NORTRIPTYLINE, A TRICYCLIC ANTIDEPRESSANT, INDUCES APOPTOSIS IN U266 MULTIPLE MYELOMA CELL LINE

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ABSTRACT

NORTRIPTYLINE, A TRICYCLIC ANTIDEPRESSANT, INDUCES APOPTOSIS IN U266 MULTIPLE MYELOMA CELL LINE

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Multiple Myeloma (MM), the second most common hematological malignancy, accounts for approximately 1% of all new cancer cases and deaths worldwide. Although the success of chemotherapy treatment has been significantly increased with the introduction of new generation drugs, bortezomib, thalidomide and lenalidomide, MM is still an incurable disease with a high rate of relapse. As exemplified by thalidomide, drug repurposing has been proved to be an effective strategy to meet the urgent need for novel anticancer agents for MM treatment.

In addition to their primary clinical use for the treatment of depression, several tricyclic antidepressants (TCA) also act as potent antitumor agents. Among these, nortriptyline (NTP), was shown to have an inhibitory effect on osteosarcoma, prostate, melanoma and bladder cancer cells. In this work, we investigated the anticancer effect and mechanism of NTP on U266 MM cell line. We first studied NTP`s *in vitro* inhibitory effect at various doses and time points. Combination potential of cisplatin-NTP pair was also investigated as part of the potency

examination. Next, cell cycle analysis was performed using propidium iodide staining which was followed by three flow cytometric apoptosis assays.

NTP showed dose and time-dependent inhibitory effect on U266 MM cell line. It had greater inhibitory effect on U266 cells than cisplatin (cis) based on the calculated IC_{50} values (26.1 μM vs 39.8 μM). Cis-NTP combination indicated strong antagonism which may have significant clinical relevance since antidepressants are commonly employed in adjuvant therapy for cancer patients. NTP arrested U266 cells at G2/M phase. It also induced apoptosis as indicated by mitochondrial membrane potential, caspase-3 and Annexin V assays. Based on these preliminary findings, therapeutic potential of NTP for MM treatment should be investigated with in-depth mechanistic studies and *in vivo* experiments.

Keywords: tricyclic antidepressant, cisplatin, combination chemotherapy, cancer, drug repurposing, apoptosis, mitochondria

ÖZ

BİR TRİSİKLİK ANTİDEPRESAN OLAN NORTRİPTİLİN'İN U266 MULTİPL MİYELOM HÜCRE HATTINDAKİ APOPTOTİK ETKİSİ

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Bütün hematolojik maligniteler arasında Multipl Miyeloma (MM) en yaygın ikinci tiptir. Dünya genelinde ortaya çıkan yeni kanser vakalarının ve ölümle sonuçlanan kanser vakalarının %1'ini MM oluşturmaktadır. Kemoterapide kullanılan bortezomib, talidomid ve lenalidomid gibi yeni nesil ilaçların sağladığı başarıya rağmen MM hala relaps olasılığı yüksek, tedavisi olmayan bir hastalıktır. Talidomid örneğinde de görüldüğü gibi ilaçların yeniden konumlandırılması (drug repurposing or repositioning) MM tedavisi için gerekli yeni anti-kanser ilaçlara olan ihtiyacı karşılayabilecek efektif bir stratejidir.

Trisiklik antidepresanların (TSA) klinikte esas kullanım amacı depresyon tedavisi olmakla beraber bir kısmı aynı zamanda antitümor potansiyeline sahip ilaçlardır. Bunlardan biri olan Nortriptilin'in (NTP) osteosarkoma, prostat, melanoma ve mesane kanserleri üzerinde büyümeyi inhibe edici etkileri daha önce yapılan calışmalarda gösterilmiştir. Bu çalışmada bir Multipl Miyeloma kanseri hücre hattı olan U266 üzerinde NTP'nin antikanser etki ve mekanizması araştırılmıştır. NTP'nin *in vitro* olarak çeşitli dozlardaki inhibe edici etkileri zamana bağlı gösterilmiştir.

