INVESTIGATION OF THE EFFECT OF AURANOFIN, CISPLATIN, AND GLIPTINS ON THE NEUROLYSIN CATALYTIC ACTIVITY

by

MANAR ALJARRAH

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE IN BIOLOGY

2025

Oakland University
Rochester, Michigan

Thesis Advisory Committee:

Vardan T. Karamyan, Ph.D., Chair
Roman Dembinski, Ph.D.
Luis Villa Diaz, Ph.D.

© Copyright by Manar Aljarrah, 2025
All rights reserved

To my mother and father

ACKNOWLEDGMENTS

I would like to express my most profound appreciation and gratitude to the following individuals for their support and encouragement throughout this journey.

First and foremost, thank you to Dr. Vardan T. Karamyan for your guidance and support throughout my research. I am feeling grateful and honored to work under your mentorship.

In addition, I would like to thank my thesis committee members, Dr. Roman Dembinski and Dr. Luis Villa Diaz, for their insightful feedback and valuable contributions. I also appreciate the collaboration of the members of the Dr. Karamyan lab. I would like to extend special thanks to Dr. Heba Elzorkany for the production and purification of recombinant neurolysin and thimet oligopeptidase used in this study, and for supporting the cell culture work.

I sincerely thank Oakland University for their support, guidance, and the invaluable opportunities that made my academic journey an amazing learning experience and School of Medicine, and NIH – for funding and support.

I would also like to thank my family, especially my parents, for their emotional support.

Lastly, I would like to extend my gratitude to my husband and son for their love, patience, and support throughout this journey.

Manar Aljarrah

ABSTRACT

INVESTIGATION OF THE EFFECT OF AURANOFIN, CISPLATIN, AND GLIPTINS ON THE NEUROLYSIN CATALYTIC ACTIVITY

by

Manar Aljarrah

Advisor: Vardan T. Karamyan, Ph.D.

Neurolysin is a zinc metallopeptidase belonging to the M3 family of endopeptidases. It can be found in different cellular components, such as the cytosol, mitochondria, and plasma membrane. It plays a crucial role in breaking down neurotoxic and cerebrotoxic neuropeptides. Neurolysin plays an important role in stroke and some cancer types. Enhancing neurolysin is deemed to be a promising therapeutic approach for stroke, while inhibiting neurolysin is believed to be a potential therapeutic approach for specific types of cancer.

This study aimed to evaluate the effects of various approved drugs, such as auranofin, cisplatin, alogliptin, linagliptin, sitagliptin, and vildagliptin, on the activity of neurolysin and related enzymes. The majority of experiments were carried out using fluorometric enzyme assays and recombinant proteins. Our results show that auranofin selectively inhibits neurolysin at nanomolar concentrations, potentially offering a previously unrecognized mechanism of action for this drug.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ACE	Angiotensin-Converting Enzyme
ACE2	Angiotensin-Converting Enzyme 2
AKT	Protein kinase B
AML	Acute Myeloid Leukemia
ASK1	Apoptosis Signaling Kinase 1
CO2	Carbon Dioxide
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DPP-4	Dipeptidyl Peptidase-4
FBS	Fetal Bovine Serum
GLP-1	Glucagon-Like Peptide-1
IC_{50}	Half-maximal Inhibitory Concentration
JNK	c-JUN N-terminal kinase
MAPK	Mitogen-Activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEP	Neprilysin
Nln	Neurolysin
PMSF	Phenylmethylsulfonyl fluoride

PTEN	Phosphatase and Tensin Homolog
QFS	Quenched Fluorescent Substrates
ROS	Reactive Oxygen Species
SH-SY5Y	Human neuroblastoma cell line
TOP	Thimet Oligopeptidase
Trx	Thioredoxin
TrxRs	Thioredoxin Reductases

CHAPTER ONE

INTRODUCTION

Neurolysin (Nln) is a peptidase that is also referred to as EC 3.4.24.16. This endopeptidase is one of the M3 family of zinc metallopeptidases that was discovered first by Frederic Checler and Emer Ferro (Checler & Ferro, 2018).

The zinc metallopeptidase family has two essential characteristics; one of them is that it has a zinc ion in the active site. This zinc ion is necessary for its catalytic activity. Also, it contains His-Glu-Xaa-Xaa-His (HEXXH) motif (where X = any amino acid residue) in its sequence, which is essential for forming part of the binding site for the zinc metal cofactor (Shrimpton et al., 2002; Brown et al., 2001). The molecular weight of Nln is 70–75 kDa, and it exists as a monomeric protein and can be inhibited by metal chelators and dithiothreitol (Dauch et al., 1995).

Nln is found in various mammalian tissues, with the highest expression levels in the brain and liver, and depending on the cell type, it is primarily localized in the cytosol, mitochondria, and plasma membrane, and can be secreted. Nln functions most effectively at a neutral pH. The primary function of Nln is the hydrolysis of bioactive peptides in the extracellular environment and mitochondria (Shrimpton et al., 2002; Teixeira et al., 2018).

Among most studied extracellular substrates of Nln are bradykinin, neurotensin, substance P, hemopressin, angiotensin I/II, dynorphin-A (1–8), and metorphamide (Rashid et al., 2014; Mentlein & Dahms, 1994). All of these peptides are hydrolyzed and inactivated by Nln except for angiotensin I, metorphamide, and dynorphin-A(1–8) (Rashid et al.,

2014). Nln converts inactive precursor peptide angiotensin I to bioactive peptide angiotensin-(1–7) and converts metorphamide and dynorphin-A (1–8) to Met- and Leu-enkephalins, respectively (Rashid et al., 2014; Karamyan, 2019; Karamyan, 2021).

Nln acts as a compensatory and neuroprotective mechanism following a stroke by reducing excitotoxicity, oxidative stress, edema formation, blood-brain barrier permeability, and neuroinflammation. It achieves this by inactivating neuro/cerebrotoxic peptides such as bradykinin, substance P, and neurotensin, and generating neuro/cerebroprotective peptides, such as angiotensin-(1–7), Leu- and Met-enkephalins (Rashid et al., 2014; Karamyan, 2019; Jayaraman et al., 2020).

Experimental studies showed that after ischemia, Nln expression is increased in primary cortical neurons in culture and in the mouse brain, impacted by stroke, suggesting its role in the response of the brain to ischemia (Rashid et al., 2014; Rashid et al., 2010).

Another study showed that inhibiting Nln with a specific inhibitor, in mice following experimental stroke (i.e., middle cerebral artery occlusion, MCAO), leads to worsening of outcomes, including increased infarct size, edema, blood-brain barrier disruption, and higher levels of pro-inflammatory cytokines. This is accompanied by the elevation of Nln substrates neurotensin, substance P, and bradykinin in the brain. Conversely, overexpressing Nln in the mouse brain using an adeno-associated viral vector (AAV2/5-CAG-Nln) before stroke provided cerebroprotection by lowering infarct volume, brain edema, and inflammation, and was accompanied by reduced levels of Nln substrate peptides (Jayaraman et al., 2020).

When it comes to neurological disorders, in addition to stroke, Nln was also hypothesized to play a role in the pathogenesis of traumatic brain injury (Karamyan, 2019) and Alzheimer's disease (Teixeira et al., 2018). Notably, these ideas have not been experimentally tested, though there is strong experimental evidence linking the activity of Nln to the function of mitochondria and Alzheimer's disease (Teixeira et al., 2018).

Amyloid beta peptide (Aβ) is an important contributing factor to the development of Alzheimer's disease. Aβ is a neurotoxic peptide that aggregates and accumulates in the brain mitochondria of Alzheimer's patients. Nln can break and degrade Aβ peptide (Teixeira et al., 2018). This study opens a new door in the treatment of Alzheimer's disease by using a small molecular modulator of Nln in order to increase Nln activity to block the formation of Aβ (Teixeira et al., 2018; Qi & Yao, 2021).

In addition, Nln was found to be overexpressed in acute myeloid leukemia (AML) (Mirali et al., 2020). Inhibition of Nln by a specific inhibitor caused a selective cytotoxic effect on AML cells without any harmful effect on normal hematopoietic cells. Additionally, it was found that Nln plays a crucial role in the formation of respiratory chain super complexes, which play a major role in oxidative metabolism, the major way AML cells generate energy. When Nln is inhibited, the respiratory chain super-complexes will not form properly, and mitochondria will be unable to produce energy. As a result of that, AML cells can't get enough energy to survive and grow. These findings highlight that inhibiting Nln could be a promising target for AML treatment (Mirali et al., 2020; Mirali & Schimmer, 2020).

Collectively, these experimental studies suggest that Nln is involved in pathogenic mechanisms of several disorders and that it may serve as a new therapeutic target for the treatment of these disorders. On the other hand, when it comes to the development of new drugs, the modulation of Nln activity is rarely considered a possibility, whether as an additional mechanism of action or an off-target (Maciag & Karamyan, 2025a; Maciag & Karamyan, 2025b). To this end, the aim of this study was to evaluate the effect of several FDA-approved drugs, including auranofin, cisplatin, alogliptin, linagliptin, sitagliptin, and vildagliptin, on the catalytic activity of Nln. The choice of these drugs was based on our knowledge about the mechanism of action of these drugs and the catalytic machinery of Nln.

Auranofin, with the brand name Ridaura, is an oral gold-containing anti-inflammatory medication that was approved by the FDA for the treatment of rheumatoid arthritis in 1985 (Wu et al., 2019; Englinger et al., 2019). It belongs to the so-called chrysotherapeutic agents, most common of which are gold(I) thiolates. The early chrysotherapeutic agents, including gold sodium thiomalate (Myochrysine) and gold sodium thioglucose (Solganol) were water-soluble and administered parenterally (Crooke et al., 1986). Discovery of auranofin replaced these early-generation drugs because it could be administered orally. The chemical structure of auranofin is 1-Thio-β-D-glucopyranosato triethylphosphine gold-2,3,4,6-tetraacetate. Looking at its chemical structure, auranofin has monomeric structure consisting of a single gold(I) center bound linearly to two ligands: one being 1-thio-β-D-glucose-2,3,4,6-tetraacetate and the other being triethylphosphine (Figure 1) (Zoppi et al., 2020).

