data of Non-parametric one-way ANOVA of HL_6

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	162.3				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9861				
Brown-Forsythe test					
F (DFn, DFd)	0.8781 (7, 16)				
P value	0.5445				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Treatment (between columns)	21624	7	3089	F (7, 16) = 162.3	P<0.0001
Residual (within columns)	304.6	16	19.04		
Total	21929	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 17 Data of Tukeys multiple comparison of HL_6

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	50.74	38.40 to 63.07	Yes	****	<0.0001	A-B		
0 vs. 5	63.89	51.56 to 76.23	Yes	****	<0.0001	A-C		
0 vs. 10	80.03	67.69 to 92.36	Yes	****	<0.0001	A-D		
0 vs. 15	87.16	74.82 to 99.49	Yes	****	<0.0001	A-E		
0 vs. 20	90.35	78.02 to 102.7	Yes	****	<0.0001	A-F		
0 vs. 25	91.93	79.59 to 104.3	Yes	****	<0.0001	A-G		
0 vs. 50	94.70	82.36 to 107.0	Yes	****	<0.0001	A-H		
1 vs. 5	13.16	0.8220 to 25.49	Yes	*	0.0323	B-C		
1 vs. 10	29.29	16.96 to 41.62	Yes	****	<0.0001	B-D		
1 vs. 15	36.42	24.09 to 48.76	Yes	****	<0.0001	B-E		
1 vs. 20	39.62	27.28 to 51.95	Yes	****	<0.0001	B-F		
1 vs. 25	41.19	28.86 to 53.53	Yes	****	<0.0001	B-G		
1 vs. 50	43.96	31.63 to 56.29	Yes	****	<0.0001	B-H		
5 vs. 10	16.13	3.799 to 28.47	Yes	**	0.0064	C-D		
5 vs. 15	23.26	10.93 to 35.60	Yes	***	0.0001	C-E		
5 vs. 20	26.46	14.13 to 38.80	Yes	****	<0.0001	C-F		
5 vs. 25	28.04	15.70 to 40.37	Yes	****	<0.0001	C-G		
5 vs. 50	30.80	18.47 to 43.14	Yes	****	<0.0001	C-H		
10 vs. 15	7.130	-5.204 to 19.46	No	Ns	0.5099	D-E		
10 vs. 20	10.33	-2.006 to 22.66	No	Ns	0.1378	D-F		
10 vs. 25	11.90	-0.4334 to 24.24	No	Ns	0.0627	D-G		
10 vs. 50	14.67	2.335 to 27.00	Yes	*	0.0142	D-H		
15 vs. 20	3.198	-9.136 to 15.53	No	Ns	0.9820	E-F		
15 vs. 25	4.771	-7.563 to 17.11	No	Ns	0.8707	E-G		

15 vs. 50	7.539	-4.795 to 19.87	No	Ns	0.4451	E-H		
20 vs. 25	1.573	-10.76 to 13.91	No	Ns	0.9998	F-G		
20 vs. 50	4.341	-7.993 to 16.68	No	Ns	0.9147	F-H		
25 vs. 50	2.768	-9.566 to 15.10	No	Ns	0.9921	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	49.26	50.74	3.563	3	3	20.14	16
0 vs. 5	100.0	36.11	63.89	3.563	3	3	25.36	16
0 vs. 10	100.0	19.97	80.03	3.563	3	3	31.77	16
0 vs. 15	100.0	12.84	87.16	3.563	3	3	34.60	16
0 vs. 20	100.0	9.646	90.35	3.563	3	3	35.87	16
0 vs. 25	100.0	8.073	91.93	3.563	3	3	36.49	16
0 vs. 50	100.0	5.305	94.70	3.563	3	3	37.59	16
1 vs. 5	49.26	36.11	13.16	3.563	3	3	5.223	16
1 vs. 10	49.26	19.97	29.29	3.563	3	3	11.63	16
1 vs. 15	49.26	12.84	36.42	3.563	3	3	14.46	16
1 vs. 20	49.26	9.646	39.62	3.563	3	3	15.73	16
1 vs. 25	49.26	8.073	41.19	3.563	3	3	16.35	16
1 vs. 50	49.26	5.305	43.96	3.563	3	3	17.45	16
5 vs. 10	36.11	19.97	16.13	3.563	3	3	6.404	16
5 vs. 15	36.11	12.84	23.26	3.563	3	3	9.235	16
5 vs. 20	36.11	9.646	26.46	3.563	3	3	10.50	16
5 vs. 25	36.11	8.073	28.04	3.563	3	3	11.13	16
5 vs. 50	36.11	5.305	30.80	3.563	3	3	12.23	16
10 vs. 15	19.97	12.84	7.130	3.563	3	3	2.830	16
10 vs. 20	19.97	9.646	10.33	3.563	3	3	4.100	16
10 vs. 25	19.97	8.073	11.90	3.563	3	3	4.724	16
10 vs. 50	19.97	5.305	14.67	3.563	3	3	5.823	16
15 vs. 20	12.84	9.646	3.198	3.563	3	3	1.269	16
15 vs. 25	12.84	8.073	4.771	3.563	3	3	1.894	16
15 vs. 50	12.84	5.305	7.539	3.563	3	3	2.993	16
20 vs. 25	9.646	8.073	1.573	3.563	3	3	0.6244	16
20 vs. 50	9.646	5.305	4.341	3.563	3	3	1.723	16
25 vs. 50	8.073	5.305	2.768	3.563	3	3	1.099	16