Kombinasyon potansiyeli çalışmalarında Sisplatin (cis) ve NTP ikilisi kullanılmıştır. Hücre döngüsü analizi ve sonrasında yapılan üç ayrı apopitoz çalışması akış sitometri (flow cytomety) tekniğiyle yapılmıştır. NTP, U266 MM hücre hattı üzerinde doz ve zamana bağlı inhibisyon etkisi göstermiştir. Bu etki, yapılan IC_{50} çalışmalarına göre kemoterapide kullanılmakta olan cis'ten daha güçlü olduğu belirlenmiştir (26 μM'a karşı 39 μM). Cis-NTP kombinasyonu güçlü bir antagonistik etkiye sahiptir. Kanser hastalarında antidepresanların yardımcı terapi amacıyla sıkça kullanılmaları sebebiyle bu sonuçlar klinik açıdan oldukça önemlidir. NTP hücre döngüsünü G2/M fazında durdurmaktadır. Aynı zamanda mitokondri membran potansiyeli, Kaspaz-3 ve Anneksin-V testleri ile apoptozu indüklediği belirlenmiştir. Elde ettiğimiz öncü verilere bakıldığında MM'nin tedavisinde kullanılmasi amacıyla NTP'nin etki mekanizması daha derinlemesine çalışılmalı ve *in vivo* çalışmalar yapılmalıdır.

Anahtar Kelimeler: trisiklik antidepresan, cisplatin, kombinasyon kemoterapi, kanser, ilaç yeniden konumlandırma, apoptoz, mitokondri

To my parents Müberra and Burhan, my sister Güneş and my cats Piraye, Huri and Nuri

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LIST OF SYMBOLS AND ABBREVIATIONS

5-HT: Serotonin receptor

7-AAD: 7-Aminoactinomycin D

CI: Combination Index

Cis: Cisplatin

h: Hour

H1: Antihistamine

IC_{50}: Half Maximal Inhibitory Concentration

KMS11: Human Myeloma Cell Line

LiCl: Lithium chloride

LP1: Human Myeloma Cell Line

mg: Milligram

min: Minute

mL: Milliliter

mM: Milli Molar

MM: Multiple Myeloma

µL: Microliter

µM: Micro Molar

nm: Nanometer

NTP: Nortriptyline

NE: Norepinephrine

PBS: Phosphate Buffer Saline

PE: Phycoerythrin

PI: Propidium Iodide

TCA: Tricyclic Antidepressants

U266: Human Multiple Myeloma Cell Line

CHAPTER 1

INTRODUCTION

1.1. Multiple Myeloma

Multiple myeloma (MM) is a cancer of plasma cells. Plasma cells are responsible for producing antibodies in the immune system. In MM, neoplastic plasma cells are abnormally accumulate too many in the bone marrow. The abnormal plasma cells produce abnormal M antibodies in the bone marrow that can cause MM pathology such as thickness of blood. The neoplastic plasma cells can also promote tumor formation in the bone or tissue [1].

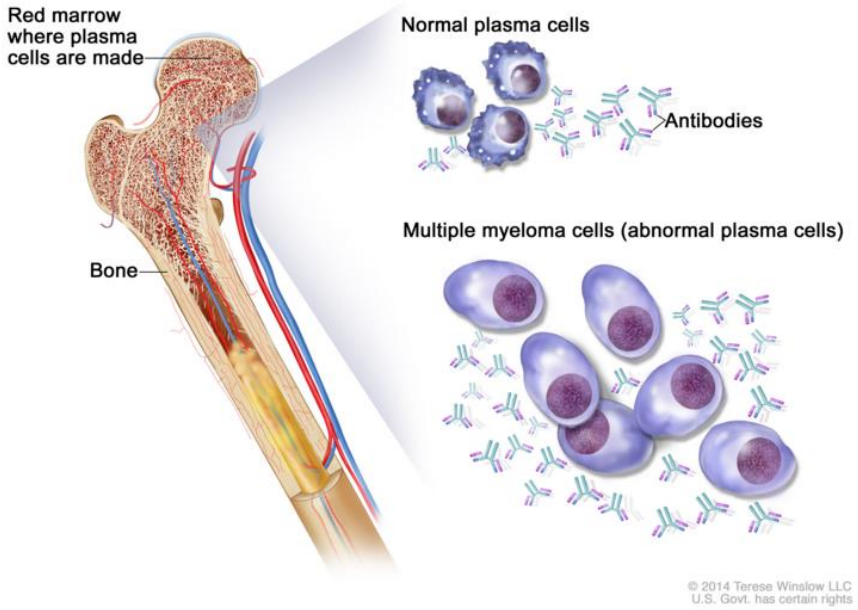

Figure 1. Normal and MM cells comparison in the bone marrow [1].