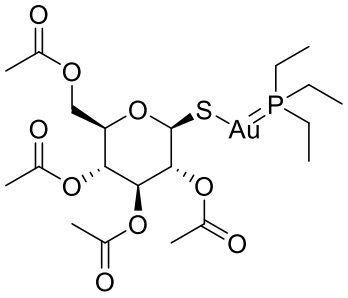

Figure 1. Chemical structure of auranofin. This image was created using ChemDraw software.

Auranofin is a prodrug, which means that it is metabolized and converted into an active drug after administration. The aurothioglucose moiety in the drug complex, which contains a sulfur donor group, is responsible for the drug's water solubility, allowing it to dissolve and circulate in the biological system. On the other hand, the other part, triethyl phosphine, which contains a phosphine ligand, makes the drug lipophilic, helping it in the crossing of cell membranes. Following oral administration and absorption, the thioglucose tetraacetate part of the molecule undergoes oxidation and hydrolysis, causing progressive deacetylation. As result, two active forms are formed – triethylphosphine-gold(I) cation and gold(I)-thioglucose species with different numbers of acetyl groups (Kupiec et al., 2019).

Auranofin and other gold(I) compounds have a high affinity for ligands containing sulfur and selenium in their structure. Proteins that have accessible side chains, such as cysteine and selenocysteine, are the perfect target for auranofin and similar drugs. The binding of these drugs to proteins results in the formation of strong gold–protein adducts, which can impact the protein's function (Nobili et al., 2010). In case of auranofin, it is the gold-triethylphosphine fragment that primarily reacts with surface-exposed cysteine and selenocysteine-containing proteins (Zoppi et al., 2020; Nobili et al., 2010).

The primary anti-rheumatoid mechanism of auranofin is its action as a pro-oxidant, disrupting the thiol-redox homeostasis in cells. Among the most studied targets is inhibition of thioredoxin reductases (TrxRs), which are selenoenzymes – TrxR1 located in the cytosol, and TrxR2, located in the mitochondria. TrxRs are involved in maintaining the cellular redox balance, working as an antioxidant defense system, regulating the levels of reactive oxygen species (ROS), and protecting cells from oxidative damage (Roder & Thomson, 2015; Saikolappan et al., 2019).

TrxRs reduce oxidized Trx **(**thioredoxin) protein, which contains a disulfide bond (S-S), by transferring electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to the oxidative Trx and breaking the disulfide bond to form reduced Trx with a free thiol group. The reduced form of Trx functions as a reducing agent, donating electrons and interacting with other oxidized cysteines in different proteins. In this process, Trx is oxidized again. Thus, the thioredoxin system regulates and controls the reduction of oxidized cysteine residues in proteins, thereby regulating the function of these proteins (Karlenius & Tonissen, 2010; Mohammadi et al., 2019; Onodera et al., 2019). Auranofin interacts with the thiol group of TrxRs and Trx, leading to their inhibition.

Among several functions, Trx1 is involved in the regulation of cell growth and proliferation via increasing AKT activity and inhibiting phosphatase and tensin homolog (PTEN) (Meuillet et al., 2004). In addition, Trx1 can translocate from the cytoplasm to the nucleus and activate specific transcription factors, such as the tumor suppressor p53 and NF-κB (Mohammadi et al., 2019). Inhibition of TrxR1 by auranofin suppresses these molecular mechanisms, resulting in anti-inflammatory and anti-rheumatoid effects.

Notably, inhibition of TrxR1 and TrxR2 by auranofin, and subsequent cytotoxicity and cell death, are also responsible for the off-label use of this drug as an anti-cancer agent. For example, Trx1 functions to prevent cell death by binding to apoptosis signaling kinase 1 (ASK1); this binding prevents ASK1 activation, and as a result, inhibits apoptosis. When Trx1 is oxidized by ROS, oxidized Trx1 dissociates from ASK1, leading to its activation. Activated ASK1 triggers cell death via mitogen-activated protein kinase (MAPK) signaling pathways, including c-JUN N-terminal kinase (JNK) and p38 MAP kinase (Saikolappan et al., 2019). Thus, inhibition of TrxR1 by auranofin leads to increased levels of ROS and oxidative stress, and activation of ASK1. This triggers JNK and p38 MAPK pathways that are responsible for inducing apoptosis, reducing cells' ability to survive, grow, and proliferate through reducing AKT activity and decreasing transcription mediated by p53 and NF-kB (Abdalbari & Telleria, 2021).

Research shows that TrxR1/Trx1 are overexpressed in a specific type of cancer, such as breast, colorectal, lung, pancreatic, ovarian, and gastric cancer. This overexpression is closely related to cancer development and poor disease prognosis. Based on these observations, auranofin is considered a promising anticancer molecule (Onodera et al., 2019; Gasdaska et al., 1994; Lim et al., 2012).

In addition, auranofin may cause cytotoxicity via the disruption of protein homeostasis (Abdalbari & Telleria, 2021), a mechanism observed detected in HepG2 liver hepatocellular carcinoma cells and MCF-7 breast cancer cells (Liu et al., 2014). This study demonstrated that the cytotoxic effect of auranofin is due to inhibiting proteasome-associated deubiquitinases (DUBs), which play a vital role in the growth of cells and the progression

of cancer (Pfoh et al., 2015). Notably, the same mechanism was also documented in chronic myeloid leukemia (Chen et al., 2014).

The second drug investigated in our study is cisplatin, which was used as a control for auranofin. While cisplatin is not a gold-containing drug, it contains another transition metal – platinum, and similar to auranofin, it is a pro-drug. After metabolic activation, cisplatin (Pt(II) form reacts with DNA and thiol-con-taining proteins, leading to anticancer effects. In our study, cisplatin was used as a com-parison to auranofin, since there are no other gold-containing FDA-approved drugs. Our studies with cisplatin showed a lack of significant effect on the activity of Nln and related enzymes, confirming that the cytotoxic effect of cisplatin does not involve Nln modula-tion.

Cisplatin is also referred to as *cis*-diamminedichloridoplatinum(II), the first platinum-based metal agent used in chemotherapy for the treatment of cancer. Cisplatin was approved by the FDA in the 1970s and is used for the treatment of several types of solid cancer, such as lung, head and neck, testicular, ovarian, and other types of cancer (Ghosh, 2019).

Cisplatin is a coordination complex with the chemical formula *cis*-$[Pt(NH_3)_2Cl_2]$, consisting of a central platinum atom with two ammine ligands and two chloride ligands. The ammonia and chloride ligands, arranged in the "cis" configuration, form a square planar geometry around the platinum atom (Figure 2) (Brown et al., 2019).

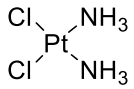

Figure 2. Chemical structure of cisplatin. This image was created using ChemDraw software.

Cisplatin contains a platinum atom that makes covalent bonds with DNA's purine bases, primarily guanine and adenine. This binding results in the formation of intra-strand and inter-strand crosslinks, which cause damage to the DNA structure and lead to strand breaks. Repairing this damage is difficult, leading to the accumulation of DNA, RNA, and protein damage, which consequently triggers cell death and apoptosis. Cisplatin is considered a DNA-damaging agent that induces cell death through various mechanisms, including direct mitochondrial toxicity and the formation of DNA cross-links (Rottenberg et al., 2021; Wang & Lippard, 2005; Kelland, 2007). In addition, similar to auranofin, cisplatin interacts with thiol groups of various proteins, including TrxR1, leading to its inactivation (Arnér et al., 2001).

While cisplatin is effective in treating various cancers, its use is associated with a high rate of morbidity and mortality associated with the described mechanism of action (Sung et al., 2021).

Lastly, in this study, we investigated a family of drugs known as gliptins - dipeptidyl peptidase 4 (DPP-4) inhibitors – used for the treatment of diabetes mellitus (DM). Since no information is available on whether any of the currently approved DPP-4 inhibitors were tested against Nln and associated peptidases during their development in pharmacological selectivity screens, we carried out focused experiments to answer this question.

DPP-4, also known as dipeptidyl-peptidase IV, peptidase 3.4.14.5, or CD26, is a serine peptidase involved in hydrolytic processing of proline-containing peptides in the body (Pereira et al., 2003). DPP-4 is most known for its hydrolysis of glucagon-like peptide-1 (GLP-1), which is a major peptide playing a crucial role in regulating blood glucose lev-

els. GLP-1 regulates glucose levels immediately after meal administration by stimulating insulin secretion, inhibiting glucagon release, delaying gastric emptying, and increasing satiety. Inhibition of DPP-4 by gliptins leads to increased levels of GLP-1, and this approach has emerged as a reliable approach to treating type 2 diabetes (Capuano et al., 2013; Drucker & Nauck, 2006). These drugs, such as alogliptin, linagliptin, sitagliptin, and vildagliptin, also lower blood pressure, lead to modest weight loss, reduce inflammatory markers and oxidative stress, and improve postprandial lipemia, providing a broad range of cardiovascular benefits (Scheen, 2013). These associated effects are most likely due to the inhibition of the hydrolysis of other bioactive peptides by DPP-4.

All gliptins are similar in their mechanism of action but differ in chemical structure, as shown in Figures 3, 4, 5, and 6, which translates to differences in pharmacokinetic properties, including metabolism, distribution, absorption, excretion, and pharmacodynamic effects. These differences result in variations in their dose, duration of action, and potency, and hence suitability for a specific patient.

Sitagliptin is a beta-amino acid-based DPP-4 inhibitor with the brand name Januvia, which was the first gliptin agent approved by the FDA in 2006. The recommended dosage is 100 mg once daily. Sitagliptin is highly selective toward DPP-4 without affinity to other DPP enzymes (DPP-8 and DPP-9) (Gallwitz, 2007; Ahrén, 2019).

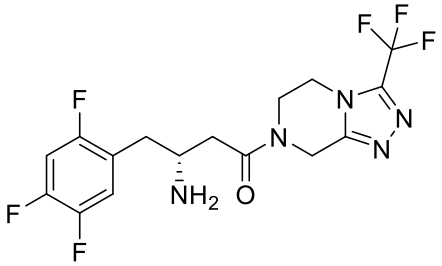

Figure 3. Chemical structure of sitagliptin. This image was created using ChemDraw software.