Table 18 Data of dose response curve for [CuL_1Cl]

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	5.062
HillSlope	-4.478
logIC_{50}	0.7043
95% CI (profile likelihood)	
IC_{50}	3.092 to 5.622
HillSlope	??? to -1.457
logIC_{50}	0.4903 to 0.7499
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9453
Sum of Squares	1942
Sy.x	9.395
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 19 Data of Non-parametric one-way ANOVA of [CuL_1Cl]

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	152.9				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9853				
Brown-Forsythe test					
F (DFn, DFd)	1.220 (7, 16)				
P value	0.3477				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Treatment (between columns)	34953	7	4993	F (7, 16) = 152.9	P<0.0001
Residual (within columns)	522.4	16	32.65		
Total	35476	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 20 Data of Tukeys multiple comparison of [CuL1Cl]

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	21.60	5.446 to 37.75	Yes	**	0.0052	A-B		
0 vs. 5	48.40	32.25 to 64.55	Yes	****	<0.0001	A-C		
0 vs. 10	96.59	80.43 to 112.7	Yes	****	<0.0001	A-D		
0 vs. 15	98.84	82.69 to 115.0	Yes	****	<0.0001	A-E		
0 vs. 20	98.43	82.27 to 114.6	Yes	****	<0.0001	A-F		
0 vs. 25	98.67	82.52 to 114.8	Yes	****	<0.0001	A-G		
0 vs. 50	97.81	81.66 to 114.0	Yes	****	<0.0001	A-H		
1 vs. 5	26.80	10.65 to 42.95	Yes	***	0.0006	B-C		
1 vs. 10	74.99	58.83 to 91.14	Yes	****	<0.0001	B-D		
1 vs. 15	77.24	61.09 to 93.39	Yes	****	<0.0001	B-E		
1 vs. 20	76.83	60.67 to 92.98	Yes	****	<0.0001	B-F		
1 vs. 25	77.07	60.92 to 93.22	Yes	****	<0.0001	B-G		
1 vs. 50	76.21	60.06 to 92.36	Yes	****	<0.0001	B-H		
5 vs. 10	48.19	32.03 to 64.34	Yes	****	<0.0001	C-D		
5 vs. 15	50.44	34.29 to 66.59	Yes	****	<0.0001	C-E		
5 vs. 20	50.03	33.87 to 66.18	Yes	****	<0.0001	C-F		
5 vs. 25	50.27	34.12 to 66.42	Yes	****	<0.0001	C-G		
5 vs. 50	49.41	33.26 to 65.56	Yes	****	<0.0001	C-H		
10 vs. 15	2.253	-13.90 to 18.41	No	Ns	0.9996	D-E		
10 vs. 20	1.840	-14.31 to 17.99	No	Ns	0.9999	D-F		
10 vs. 25	2.086	-14.07 to 18.24	No	Ns	0.9998	D-G		
10 vs. 50	1.224	-14.93 to 17.38	No	Ns	>0.9999	D-H		
15 vs. 20	-0.4130	-16.57 to 15.74	No	Ns	>0.9999	E-F		
15 vs. 25	-0.1673	-16.32 to 15.98	No	Ns	>0.9999	E-G		
15 vs. 50	-1.028	-17.18 to 15.12	No	Ns	>0.9999	E-H		
20 vs. 25	0.2457	-15.91 to 16.40	No	Ns	>0.9999	F-G		
20 vs. 50	-0.6154	-16.77 to 15.54	No	Ns	>0.9999	F-H		
25 vs. 50	-0.8611	-17.01 to 15.29	No	Ns	>0.9999	G-H		