Progression of the disease could take a long time to cause symptoms and MM is often diagnosed at late stages. One of the most common sign of MM at late stages is bone

pain. The other following symptoms are anemia, numbness, weakness, frequent infections and bleeding [1].

MM is a complex hematological malignancy in which interaction of neoplastic B-cells with the bone marrow microenvironment plays a critical role in the progression of the disease. Pathogenesis include skeletal destruction and extends to other organs and systems such as kidneys, brain and immune system. MM is the second most common blood cancer (10% of all blood cancers) after non-Hodgkin's lymphoma and accounts for approximately 1% of all new cancer cases. The median age of patients at the time of diagnosis is around 65 years [1]. Effective combination chemotherapy including proteasome inhibitors (bortezomib) and immunomodulatory agents (lenalidomid) has significantly increased the survival rate of the patients [2, 3]. However, high rate of relapse especially due to multidrug resistance requires addition of new drugs to existing chemotherapy strategies.

1.2. Drug Repositioning

Development of new cancer agents is an urgent necessity. Traditional drug discovery approach is frequently fail because of various reasons such as high cost, poor safety or bioavailability. Overall steps of drug discovery and development steps take on average 13 years research and cost approximately US$ 2 billion to provide to cancer patients for only one drug. Repositioning of existing drug is an alternative approach as a solution for traditional drug discovery and development. The main advantage of drug repositioning is that the FDA standards such as pharmacodynamics and pharmacokinetic are investigated during Phase I trials. Hence, existing drug could pass Phase II and II trials and the cost reduces significantly comparing the other strategy [4].

Table 1. Original and new anticancer indications of repurposed drugs [4].

Drug	Orgininal Indication	New anticancer indication
Thalidomide	Antiemetic for pregnancy	Multiple myeloma
Aspirin	Analgesic, antipyretic	Colorectal cancer
Valproic acid	Antiepileptic	Leukemia, solid tumors
Celecoxib	Osteoarthritis, rheumatoid arthritis	Colorectal cancer, lung cancer
Statins	Myocardial infarction	Prostate cancer, leukemia
Metformin	Diabetes mellitus	Breast, adenocarcinoma, prostate, colorectal cancer
Rapamycin	Immunosuppressant	Colorectal cancer, lymphoma, leukemia
Methotrexate	Acute leukemia	Osteosarcoma, breast cancer, Hodgkin lymphoma
Zoledronic acid	Anti-bone resorption	Multiple myeloma, prostate cancer, breast cancer
Leflunomide	Rheumatoid arthritis	Prostate cancer
Wortmannin	Antifungal	Leukemia
Minocycline	Acne	Ovarian cancer, glioma
Vesnarinone	Cardioprotective	Oral cancer, leukemia, lymphoma
Thiocolchicoside	Muscle relaxant	Leukemia, multiple myeloma
Nitroxoline	Antibiotic	Bladder, breast cancer
Noscapine	Antitussive, antimalarial, analgesic	Multiple cancer types

1.3. Tricyclic Antidepressants

Tricyclic antidepressants (TCA) belong to a broad class of psychoactive drugs. In 80s, Tricyclic antidepressants were major treatment of depression. Its mechanism of action has been shown in rabbit brain on α-noradergenic receptors. There is a correlation between selective therapeutic and psychomotor action in depression with binding affinities of TCAs (imipramine, clomipramine, amitriptyline, nortriptyline, protriptyline, desipramine) to α-noradrenergic receptors [5].