Vildagliptin is a cyanopyrrolidine-based DPP-4 inhibitor with the brand name Galvus, approved in the European Union in 2007; however, it has not been approved by the FDA. Vildagliptin is a highly selective inhibitor of DPP-4, with a stronger affinity for DPP-4 compared to DPP-8 and DPP-9. The recommended dosage is 50 mg twice daily or once, depending on the medication it is combined with. If it is used in combination with metformin or a thiazolidinedione, the dose is 50 mg twice daily. However, when used in combination with a sulfonylurea, the recommended dosage is 50 mg once daily. This drug has a neutral effect on weight, with a low risk of hypoglycemia (Mathieu & Degrande, 2008; Scheen, 2012; Keating, 2014).

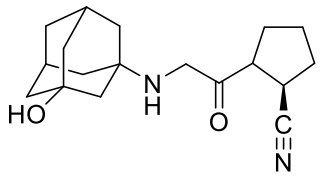

Figure 4.Chemical structure of vildagliptin. This image was created using ChemDraw software.

Linagliptin is a xanthine-based DPP-4 inhibitor with the brand name Tradjenta, approved by the FDA in 2011. It is the most selective and highly potent agent in the gliptin family. Linagliptin has a 40,000-fold higher affinity for DPP-4 than DPP-8 and a 10,000-fold higher affinity for DPP-4 than DPP-9. The recommended dosage is 5 mg once daily. Linagliptin is the only DPP-4 inhibitor that is eliminated by the non-renal route (Gallwitz, 2013; Mahfuz ul Alam et al., 2023).

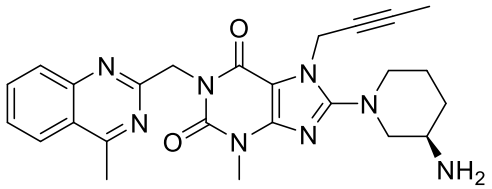

Figure 5. Chemical structure of linagliptin. This image was created using ChemDraw software.

Alogliptin is a quinazolinone-based DPP-4 inhibitor with the brand name Nesina. It is a highly selective and potent gliptin. Alogliptin has a 10,000-fold higher affinity for DPP-4 than DPP-2, DPP-8, and DPP-9. The recommended dosage is 25 mg once daily (Scott, 2010).

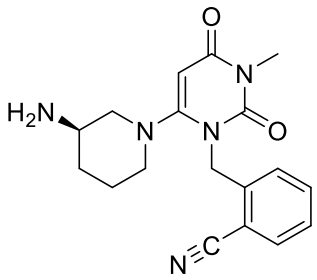

Figure 6. Chemical structure of alogliptin. This image was created using ChemDraw software.

CHAPTER TWO

METHODS

ENZYME ASSAYS USING RECOMBINANT PROTEINS

The effect of the drugs discussed above on the activity of Nln and associated enzymes was measured in fluorometric enzymatic assays used in our publications (Jayaraman et al., 2021; Rahman et al., 2021; Esfahani et al., 2022a). In brief, a fixed concentration of recombinant rat or human Nln (0.3 or 0.5 nM final assay) was dissolved in artificial cerebrospinal fluid (aCSF; NaCl 126 mM, $NaHCO_3$ 26 mM, KCl 3 mM, KH_2PO_4 1.4 mM, HEPES 25 mM, glucose 4 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 1.4 mM, $ZnSO_4$ 0.2 μM, pH 7.2) containing Triton X-100 (0.01% final assay concentration), and preincubated with 0.3 nM – 10 μM concentrations of auranofin for 10 minutes at 37 °C in a 96-well plate. The enzymatic reaction was started by the addition of a fluorogenic substrate for Nln (Mca-Pro-Leu-Gly-Pro-D-Lys(Dnp)-OH) in a final assay volume of 100 μL. Cisplatin, alogliptin, linagliptin, sitagliptin, and vildagliptin were tested at 0.1 μM – 300 μM final assay concentrations. The enzymatic reaction was continuously monitored for 20 minutes, while measuring fluorescence once per minute. Control wells contained the vehicle used to dissolve the drugs (aCSF). All experimental samples were tested in duplicates. Each assay was repeated four times to ensure reproducibility and accuracy of the data.

The activity of recombinant human thimet oligopeptidase (TOP) was measured in the same way as for Nln, except that dithiothreitol was present at a final assay concentration of 0.1 mM (Jayaraman et al., 2021). The activities of recombinant human ACE, NEP, and ACE2 were measured similarly to Nln, except that different fluorogenic substrates were

used. For ACE2, we used Mca-Ala-Pro-Lys(Dnp), and for ACE and NEP, we used Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (Jayaraman et al., 2021; Esfahani et al., 2022a). Rat and human Nln, and human TOP were produced in-house using our established protocols (Wangler et al., 2016; Jayaraman et al., 2021). Recombinant human NEP, ACE and ACE2 were purchased from R&D Systems.

CELL CULTURE AND TREATMENT OF THE CELLS WITH AURANOFIN

The SH-SY5Y cell line was used to evaluate the effect of auranofin on Nln activity. The SH-SY5Y cell line is a human neuroblastoma cell line widely used in biomedical research, both as a model for brain cancer cells and neuronal cells (Yusuf et al., 2013; Lopez-Suarez et al., 2022).

SH-SY5Y cells were purchased from ATCC, expanded to the 2nd and 3rd generation, and cryopreserved following ATCC protocols. For our experiments, the frozen SH-SY5Y cells were thawed in a 37 °C water bath for 2 minutes, resuspended in pre-warmed growth media consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, seeded in a T-75 flask, and cultured in a humidified incubator at 37 °C in the atmosphere air supplemented with 5% CO_2. The medium was renewed every 2–3 days until ~80% confluency, followed by subculturing in 6-well plates or 100 mm dishes. At approximately 90% confluency, the cells were treated with auranofin. Before the treatment, the entire well/dish medium was replaced with fresh medium containing 2% FBS, followed by the addition of 50 or 300 µL of auranofin solution to reach a 0.03 µM to 300 µM final concentration.

The well plate/dish was gently rotated to facilitate the diffusion of the drug and placed in the humidified incubator for 1 hour.

Measurement of total cellular Nln activity: After the 1-hour incubation of cells with auranofin in 6-well plates, the cells were scraped gently but quickly and transferred into a microtube. The cells were then centrifuged at 1000 × g for 10 minutes, the supernatant was removed, and the cell pellet was resuspended in 250 µL of aCSF. After resuspending the cells, they were sonicated using a probe sonicator for 1 second, twice at 20% amplitude/intensity. Next, 20 µL of the cell suspension was transferred into a new tube containing 110 µL of aCSF. The enzyme assay was then carried out described above, with the exception that activity of each sample was measured both in the absence and presence of Nln inhibitor Pro-Ile (10 mM final assay concentration), as described in our previous publications (Rashid et al., 2014; Jayaraman et al., 2020).

Measurement of mitochondrial Nln activity: One hour after the auranofin treatment of cells cultured in 100 mm dishes, the culture medium was aspirated to leave ~1 mL, followed by scraping of the cells, centrifugation (2000 × g for 10 min at 6 °C), and removal of the supernatant. Next, the cell pellet was resuspended in 500 µL of hypotonic aCSF (1 part aCSF + 2 parts water), followed by gentle vortexing and another centrifugation at 2000 × g for 10 min at 6 °C. After this second centrifugation, the supernatant was again discarded, and the pellet was resuspended in 500µl of hypotonic aCSF, followed by gentle vortexing. Next, the tube containing the cells was placed in well-packed ice for 10 minutes, followed by sonication for 1 second at 50% amplitude/intensity using a probe sonicator. After sonication, 5 µl of phenylmethylsulfonyl fluoride (PMSF) from a 50 mM

stock solution was added to the suspension, and the tube was immediately vortexed thoroughly. The tube was centrifuged again at 2000 × g for 10 min at 6 °C, and the supernatant was transferred to a new, chilled tube and kept immersed in well-packed ice box. The pellet was resuspended again in 500 µL of hypotonic aCSF, followed by the addition of 5 µL PMSF (50 mM) and immediate vortexing, and centrifugation (2000 × g for 10 min at 6 °C). The supernatant was combined with the prior supernatant, and the tube was centrifuged at 12000 × g for 15 min at 6 °C to separate mitochondria from other organelles. The resulting pellet was resuspended in 500 µl of hypotonic aCSF, followed by sonication in a water bath sonicator for 30 seconds. The tube was vortexed and sonicated once again, and the resulting suspension was used to measure the activity of Nln as described above for the total cellular Nln activity.

DATA ANALYSIS APPROACH

For each enzymatic reaction, the slope of the line that represents the initial velocity (V_0) for the reaction progress curve was calculated in Excel. To calculate IC_{50} values, V_0 values for enzymatic reactions in the presence of varying concentrations of the drugs were expressed as a percentage within each experiment, with 100% being V_0 of the enzymatic reaction in the absence of any drug (i.e., basal activity with vehicle control). IC_{50} values were calculated in GraphPad Prism by fitting $\%V_0$ values into a nonlinear regression model for the three-parameter log(inhibitor) versus response equation. GraphPad Prism was also used to create all graphs. Data are presented as mean ± S.E.M.

CHAPTER THREE

RESULTS

The concentration-dependent effects of auranofin on the catalytic activity of recombinant peptidases are presented in Figure 7. The results show that auranofin inhibits rat and human Nln, with IC_{50} values of 121 nM and 63 nM, respectively. It showed no significant effect on the activity of ACE, ACE2, or NEP, and inhibited TOP only at high concentrations (IC_{50} >10 μM), suggesting >150-fold selectivity in inhibiting human Nln than TOP.

Additionally, our experiments carried out in SH-SY5Y cells showed that auranofin inhibits total cellular Nln with an IC_{50} value of 4.8 μM (Figure 8), whereas the potency for inhibiting mitochondrial Nln activity in the same cells was even lower (IC_{50} = 31 μM; Figure 9).