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	78.40	21.60	4.665	3	3	6.547	16
0 vs. 5	100.0	51.60	48.40	4.665	3	3	14.67	16
0 vs. 10	100.0	3.415	96.59	4.665	3	3	29.28	16
0 vs. 15	100.0	1.162	98.84	4.665	3	3	29.96	16
0 vs. 20	100.0	1.575	98.43	4.665	3	3	29.84	16
0 vs. 25	100.0	1.329	98.67	4.665	3	3	29.91	16
0 vs. 50	100.0	2.190	97.81	4.665	3	3	29.65	16
1 vs. 5	78.40	51.60	26.80	4.665	3	3	8.124	16
1 vs. 10	78.40	3.415	74.99	4.665	3	3	22.73	16
1 vs. 15	78.40	1.162	77.24	4.665	3	3	23.41	16
1 vs. 20	78.40	1.575	76.83	4.665	3	3	23.29	16
1 vs. 25	78.40	1.329	77.07	4.665	3	3	23.36	16
1 vs. 50	78.40	2.190	76.21	4.665	3	3	23.10	16
5 vs. 10	51.60	3.415	48.19	4.665	3	3	14.61	16
5 vs. 15	51.60	1.162	50.44	4.665	3	3	15.29	16
5 vs. 20	51.60	1.575	50.03	4.665	3	3	15.16	16
5 vs. 25	51.60	1.329	50.27	4.665	3	3	15.24	16
5 vs. 50	51.60	2.190	49.41	4.665	3	3	14.98	16
10 vs. 15	3.415	1.162	2.253	4.665	3	3	0.6829	16
10 vs. 20	3.415	1.575	1.840	4.665	3	3	0.5577	16
10 vs. 25	3.415	1.329	2.086	4.665	3	3	0.6322	16
10 vs. 50	3.415	2.190	1.224	4.665	3	3	0.3712	16
15 vs. 20	1.162	1.575	-0.4130	4.665	3	3	0.1252	16
15 vs. 25	1.162	1.329	-0.1673	4.665	3	3	0.05072	16
15 vs. 50	1.162	2.190	-1.028	4.665	3	3	0.3118	16
20 vs. 25	1.575	1.329	0.2457	4.665	3	3	0.07448	16
20 vs. 50	1.575	2.190	-0.6154	4.665	3	3	0.1866	16
25 vs. 50	1.329	2.190	-0.8611	4.665	3	3	0.2610	16

Table 21 Data of dose response curve for [CuL_3Cl]

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC_{50}	8.687
HillSlope	-1.807
$logIC_{50}$	0.9389
95% CI (profile likelihood)	
IC_{50}	6.812 to 10.55
HillSlope	-2.525 to -1.285
$logIC_{50}$	0.8333 to 1.023
Goodness of Fit	
Degrees of Freedom	22

R squared	0.9249
Sum of Squares	2465
Sy.x	10.59
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	
# of X values	24
# Y values analyzed	24

Table 22 Data of Non-parametric one-way ANOVA of $[CuL_3Cl]$

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	898.8				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9975				
Brown-Forsythe test					
F (DFn, DFd)	0.6102 (7, 16)				
P value	0.7397				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	32750	7	4679	F (7, 16) = 898.8	P<0.0001
Residual (within columns)	83.29	16	5.205		
Total	32833	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

Table 23 Data of Tukeys multiple comparison of [CuL_3Cl]