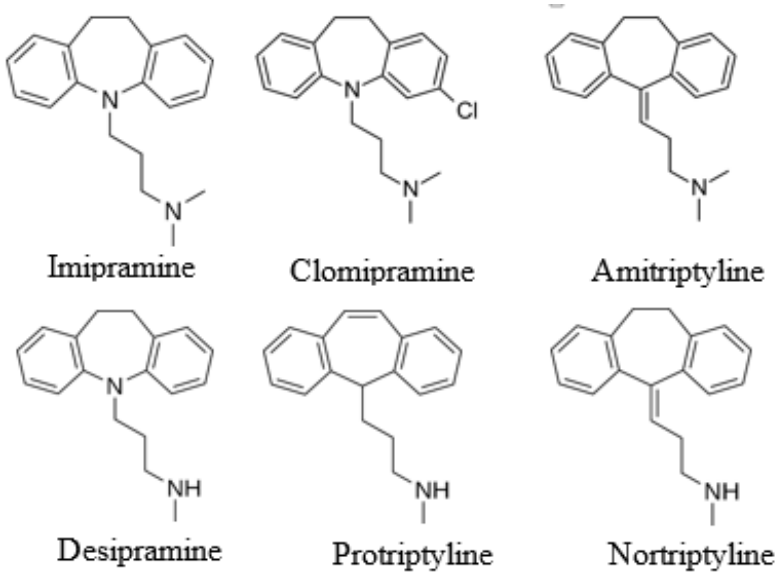

Figure 2. Structure of TCAs.

Past two decade, it was found that TCAs have more than five activities at the same time such as a reuptake of norepinephrine inhibitor activity, a reuptake of serotonin inhibitor activity, an anticholinergic-antimuscarinic activity, an α1-adrenergic antagonist activity, and an antihistamine (H1) activity. Result of overdose usage, they inhibit sodium channels and induce cardiac disorders such as cardiac arrhythmias and seizures. However, TCAs prescribe as reuptake of serotonin and norepinephrine inhibitor for depression treatment [35].

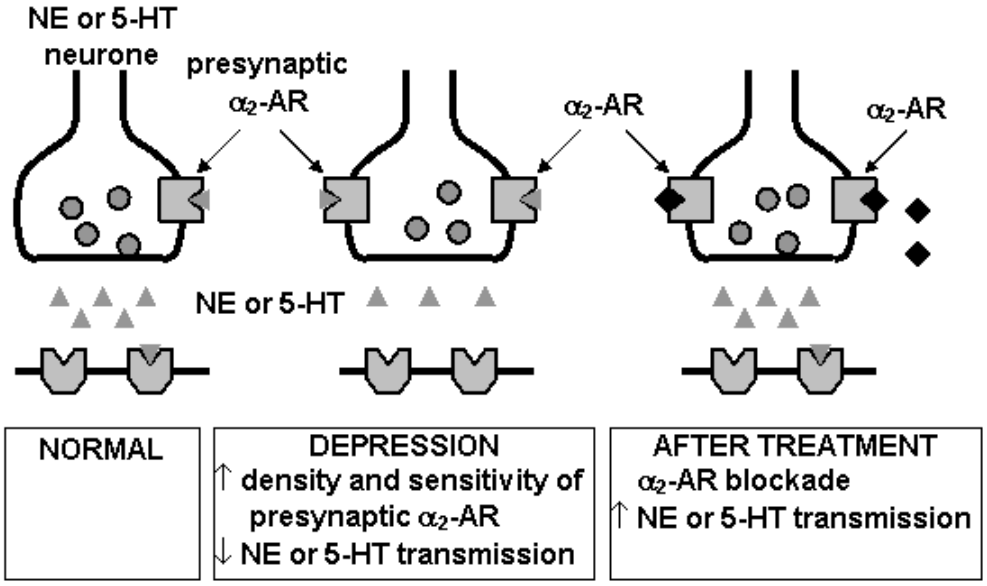

Figure 3. Mechanism of action of TCAs on α_2- adrenergic reseptor (α_2- AR). NORMAL: Norepinephrine (NE) or serotonin neurone in healthy state. DEPRESSION: α-2 adrenergic receptor density and sensitivity of presynaptic increase in NE or 5-HT neurone during depression. AFTER TREATMENT: TCAs blockade the α_2- AR and NE or 5-HT transmission becomes normal [6].

In early studies, it was thought that TCAs has been increased risk of various cancers types. However, further studies showed that TCAs interestingly show potent *in vitro* and *in vivo* anticancer effect on a large variety of tumor cells such as colon, osteosarcoma, prostate, glioma, skin squamous carcinoma and multiple myeloma [7].