The concentration-dependent effects of cisplatin on the catalytic activity of recombinant peptidases are presented in Figure 10. Our results indicate that cisplatin is a much less potent Nln inhibitor – IC_{50} values for rat and human Nln are 86.8 μM and 784 μM, respectively. Notably, we did not observe any significant inhibition of TOP, ACE, ACE2, or NEP in the presence of even high cisplatin concentrations (Figure 10).

Lastly, the concentration-dependent effects of gliptins on the catalytic activity of the recombinant peptidases are presented in Figures 11, 12, 13, and 14. Our results indicate that alogliptin, vildagliptin, and sitagliptin have no substantial effect on the activity of Nln and the replated peptidases. In the case of linagliptin, we observed weak inhibition with

some of the enzymes, with calculated IC_{50} values ranging from 270 µM and higher (Figure 14).

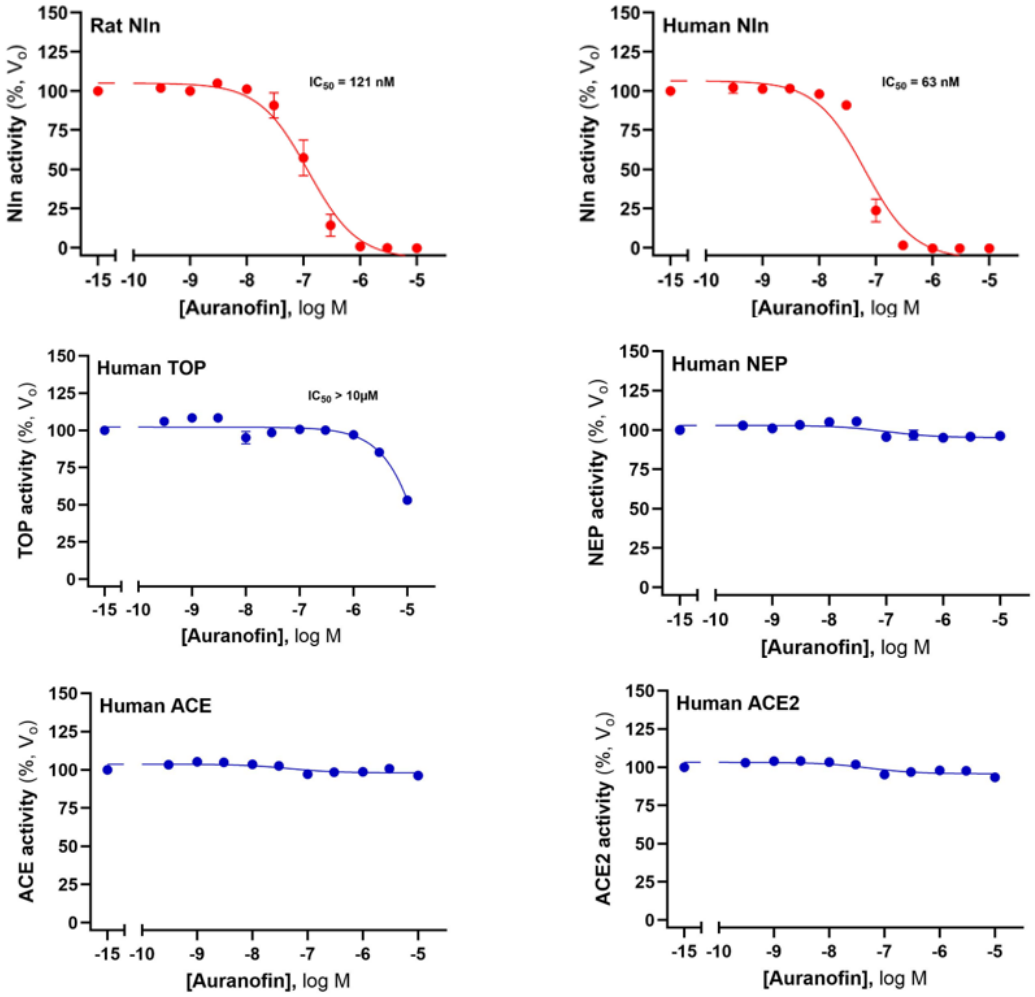

Figure 7. The effect of auranofin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of auranofin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of auranofin is set to 100% on the Y-axis, and −15 on the X-axis. Auranofin inhibited rat and human Nln with IC_{50} values of 121 nM (95% CI: 90.5 - 162 nM) and 63 nM (95% CI: 49 - 82 nM), respectively, while having negligible or no effect on TOP, ACE, ACE2, and NEP.*

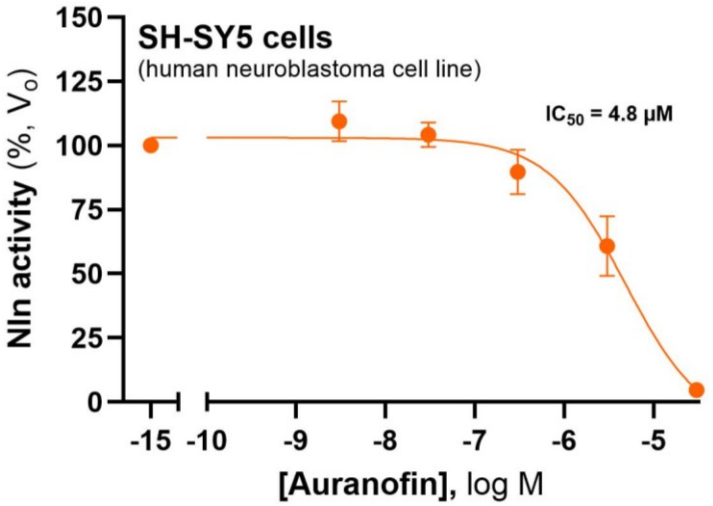

Figure 8. The effect of auranofin on the catalytic activity of total cellular Nln in SH-SY5 cells. *The concentration-dependent effect of auranofin on the hydrolysis of the fluorogenic substrate by Nln in the SH-SY5 cell lysate is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). The initial velocity of the hydrolysis in the absence of auranofin is set to 100% on the Y-axis, and −15 on the X-axis. Auranofin inhibited Nln activity in SH-SY5Y cells with an IC_{50} value of 4.8 µM (95% CI: 2.1 – 12.0 µM).*

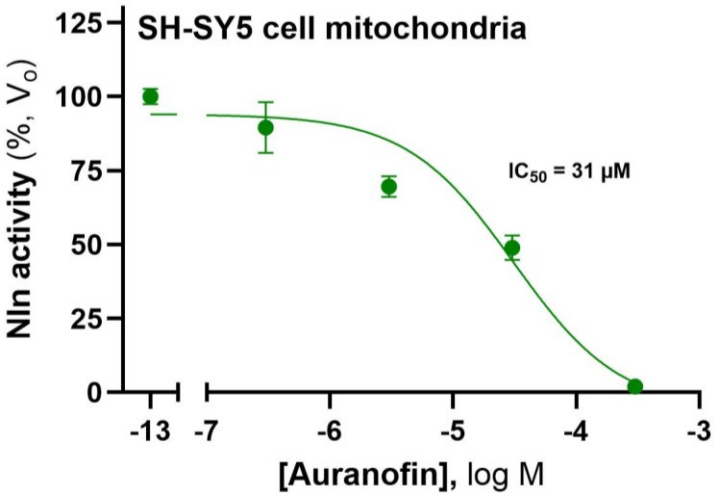

Figure 9. The effect of auranofin on the catalytic activity of mitochondrial Nln in SH-SY5 cells. *The concentration-dependent effect of auranofin on the hydrolysis of the fluorogenic substrate by mitochondrial Nln in the SH-SY5 cells is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). The initial velocity of the hydrolysis in the absence of auranofin is set to 100% on the Y-axis, and −13 on the X-axis. Auranofin inhibited mitochondrial Nln activity with an IC_{50} value of 31 µM (95% CI: 14.4 - 60.8 µM).*