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
0 vs. 1	11.86	5.409 to 18.31	Yes	***	0.0002	A-B		
0 vs. 5	36.51	30.06 to 42.96	Yes	****	<0.0001	A-C		
0 vs. 10	44.84	38.39 to 51.29	Yes	****	<0.0001	A-D		
0 vs. 15	60.09	53.64 to 66.54	Yes	****	<0.0001	A-E		
0 vs. 20	92.94	86.49 to 99.39	Yes	****	<0.0001	A-F		
0 vs. 25	99.80	93.35 to 106.2	Yes	****	<0.0001	A-G		
0 vs. 50	101.0	94.59 to 107.5	Yes	****	<0.0001	A-H		
1 vs. 5	24.65	18.20 to 31.10	Yes	****	<0.0001	B-C		
1 vs. 10	32.98	26.54 to 39.43	Yes	****	<0.0001	B-D		
1 vs. 15	48.23	41.78 to 54.68	Yes	****	<0.0001	B-E		
1 vs. 20	81.08	74.63 to 87.53	Yes	****	<0.0001	B-F		
1 vs. 25	87.94	81.49 to 94.39	Yes	****	<0.0001	B-G		
1 vs. 50	89.18	82.73 to 95.63	Yes	****	<0.0001	B-H		
5 vs. 10	8.337	1.888 to 14.79	Yes	**	0.0071	C-D		
5 vs. 15	23.59	17.14 to 30.04	Yes	****	<0.0001	C-E		
5 vs. 20	56.43	49.99 to 62.88	Yes	****	<0.0001	C-F		
5 vs. 25	63.29	56.84 to 69.74	Yes	****	<0.0001	C-G		
5 vs. 50	64.53	58.08 to 70.98	Yes	****	<0.0001	C-H		
10 vs. 15	15.25	8.799 to 21.70	Yes	****	<0.0001	D-E		
10 vs. 20	48.10	41.65 to 54.55	Yes	****	<0.0001	D-F		
10 vs. 25	54.95	48.50 to 61.40	Yes	****	<0.0001	D-G		
10 vs. 50	56.19	49.74 to 62.64	Yes	****	<0.0001	D-H		
15 vs. 20	32.85	26.40 to 39.30	Yes	****	<0.0001	E-F		
15 vs. 25	39.70	33.25 to 46.15	Yes	****	<0.0001	E-G		
15 vs. 50	40.94	34.49 to 47.39	Yes	****	<0.0001	E-H		
20 vs. 25	6.855	0.4053 to 13.30	Yes	*	0.0331	F-G		
20 vs. 50	8.096	1.646 to 14.55	Yes	**	0.0091	F-H		
25 vs. 50	1.241	-5.209 to 7.690	No	ns	0.9969	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	88.14	11.86	1.863	3	3	9.002	16
0 vs. 5	100.0	63.49	36.51	1.863	3	3	27.71	16
0 vs. 10	100.0	55.16	44.84	1.863	3	3	34.04	16
0 vs. 15	100.0	39.91	60.09	1.863	3	3	45.62	16

0 vs. 20	100.0	7.059	92.94	1.863	3	3	70.56	16
0 vs. 25	100.0	0.2046	99.80	1.863	3	3	75.76	16
0 vs. 50	100.0	-1.036	101.0	1.863	3	3	76.70	16
1 vs. 5	88.14	63.49	24.65	1.863	3	3	18.71	16
1 vs. 10	88.14	55.16	32.98	1.863	3	3	25.04	16
1 vs. 15	88.14	39.91	48.23	1.863	3	3	36.62	16
1 vs. 20	88.14	7.059	81.08	1.863	3	3	61.55	16
1 vs. 25	88.14	0.2046	87.94	1.863	3	3	66.76	16
1 vs. 50	88.14	-1.036	89.18	1.863	3	3	67.70	16
5 vs. 10	63.49	55.16	8.337	1.863	3	3	6.329	16
5 vs. 15	63.49	39.91	23.59	1.863	3	3	17.91	16
5 vs. 20	63.49	7.059	56.43	1.863	3	3	42.84	16
5 vs. 25	63.49	0.2046	63.29	1.863	3	3	48.05	16
5 vs. 50	63.49	-1.036	64.53	1.863	3	3	48.99	16
10 vs. 15	55.16	39.91	15.25	1.863	3	3	11.58	16
10 vs. 20	55.16	7.059	48.10	1.863	3	3	36.51	16
10 vs. 25	55.16	0.2046	54.95	1.863	3	3	41.72	16
10 vs. 50	55.16	-1.036	56.19	1.863	3	3	42.66	16
15 vs. 20	39.91	7.059	32.85	1.863	3	3	24.94	16
15 vs. 25	39.91	0.2046	39.70	1.863	3	3	30.14	16
15 vs. 50	39.91	-1.036	40.94	1.863	3	3	31.08	16
20 vs. 25	7.059	0.2046	6.855	1.863	3	3	5.204	16
20 vs. 50	7.059	-1.036	8.096	1.863	3	3	6.146	16
25 vs. 50	0.2046	-1.036	1.241	1.863	3	3	0.9419	16