1.3.1. Nortriptyline

Nortriptyline (NTP), a classic TCA. It is using for treatment of depressive disorders, pain, irritable bowel disease, insomnia, agitation and insomnia, migraine prophylaxis and sleeping disorders. NTP's pharmacodynamics properties are similar to protriptyline. NTP is dibenzocycloheptene type TCA. NTP is active metabolite of amitriptyline. The various targets of NTP are identified in human [8].

NTP also displays antineoplastic effect on osteosarcoma, prostate, melanoma and bladder cancer [9-12]. Pan et al showed that NTP inhibits proliferation PC3 investigated the therapeutic potential of NTP on bladder cancer and observed cell cycle

arrest, intrinsic and extrinsic apoptosis, increase in reactive oxygen species production and suppression of tumor growth in mice [12].

NTP inhibits opioid receptors, Ca+2-activated K+ channel, priming of human neutrophils, and human cytochrome P450 enzymes [9, 10].

Table 2. List of NTP targets in human [8].

Targets	Action
Sodium-dependent noradrenaline transporter	inhibitor
Sodium-dependent serotonin transporter	inhibitor
5-hydroxytryptamine receptor 2A	antagonist
5-hydroxytryptamine receptor 1A	antagonist
Histamine H1 receptor	antagonist
Alpha-1A adrenergic receptor	antagonist
Alpha-1D adrenergic receptor	antagonist
Muscarinic acetylcholine receptor M1	antagonist
Muscarinic acetylcholine receptor M2	antagonist
Muscarinic acetylcholine receptor M3	antagonist
Muscarinic acetylcholine receptor M4	antagonist
Muscarinic acetylcholine receptor M5	antagonist
5-hydroxytryptamine receptor 2C	antagonist
5-hydroxytryptamine receptor 6	binder
Alpha-1B adrenergic receptor	antagonist
Alpha-2 adrenergic receptor	antagonist
Beta adrenergic receptor	antagonist
D(2) dopamine receptor	antagonist
Sigma receptor	binder
5-hydroxytryptamine receptor 1C	antagonist

Inhibitory effect of NTP determined in various cancer type. NTP has strong affinity with 9 μM IC50 value in human cutaneous melanoma cells when we compared with clomipramine (27 μM) and amitriptyline (33 μM). It also show growth inhibition effect and IC50 calculated as 35 μM on human osteosarcoma cell. NTP induces apoptosis in PC3 cells and IC50 value determined as 450 μM. [12].

1.4 Aim of This Study

Based on previous findings on its antitumor potential and results from our preliminary screening studies, we aimed to investigate NTP`s potency and mechanism of anticancer effect on U266 multiple myeloma cell line.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Clomipramine, imipramine, amitriptyline, maprotiline and mianserin were purchased from Alfa Aesar (Lancashire, United Kingdom). Opipramol, protriptyline, nortriptyline, desipramine and cisplatin from Sigma-Aldrich were obtained from Sigma-Aldrich (Taufkirchen, Germany). Antidepressant and cisplatin solutions were prepared in deionized water and 0.9% NaCl, respectively.

2.2 Cell Culture

Human myeloma cell line U266 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2.5 μg/mL plasmocin prophylactic at 37˚C and 5% CO_2. Drug treatment was done at 10^5 cells/mL cell density in all experiments. Unless specified otherwise, cell harvesting was performed at 400 g for 5 min.

2.3 Cell Viability Assays

Drug cytotoxicity screening, potency (IC_{50}) determination, time-response and combination assays were completed using Promega (Madison, USA) CellTiter-Blue Cell Viability Assay. For each measurement, five technical replicates were prepared. At the end of the treatment time, assay reagent was added and plates were incubated for 4 h. Measurements were taken with Molecular Devices (Sunnyvale, USA) SpectraMax Paradigm fluorescence plate reader at 555 nm excitation and 595 nm

emission settings. IC_{50} values were calculated on GraphPad (La Jolla, USA) Prism v5.0 using non-linear curve fitting. Combination index values were calculated using ComboSyn Inc. (Paramus, USA) CompuSyn software [13].

2.4 Flow Cytometry

Cell cycle analysis and apoptosis assays were carried out on BD Biosciences (San Diego, USA) Accuri C6 flow cytometer. Flow rate was set to medium (35 μL/min) and 10^4 events were recorded for each measurement.