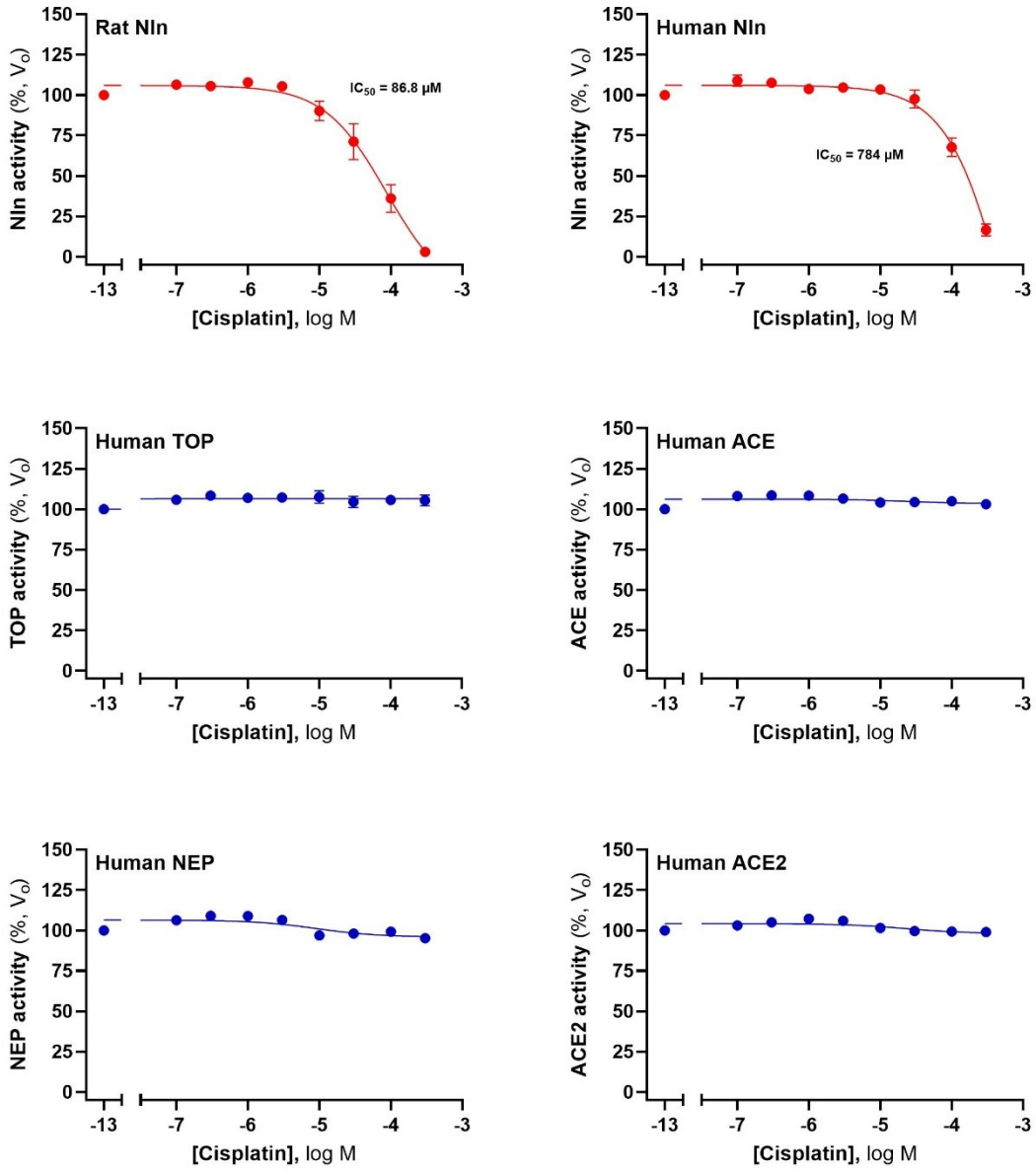

Figure 10. The effect of cisplatin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of cisplatin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of cisplatin is set to 100% on the Y-axis, and −13 on the X-axis. Cisplatin inhibited rat and human Nln with IC_{50} values of 86.8 µM (95% CI: 52.4 - 151 µM) and 784 µM (95% CI: 330 - 1203 µM), respectively, while having no effects on TOP, ACE, ACE2, and NEP.*

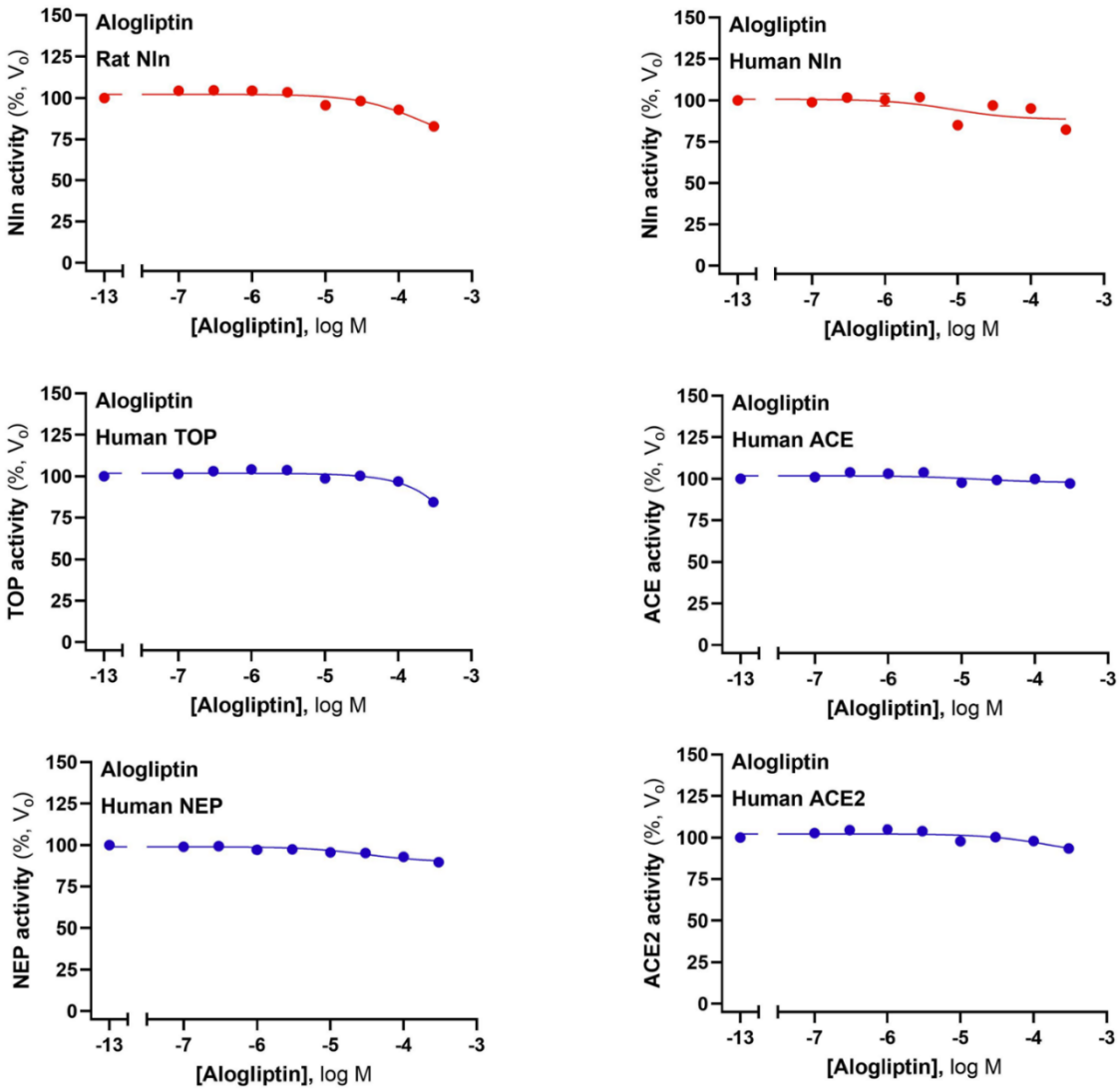

Figure 11. The effect of alogliptin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of alogliptin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of alogliptin is set to 100% on the Y-axis, and −13 on the X-axis. No substantial effect of alogliptin on the activity of Nln, TOP, ACE, ACE2, and NEP was observed.*

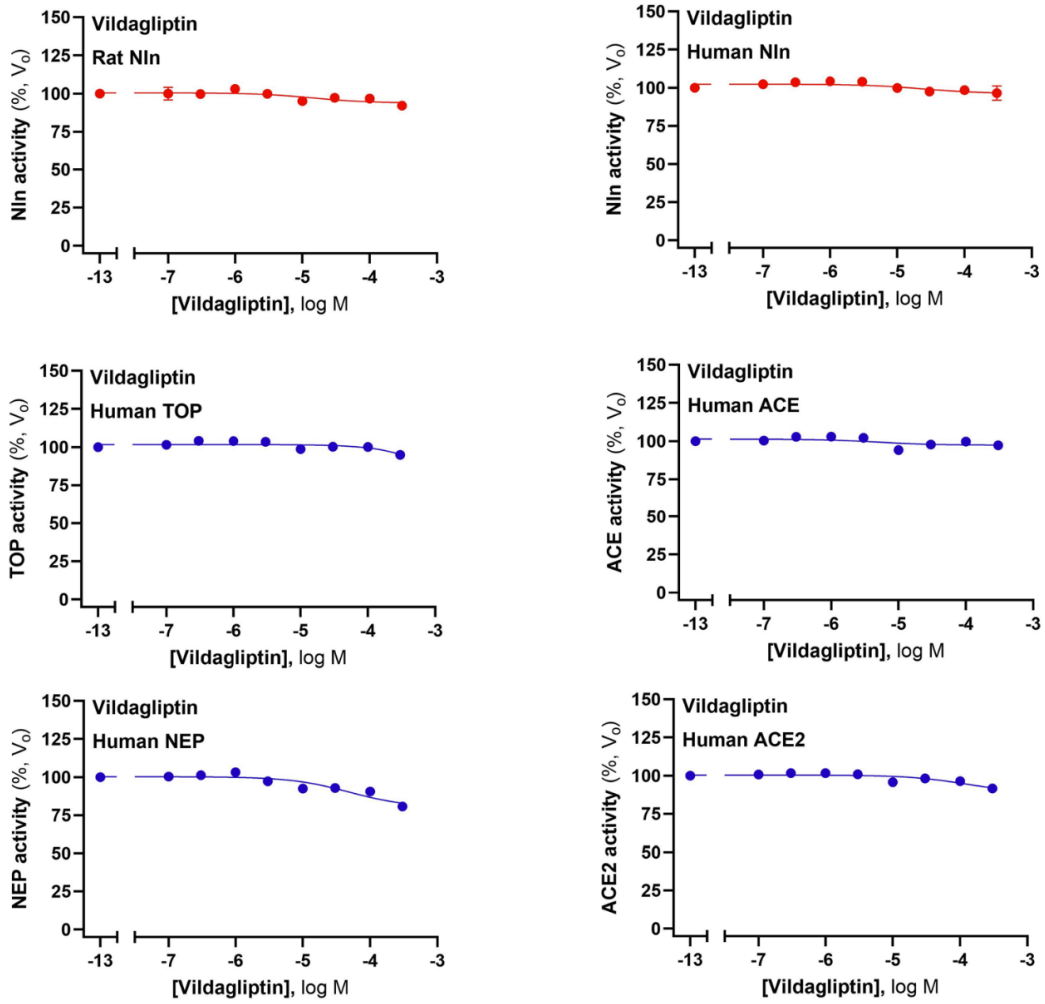

Figure 12. The effect of vildagliptin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of vildagliptin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of vildagliptin is set to 100% on the Y-axis, and −13 on the X-axis. No substantial effect of vildagliptin on the activity of Nln, TOP, ACE, ACE2, and NEP was observed.*