Table 24 Data of dose response curve for [CuL_4Cl]

[Inhibitor] vs. normalized response -- Variable slope	
Best-fit values	
IC50	0.08406
HillSlope	-0.9542
logIC50	-1.075
95% CI (profile likelihood)	
IC_{50}	0.02466 to 0.2314
HillSlope	-1.593 to -0.6730
$logIC_{50}$	-1.608 to -0.6357
Goodness of Fit	
Degrees of Freedom	22
R squared	0.9982
Sum of Squares	46.74
Sy.x	1.458
Constraints	
IC_{50}	$IC_{50} > 0$
Number of points	

# of X values	24
# Y values analyzed	24

Table 25 Data of Non-parametric one-way ANOVA of [CuL_4Cl]

Table Analyzed	Data 2				
Data sets analyzed	0-50μM(con)				
ANOVA summary					
F	1273				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9982				
Brown-Forsythe test					
F (DFn, DFd)	1.736 (7, 16)				
P value	0.1708				
P value summary	Ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	25418	7	3631	F (7, 16) = 1273	P<0.0001
Residual (within columns)	45.63	16	2.852		
Total	25464	23			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	24				

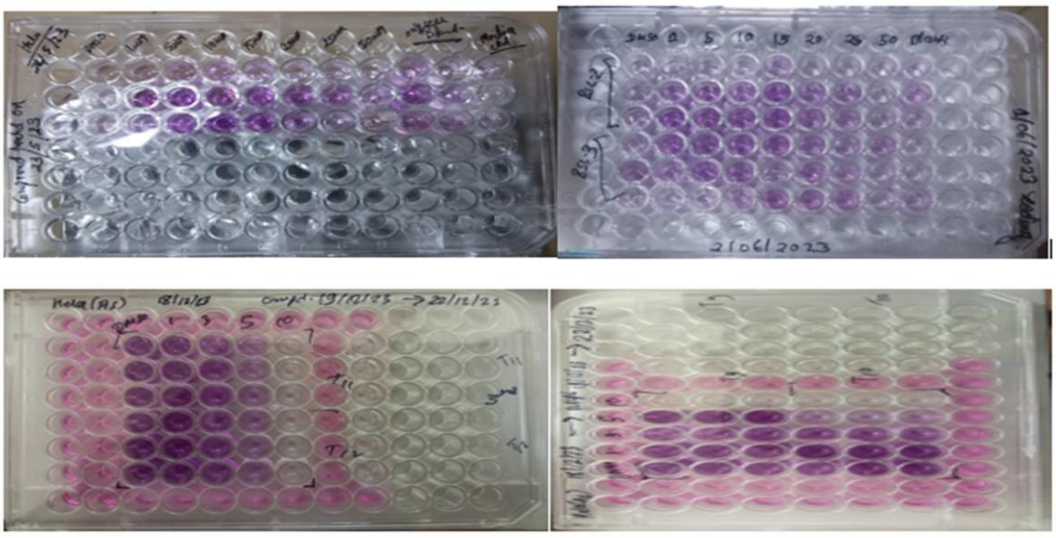
Table 26 Data of Tukeys multiple comparison of [CuL_4Cl]

Number of families	1							
Number of comparisons per family	28							
Alpha	0.05							
Tukey's multiple	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			