2.5 Cell Cycle Analysis

Cells were treated with 15 μM NTP for 24 h and harvested. After a cold PBS wash, samples were fixed with 70% ethanol and kept for 2 h on ice. Following centrifugation at 800 g for 5 min and PBS wash, cells were stained with propidium iodide (25 μg/mL) in PBS (30 min, 37˚C). Staining solution also contained 3 mg/mL RNAse. Samples were then analyzed on flow cytometer.

2.6 Apoptosis Assays

Cells were treated with 30 μM NTP and analyzed at 12, 24 and 48 h time points with flow cytometry. Samples were prepared as described in the manufacturer`s procedures. To study the effect of NTP treatment on mitochonrial membrane polarization, BD Biosciences (San Diego, USA) MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit was used. Active Caspase-3 and phosphatidylserine detection was achieved with PE Active Caspase-3 Apoptosis Kit and Annexin V Apoptosis Detection Kit I from the same manufacturer.

2.7 Statistical Analysis

For cytotoxicity screening, time-response and Annexin-V assays, statistical significance of results were analyzed using GraphPad Prism one-way ANOVA with

Tukey post test module. Other experiments were evaluated using unpaired t-test with two-tails. Significance of differences were marked on the figures with asterisks.

CHAPTER 3

RESULTS

3.1 Potency Screening of Selected Antidepressants

As a preliminary work for this study, we screened seven tricyclic (imipramine, clomipramine, amitriptyline, opipramol, desipramine, protriptyline, nortriptyline) and two tetracyclic (maprotiline, mianserin) antidepressants for their effect on the viability of U266 multiple myeloma cell line using CellTiter-Blue cell viability assay. The assay principle is based on the ability of live cells to metabolize resazurin reagent into a fluorescent end product (resorufin). Each drug was applied at 100 μM for 24 h. except mianserin, all tested TCAs and maprotiline showed significantinhibitory effect on cell growth (Fig. 4).

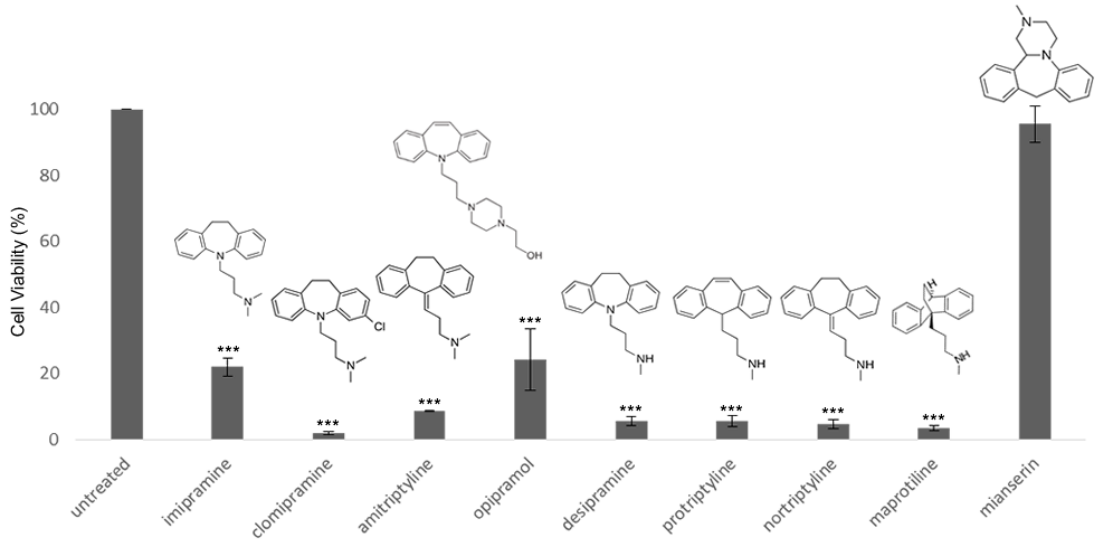

Figure 4. Effect of selected antidepressants (100 μM, 24 h) on viability of U266 cells as determined by CellTiter-Blue cell viability assay. Drug structures are shown above the corresponding bars. Asterisks denote statistical significance at $p<0.0001$ (n=2).