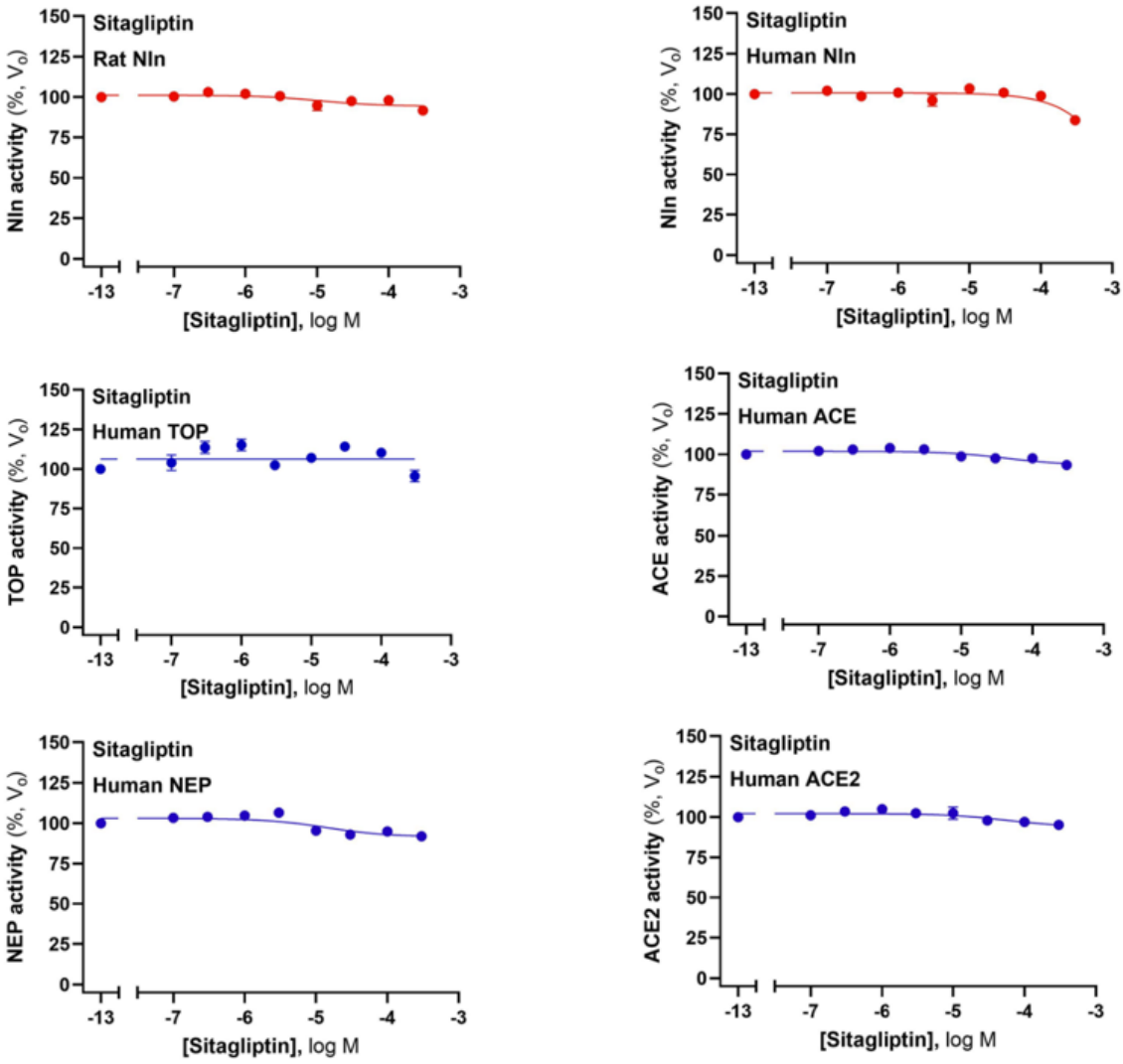

Figure 13. The effect of sitagliptin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of sitagliptin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of sitagliptin is set to 100% on the Y-axis, and −13 on the X-axis. No substantial effect of sitagliptin on the activity of Nln, TOP, ACE, ACE2, and NEP was observed.*

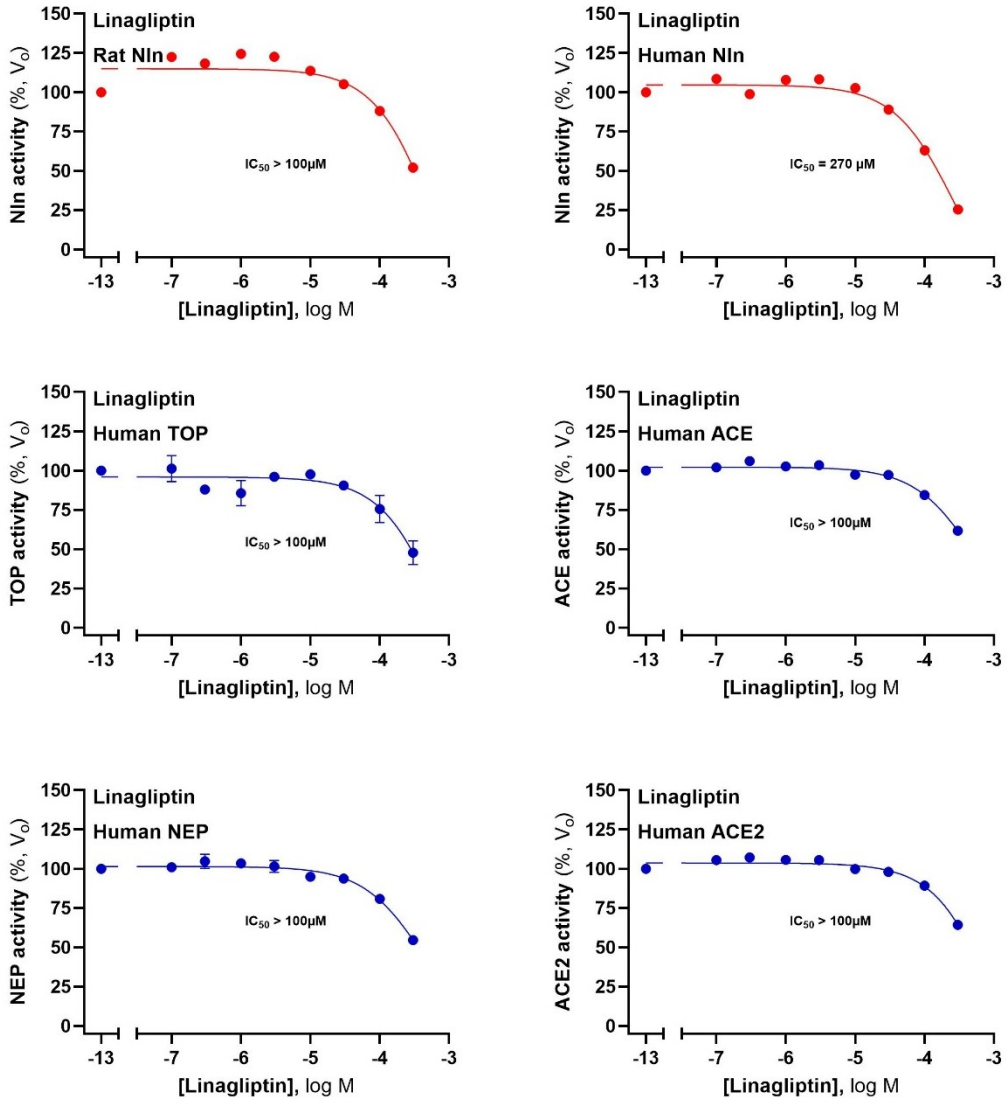

Figure 14. The effect of linagliptin on the catalytic activity of recombinant peptidases. *The concentration-dependent effect of linagliptin on the hydrolysis of the fluorogenic substrate by the respective recombinant peptidase is shown (mean ± SEM, n = 4 independent experiments with duplicate samples for each condition). In all panels, the initial velocity of the hydrolysis in the absence of linagliptin is set to 100% on the Y-axis, and −13 on the X-axis. Linagliptin inhibited human Nln with IC_{50} value of 270 μM (95% CI: 158 - 569 μM), while having negligible or no effect on rat Nln, and human TOP, ACE, ACE2, and NEP.*

CHAPTER FOUR

DISCUSSION

As discussed in detail in the Introduction section of this thesis, several experimental studies have confirmed the involvement of Nln in the pathogenesis of ischemic stroke, certain types of cancer, progression of Alzheimer's disease and traumatic brain injury, and sensation of pain (Rashid et al., 2014; Jayaraman et al., 2020; Mirali & Schimmer, 2020; Teixeira et al., 2018; Karamyan, 2019; Hines et al., 2014). Based on this, Nln is viewed as a new therapeutic target, and efforts have been made to develop both selective inhibitors and enhancers of this enzyme (Karamyan, 2019; Esfahani et al., 2022b; Hines et al., 2014; Checler & Ferro, 2018). In addition, pharmacological selectivity studies employed during the development of new small-molecule drugs rarely take into consideration the possible modulation of Nln activity and its related enzymes (Maciag & Karamyan, 2025a; Maciag & Karamyan, 2025b). To this end, this study was designed to evaluate the effect of several FDA-approved drugs, including auranofin, cisplatin, alogliptin, linagliptin, sitagliptin, and vildagliptin, on the catalytic activity of Nln and related peptidases.

The results of our study indicate that auranofin strongly inhibits recombinant Nln with nanomolar potency (IC_{50} value for rat and human Nln is 121 nM and 63 nM, respectively), while not having substantial effects on related peptidases TOP, ACE, ACE2, and NEP. In experiments carried out in SH-SY5Y cells, we confirmed inhibition of total cellular Nln with a calculated IC_{50} value of 4.8 μM. Lastly, the potency to inhibit mitochondrial Nln in the same cells was recorded to be around 31 μM. The results of the SH-

SY5Y cell experiments are not surprising given the reactivity of auranofin described in the introduction section, and the possible reduction of its available concentrations in the cell, and a further decrease when it reaches the mitochondria. In addition to the competition from other thiol-containing proteins, cellular uptake and intracellular metabolism may also contribute to the documented drop in potency (increasing IC_{50} values) from recombinant protein to cellular Nln to mitochondrial Nln. It is noteworthy that the documented potency of mitochondrial Nln inhibition by auranofin is likely an underestimation. This is because of the technical requirements to prepare mitochondria-enriched preparations for the Nln assay, which takes close to an hour and involves dilutions of the sample, leading to likely underestimation of Nln inhibition.