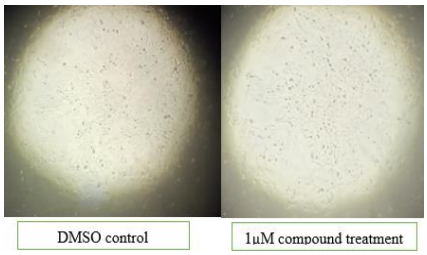
comparisons test								
0 vs. 1	91.41	86.64 to 96.18	Yes	****	<0.0001	A-B		
0 vs. 5	97.93	93.16 to 102.7	Yes	****	<0.0001	A-C		
0 vs. 10	98.76	93.99 to 103.5	Yes	****	<0.0001	A-D		
0 vs. 15	99.32	94.55 to 104.1	Yes	****	<0.0001	A-E		
0 vs. 20	99.63	94.86 to 104.4	Yes	****	<0.0001	A-F		
0 vs. 25	100.0	95.26 to 104.8	Yes	****	<0.0001	A-G		
0 vs. 50	99.50	94.72 to 104.3	Yes	****	<0.0001	A-H		
1 vs. 5	6.521	1.747 to 11.30	Yes	**	0.0043	B-C		
1 vs. 10	7.355	2.581 to 12.13	Yes	**	0.0013	B-D		
1 vs. 15	7.914	3.140 to 12.69	Yes	***	0.0006	B-E		
1 vs. 20	8.221	3.447 to 13.00	Yes	***	0.0004	B-F		
1 vs. 25	8.622	3.849 to 13.40	Yes	***	0.0002	B-G		
1 vs. 50	8.087	3.313 to 12.86	Yes	***	0.0005	B-H		
5 vs. 10	0.8333	-3.941 to 5.607	No	ns	0.9983	C-D		
5 vs. 15	1.392	-3.381 to 6.166	No	ns	0.9661	C-E		
5 vs. 20	1.700	-3.074 to 6.474	No	ns	0.9100	C-F		
5 vs. 25	2.101	-2.673 to 6.875	No	ns	0.7848	C-G		
5 vs. 50	1.566	-3.208 to 6.340	No	ns	0.9388	C-H		
10 vs. 15	0.5592	-4.215 to 5.333	No	ns	0.9999	D-E		
10 vs. 20	0.8666	-3.907 to 5.640	No	ns	0.9978	D-F		
10 vs. 25	1.268	-3.506 to 6.042	No	ns	0.9795	D-G		
10 vs. 50	0.7326	-4.041 to 5.506	No	ns	0.9992	D-H		
15 vs. 20	0.3075	-4.466 to 5.081	No	ns	>0.9999	E-F		
15 vs. 25	0.7087	-4.065 to 5.483	No	ns	0.9994	E-G		
15 vs. 50	0.1734	-4.600 to 4.947	No	ns	>0.9999	E-H		
20 vs. 25	0.4012	-4.373 to 5.175	No	ns	>0.9999	F-G		
20 vs. 50	-0.1341	-4.908 to 4.640	No	ns	>0.9999	F-H		
25 vs. 50	-0.5353	-5.309 to 4.239	No	ns	>0.9999	G-H		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
0 vs. 1	100.0	8.590	91.41	1.379	3	3	93.75	16
0 vs. 5	100.0	2.069	97.93	1.379	3	3	100.4	16
0 vs. 10	100.0	1.236	98.76	1.379	3	3	101.3	16
0 vs. 15	100.0	0.6764	99.32	1.379	3	3	101.9	16
0 vs. 20	100.0	0.3690	99.63	1.379	3	3	102.2	16
0 vs. 25	100.0	-0.03227	100.0	1.379	3	3	102.6	16
0 vs. 50	100.0	0.5031	99.50	1.379	3	3	102.0	16
1 vs. 5	8.590	2.069	6.521	1.379	3	3	6.688	16
1 vs. 10	8.590	1.236	7.355	1.379	3	3	7.543	16
1 vs. 15	8.590	0.6764	7.914	1.379	3	3	8.117	16
1 vs. 20	8.590	0.3690	8.221	1.379	3	3	8.432	16
1 vs. 25	8.590	-0.03227	8.622	1.379	3	3	8.843	16
1 vs. 50	8.590	0.5031	8.087	1.379	3	3	8.294	16
5 vs. 10	2.069	1.236	0.8333	1.379	3	3	0.8546	16