Nevertheless, our results offer a previously unrecognized mechanism of action for auranofin, which may explain both anticancer (e.g., inhibition of mitochondrial Nln) and perhaps the side effects of this drug. Further studies will be required to expand this discovery and elucidate this new mechanism of auranofin action in vivo.

In parallel to auranofin, we also studied the effects of cisplatin on the activity of Nln and related enzymes TOP, ACE, ACE2, and NEP. While cisplatin is not a gold-containing drug, it contains another transition metal – platinum, and similar to auranofin, it is a prodrug. After metabolic activation, cisplatin (Pt(II) form reacts with DNA and thiol-containing proteins, leading to anticancer effects. In our study, cisplatin was used as a comparison to auranofin, since there are no other gold-containing FDA-approved drugs. Our studies with cisplatin showed a lack of significant effect on the activity of Nln and related enzymes, confirming that the cytotoxic effect of cisplatin does not involve Nln modula-

tion. These results also suggest that our observations with auranofin are likely specific to the gold atom. However, there is a chance that in our assay buffer (aCSF) cisplatin is minimally transformed to an active form and therefore it does not interact with Nln or the rest of the enzymes. Future studies addressing this question are warrantied.

Lastly, we have also studied several gliptins and revealed that they do not affect the catalytic activity of Nln and related enzymes. This new knowledge confirms that gliptins are selective for dipeptidyl-peptidase IV (aka, DPP-4) and that none of the therapeutic or side effects of these drugs can be assigned to inhibition of these peptidases.

Thus, this study shows for the first time a new mechanism of action for auranofin and expands the selectivity profile for cisplatin and several gliptins, confirming that they do not affect the activity of Nln and related enzymes. Future studies will aim to investigate the involvement of Nln in the effects of auranofin in appropriate animal models.

REFERENCES

Abdalbari, F. H., & Telleria, C. M. (2021). The gold complex auranofin: new perspectives for cancer therapy. *Discover Oncology, 12*(1), 42. https://doi.org/10.1007/s12672-021-00439-0

Ahrén, B. (2019). DPP-4 Inhibition and the Path to Clinical Proof. *Frontiers in Endocrinology, 10*, 376. https://doi.org/10.3389/fendo.2019.00376

Arnér, E. S., Nakamura, H., Sasada, T., Yodoi, J., Holmgren, A., & Spyrou, G. (2001). Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by cis-diamminedichloroplatinum (II) and its major metabolite, the glutathione-platinum complex. *Free Radical Biology & Medicine, 31*(10), 1170–1178. https://doi.org/10.1016/s0891-5849(01)00698-0

Brown, A., Kumar, S., & Tchounwou, P. B. (2019). Cisplatin-Based Chemotherapy of Human Cancers. *Journal of Cancer Science & Therapy, 11*(4), 97.

Brown, C. K., Madauss, K., Lian, W., Beck, M. R., Tolbert, W. D., & Rodgers, D. W. (2001). Structure of neurolysin reveals a deep channel that limits substrate access. *Proceedings of the National Academy of Sciences of the United States of America, 98*(6), 3127–3132. https://doi.org/10.1073/pnas.051633198

Capuano, A., Sportiello, L., Maiorino, M. I., Rossi, F., Giugliano, D., & Esposito, K. (2013). Dipeptidyl peptidase-4 inhibitors in type 2 diabetes therapy--focus on

alogliptin. *Drug Design, Development and Therapy, 7*, 989–1001. https://doi.org/10.2147/DDDT.S37647

Checler, F., & Ferro, E. S. (2018). Neurolysin: From Initial Detection to Latest Advances. *Neurochemical Research, 43*(11), 2017–2024. https://doi.org/10.1007/s11064-018-2624-6

Chen, X., Shi, X., Wang, X., & Liu, J. (2014). Novel use of old drug: Anti-rheumatic agent auranofin overcomes imatinib-resistance of chronic myeloid leukemia cells. *Cancer Cell & Microenvironment, 1*(6), e415. https://doi.org/10.14800/ccm.415

Crooke, S. T., Snyder, R. M., Butt, T. R., Ecker, D. J., Allaudeen, H. S., Monia, B., & Mirabelli, C. K. (1986). Cellular and molecular pharmacology of auranofin and related gold complexes. *Biochemical Pharmacology, 35*(20), 3423–3431. https://doi.org/10.1016/0006-2952(86)90608-8

Dauch, P., Vincent, J. P., & Checler, F. (1995). Molecular cloning and expression of rat brain endopeptidase 3.4.24.16. *Journal of Biological Chemistry, 270*(45), 27266-27271. https://doi.org/10.1074/jbc.270.45.27266

Drucker, D. J., & Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet (London, England), 368*(9548), 1696–1705. https://doi.org/10.1016/S0140-6736(06)69705-5

Englinger, B., Pirker, C., Heffeter, P., Terenzi, A., Kowol, C. R., Keppler, B. K., & Berger, W. (2019). Metal Drugs and the Anticancer Immune Response. *Chemical Reviews, 119*(2), 1519–1624. https://doi.org/10.1021/acs.chemrev.8b00396

Esfahani, S. H., Abbruscato, T. J., Trippier, P. C., & Karamyan, V. T. (2022b). Small molecule neurolysin activators, potential multi-mechanism agents for ischemic stroke therapy. *Expert Opinion on Therapeutic Targets, 26*(5), 401–404. https://doi.org/10.1080/14728222.2022.20

Esfahani, S. H., Jayaraman, S., & Karamyan, V. T. (2022a). Is Diminazene an Angiotensin-Converting Enzyme 2 (ACE2) Activator? Experimental Evidence and Implications. *The Journal of Pharmacology and Experimental Therapeutics, 383*(2), 149–156. https://doi.org/10.1124/jpet.122.001339

Gallwitz, B. (2007). Review of sitagliptin phosphate: a novel treatment for type 2 diabetes. *Vascular Health and Risk Management, 3*(2), 203–210. https://doi.org/10.2147/vhrm.2007.3.2.203

Gallwitz, B. (2013). Emerging DPP-4 inhibitors: focus on linagliptin for type 2 diabetes. *Diabetes, Metabolic Syndrome and Obesity : Targets and Therapy, 6*, 1–9. https://doi.org/10.2147/DMSO.S23166

Gasdaska, P. Y., Oblong, J. E., Cotgreave, I. A., & Powis, G. (1994). The predicted amino acid sequence of human thioredoxin is identical to that of the autocrine growth factor human adult T-cell derived factor (ADF): thioredoxin mRNA is

elevated in some human tumors. *Biochimica et Biophysica Acta, 1218*(3), 292–296. https://doi.org/10.1016/0167-4781(94)90180-5

Ghosh, S. (2019). Cisplatin: The first metal based anticancer drug. *Bioorganic Chemistry, 88*, 102925. https://doi.org/10.1016/j.bioorg.2019.102925

Hines, C. S., Ray, K., Schmidt, J. J., Xiong, F., Feenstra, R. W., Pras-Raves, M., de Moes, J. P., Lange, J. H., Melikishvili, M., Fried, M. G., Mortenson, P., Charlton, M., Patel, Y., Courtney, S. M., Kruse, C. G., & Rodgers, D. W. (2014). Allosteric inhibition of the neuropeptidase neurolysin. *The Journal of Biological Chemistry, 289*(51), 35605–35619. https://doi.org/10.1074/jbc.M114.620930

Jayaraman, S., Al Shoyaib, A., Kocot, J., Villalba, H., Alamri, F. F., Rashid, M., Wangler, N. J., Chowdhury, E. A., German, N., Arumugam, T. V., Abbruscato, T. J., & Karamyan, V. T. (2020). Peptidase neurolysin functions to preserve the brain after ischemic stroke in male mice. *Journal of Neurochemistry, 153*(1), 120–137. https://doi.org/10.1111/jnc.14864

Jayaraman, S., Kocot, J., Esfahani, S. H., Wangler, N. J., Uyar, A., Mechref, Y., Trippier, P. C., Abbruscato, T. J., Dickson, A., Aihara, H., Ostrov, D. A., & Karamyan, V. T. (2021). Identification and Characterization of Two Structurally Related Dipeptides that Enhance Catalytic Efficiency of Neurolysin. *The Journal of Pharmacology and Experimental Therapeutics, 379*(2), 191–202. https://doi.org/10.1124/jpet.121.000840

Karamyan, V. T. (2019). Peptidase neurolysin is an endogenous cerebroprotective mechanism in acute neurodegenerative disorders. *Medical Hypotheses, 131*, 109309. https://doi.org/10.1016/j.mehy.2019.109309

Karamyan, V. T. (2021). The role of peptidase neurolysin in neuroprotection and neural repair after stroke. *Neural Regeneration Research, 16*(1), 21–25. https://doi.org/10.4103/1673-5374.284904

Karlenius, T. C., & Tonissen, K. F. (2010). Thioredoxin and Cancer: A Role for Thioredoxin in all States of Tumor Oxygenation. *Cancers, 2*(2), 209–232. https://doi.org/10.3390/cancers2020209

Keating, G. M. (2014). Vildagliptin: a review of its use in type 2 diabetes mellitus. *Drugs, 74*(5), 587–610. https://doi.org/10.1007/s40265-014-0199-3

Kelland, L. (2007). The resurgence of platinum-based cancer chemotherapy. *Nature Reviews. Cancer, 7*(8), 573–584. https://doi.org/10.1038/nrc2167

Kupiec, M., Ziółkowski, R., Massai, L., Messori, L., & Pawlak, K. (2019). The electrochemical profiles of Auranofin and Aubipyc, two representative medicinal gold compounds: A comparative study. *Journal of Inorganic Biochemistry, 198*, 110714. https://doi.org/10.1016/j.jinorgbio.2019.110714

Lim, J. Y., Yoon, S. O., Hong, S. W., Kim, J. W., Choi, S. H., & Cho, J. Y. (2012). Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric

cancer recurrence. *World Journal of Gastroenterology, 18*(39), 5581–5588. https://doi.org/10.3748/wjg.v18.i39.5581