5 vs. 15	2.069	0.6764	1.392	1.379	3	3	1.428	16
5 vs. 20	2.069	0.3690	1.700	1.379	3	3	1.743	16
5 vs. 25	2.069	-0.03227	2.101	1.379	3	3	2.155	16
5 vs. 50	2.069	0.5031	1.566	1.379	3	3	1.606	16
10 vs. 15	1.236	0.6764	0.5592	1.379	3	3	0.5735	16
10 vs. 20	1.236	0.3690	0.8666	1.379	3	3	0.8889	16
10 vs. 25	1.236	-0.03227	1.268	1.379	3	3	1.300	16
10 vs. 50	1.236	0.5031	0.7326	1.379	3	3	0.7513	16
15 vs. 20	0.6764	0.3690	0.3075	1.379	3	3	0.3153	16
15 vs. 25	0.6764	-0.03227	0.7087	1.379	3	3	0.7269	16
15 vs. 50	0.6764	0.5031	0.1734	1.379	3	3	0.1778	16
20 vs. 25	0.3690	-0.03227	0.4012	1.379	3	3	0.4115	16
20 vs. 50	0.3690	0.5031	-0.1341	1.379	3	3	0.1375	16
25 vs. 50	-0.03227	0.5031	-0.5353	1.379	3	3	0.5490	16

Pictures of doing laboratory work

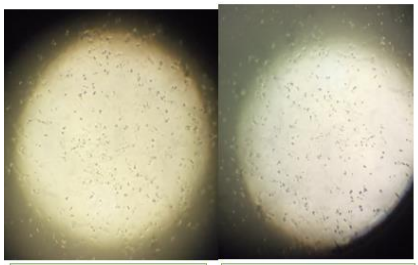


MTT assay result after treatment of DMSO reagent



DMSO control

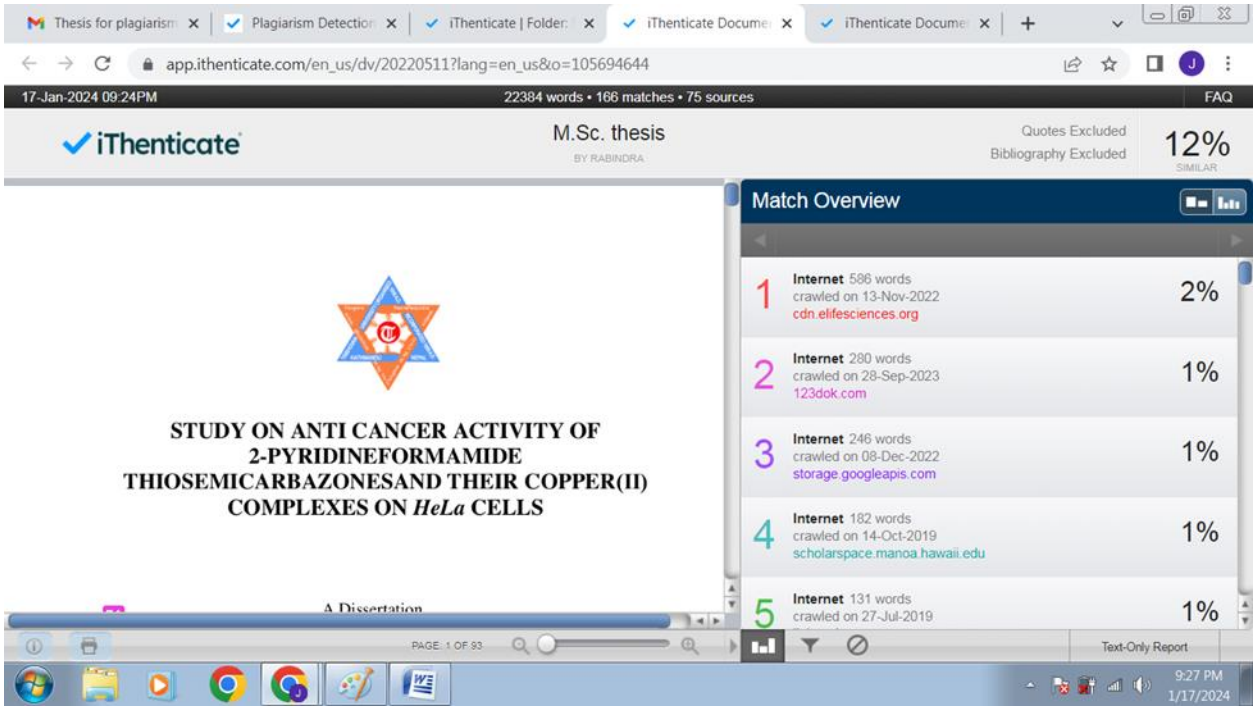
1uM compound treatment



5µM compound treatment

10 µM compound treatment

Figure:Glimpse of doing lab work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