Liu, N., Li, X., Huang, H., Zhao, C., Liao, S., Yang, C., Liu, S., Song, W., Lu, X., Lan, X., Chen, X., Yi, S., Xu, L., Jiang, L., Zhao, C., Dong, X., Zhou, P., Li, S., Wang, S., Shi, X., & Liu, J. (2014). Clinically used antirheumatic agent auranofin is a proteasomal deubiquitinase inhibitor and inhibits tumor growth. *Oncotarget, 5*(14), 5453–5471. https://doi.org/10.18632/oncotarget.2113

Lopez-Suarez, L., Awabdh, S. A., Coumoul, X., & Chauvet, C. (2022). The SH-SY5Y human neuroblastoma cell line, a relevant in vitro cell model for investigating neurotoxicology in human: Focus on organic pollutants. *Neurotoxicology, 92*, 131–155. https://doi.org/10.1016/j.neuro.2022.07.008

Maciag, M., & Karamyan, V. T. (2025a). Enzymes in secondary pharmacology screening panels: is there room for improvement? *Nature Reviews. Drug Discovery, 24*(6), 480–481. https://doi.org/10.1038/s41573-025-01173-w

Maciag, M., & Karamyan, V. T. (2025b). The Missing Enzymes: A Call to Update Pharmacological Profiling Practices for Better Drug Safety Assessment. *Journal of Medicinal Chemistry, 68*(8), 7854–7865. https://doi.org/0.1021/acs.jmedchem.4c02228

Mahfuz ul Alam, A., Mamun, I. R., Nahar, N., & Shoeb, M. (2023). A Quality by Design (QBD) approach for the development and validation of RP-HPLC method for the quantification of linagliptin tablets. *International Journal of Pharmaceutical*

Chemistry and Analysis, 10(4), 281–289. https://doi.org/10.18231/j.ijpca.2023.047

Mathieu, C., & Degrande, E. (2008). Vildagliptin: a new oral treatment for type 2 diabetes mellitus. *Vascular Health and Risk Management, 4*(6), 1349–1360. https://doi.org/10.2147/vhrm.s3005

Mentlein, R., & Dahms, P. (1994). Endopeptidases 24.16 and 24.15 are responsible for the degradation of somatostatin, neurotensin, and other neuropeptides by cultivated rat cortical astrocytes. *Journal of Neurochemistry, 62*(1), 27–36. https://doi.org/10.1046/j.1471-4159.1994.62010027.x

Meuillet, E. J., Mahadevan, D., Berggren, M., Coon, A., & Powis, G. (2004). Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity. *Archives of Biochemistry and Biophysics, 429*(2), 123–133. https://doi.org/10.1016/j.abb.2004.04.020

Mirali, S., & Schimmer, A. (2020). Targeting neurolysin in acute myeloid leukemia. *Molecular & Cellular Oncology, 7*(4), 1761243. https://doi.org/10.1080/23723556.2020.1761243

Mirali, S., Botham, A., Voisin, V., Xu, C., St-Germain, J., Sharon, D., Hoff, F., Qiu, Y., Hurren, R., Gronda, M., Jitkova, Y., Nachmias, B., MacLean, N., Wang, X., Arruda, A., Minden, M., Horton, T., Kornblau, S., Chan, S., Bader, G., & Schimmer, A. (2020). The mitochondrial peptidase, neurolysin, regulates

respiratory chain supercomplex formation and is necessary for AML viability. *Science Translational Medicine, 12*(538), eaaz8264. https://doi.org/10.1126/scitranslmed.aaz8264

Mohammadi, F., Soltani, A., Ghahremanloo, A., Javid, H., & Hashemy, S. I. (2019). The thioredoxin system and cancer therapy: a review. *Cancer Chemotherapy and Pharmacology, 84*(5), 925–935. https://doi.org/10.1007/s00280-019-03912-4

Nobili, S., Mini, E., Landini, I., Gabbiani, C., Casini, A., & Messori, L. (2010). Gold compounds as anticancer agents: chemistry, cellular pharmacology, and preclinical studies. *Medicinal Research Reviews, 30*(3), 550–580. https://doi.org/10.1002/med.20168

Onodera, T., Momose, I., & Kawada, M. (2019). Potential Anticancer Activity of Auranofin. *Chemical & Pharmaceutical Bulletin, 67*(3), 186–191. https://doi.org/10.1248/cpb.c18-00767

Pereira, D. A., Gomes, L., El-Cheikh, M. C., & & Borojevic, R. (2003). Dipeptidyl peptidase IV (CD26) activity in the hematopoietic system: differences between the membrane-anchored and the released enzyme activity. *Brazilian Journal of Medical and Biological Research = Revista Brasileira de Pesquisas Medicas e Biologica, 36*(5), 567–578. https://doi.org/10.1590/s0100-879x2003000500003

Pfoh, R., Lacdao, I. K., & Saridakis, V. (2015). Deubiquitinases and the new therapeutic opportunities offered to cancer. *Endocrine-Related Cancer, 22*(1), T35–T54. https://doi.org/10.1530/ERC-14-0516

Qi, J., & Yao, L. (2021). Modulators of neurolysin: promising agents for the treatment of tumor and neurological diseases. *Medicinal Chemistry Research, 30*(7), 1328–1333. https://doi.org/10.1007/s00044-021-02761-2

Rahman, M. S., Kumari, S., Esfahani, S. H., Nozohouri, S., Jayaraman, S., Kinarivala, N., Kocot, J., Baez, A., Farris, D., Abbruscato, T. J., Karamyan, V. T., & Trippier, P. C. (2021). Discovery of First-in-Class Peptidomimetic Neurolysin Activators Possessing Enhanced Brain Penetration and Stability. *Journal of Medicinal Chemistry, 64*(17), 12705–12722. https://doi.org/10.1021/acs.jmedchem.1c00759

Rashid, M., Arumugam, T. V., & Karamyan, V. T. (2010). Association of the novel non-AT1, non-AT2 angiotensin binding site with neuronal cell death. *The Journal of Pharmacology and Experimental Therapeutics, 335*(3), 754–761. https://doi.org/10.1124/jpet.110.171439

Rashid, M., Wangler, N. J., Yang, L., Shah, K., Arumugam, T. V., Abbruscato, T. J., & Karamyan, V. T. (2014). Functional up-regulation of endopeptidase neurolysin during post-acute and early recovery phases of experimental stroke in mouse brain. *Journal of Neurochemistry, 129*(1), 179–189. https://doi.org/10.1111/jnc.12513

Roder, C., & Thomson, M. J. (2015). Auranofin: repurposing an old drug for a golden new age. *Drugs in R&D, 15*(1), 13–20. https://doi.org/10.1007/s40268-015-0083-y

Rottenberg, S., Disler, C., & Perego, P. (2021). The rediscovery of platinum-based cancer therapy. *Nature Reviews. Cancer, 21*(1), 37–50. https://doi.org/10.1038/s41568-020-00308-y

Saikolappan, S., Kumar, B., Shishodia, G., Koul, S., & Koul, H. K. (2019). Reactive oxygen species and cancer: A complex interaction. *Cancer Letters, 452*, 132–143. https://doi.org/10.1016/j.canlet.2019.03.020

Scheen, A. J. (2012). A review of gliptins in 2011. *Expert Opinion on Pharmacotherapy, 13*(1), 81–99. https://doi.org/10.1517/14656566.2012.642866

Scheen, A. J. (2013). Cardiovascular effects of gliptins. *Nature Reviews. Cardiology, 10*(2), 73–84. https://doi.org/10.1038/nrcardio.2012.183

Scott, L. J. (2010). Alogliptin: a review of its use in the management of type 2 diabetes mellitus. *Drugs, 70*(15), 2051–2072. https://doi.org/10.2165/11205080-000000000-00000

Shrimpton, C. N., Smith, A. I., & Lew, R. A. (2002). Soluble metalloendopeptidases and neuroendocrine signaling. *Endocrine Reviews, 23*(5), 647–664. https://doi.org/10.1210/er.2001-0032

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians, 71*(3), 209–249. https://doi.org/10.3322/caac.21660

Teixeira, P. F., Masuyer, G., Pinho, C. M., Branca, R. M., Kmiec, B., Wallin, C., Wärmländer, S. K., Berntsson, R. P., Ankarcrona, M., Gräslund, A., Lehtiö, J., Stenmark, P., & Glaser, E. (2018). Mechanism of Peptide Binding and Cleavage by the Human Mitochondrial Peptidase Neurolysin. *Journal of Molecular Biology, 430*(3), 348–362. https://doi.org/10.1016/j.jmb.2017.11.011

Wang, D., & Lippard, S. J. (2005). Cellular processing of platinum anticancer drugs. *Nature Reviews. Drug Discovery, 4*(4), 307–320. https://doi.org/10.1038/nrd1691

Wangler, N. J., Jayaraman, S., Zhu, R., Mechref, Y., Abbruscato, T. J., Bickel, U., & Karamyan, V. T. (2016). Preparation and preliminary characterization of recombinant neurolysin for in vivo studies. *Journal of Biotechnology, 234*, 105–115. https://doi.org/10.1016/j.jbiotec.2016.07.007

Wu, B., Yang, X., & Yan, M. (2019). Synthesis and Structure-Activity Relationship Study of Antimicrobial Auranofin against ESKAPE Pathogens. *Journal of Medicinal Chemistry, 62*(17), 7751–7768. https://doi.org/10.1021/acs.jmedchem.9b00550

Yusuf, M., Leung, K., Morris, K. J., & Volpi, E. V. (2013). Comprehensive cytogenomic profile of the in vitro neuronal model SH-SY5Y. *Neurogenetics, 14*(1), 63–70. https://doi.org/10.1007/s10048-012-0350-9

Zoppi, C., Messori, L., & Pratesi, A. (2020). ESI MS studies highlight the selective interaction of Auranofin with protein free thiols. *Dalton Transactions*

(Cambridge, England : 2003), 49(18), 5906–5913.

https://doi.org/10.1039/d0dt00283f