Imipramine and opipramol exhibit relatively weaker inhibition when compared to others. The side chain containing 3-Carbon and N atoms seems to have a critical rolesince its drastic modification (opipramol) or removal (mianserin) diminished the effect on cell viability. Among the positive hits, we selected NTP, which is relatively cheaper and least studied, for further *in vitro* characterization. To our knowledge, this is the first report investigating the mechanism of NTP effect on U266 multiple myeloma cell line.

Nortriptyline shows dose and time-dependent toxicity on U266 cells. Dose and time-dependence of the NTP`s effect on U266 cells were examined in 1 μM to 120 μM range and at 12, 24 and 48 h. We also calculated the half-maximal inhibitory concentration (IC_{50}) at 24 h. Potency of cisplatin (cis), an anticancer drug currently in clinical use for MM treatment, was also determined for comparison. IC_{50} of NTP and cis were determined as 26.14 ± 1.0 and 39.81 ± 9.9 μM from the dose response curves (Fig. 5A and 2B). The solubility problem of cis made it quite difficult to reduce the variation among the biological replicates resulting in a higher error in calculation. Keeping this in mind, NTP seems to be a more potent agent against MM than cis. As expected, longer NTP treatment corresponds to higher inhibitory effect on the cell viability (Fig. 5C).

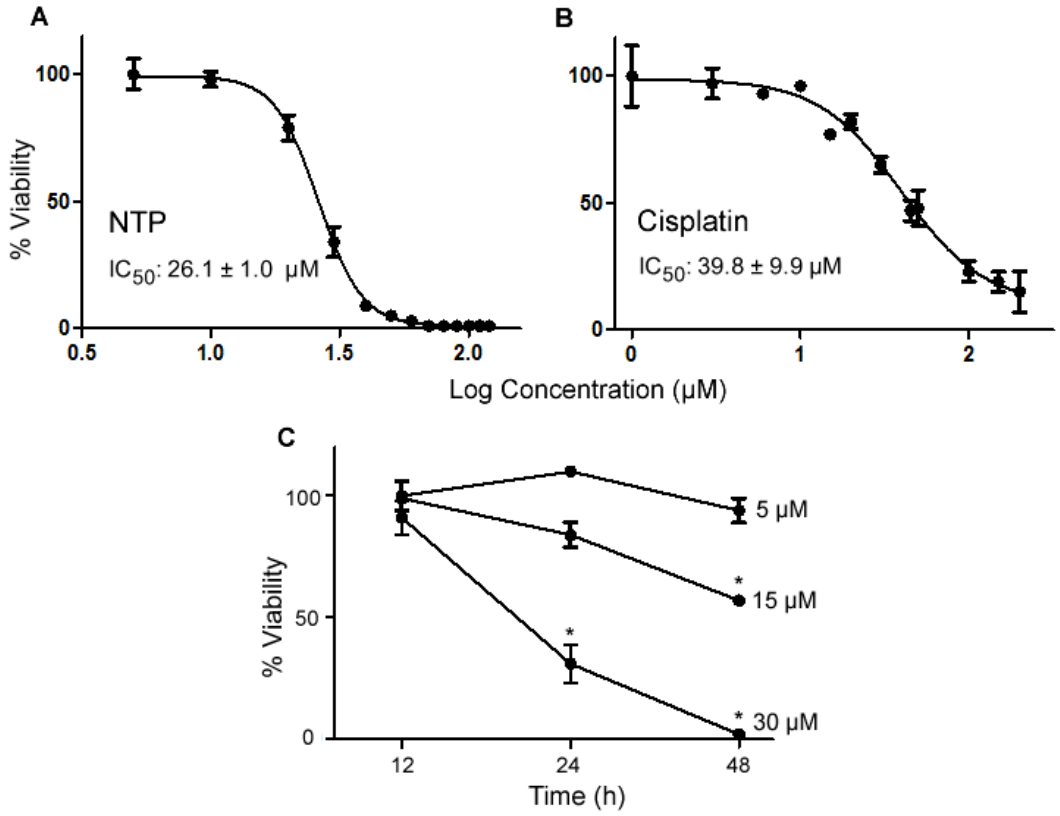

Figure 5. Dose and time response of NTP`s inhibitory effect on U266 cell viability. A) Dose response and potency (IC_{50}) determination of nortriptyline and cisplatin (n=3). Concentration ranges for nortriptyline and cisplatin were 1.0 to 120 µM and 1.0 to 300 µM, respectively. B) Time response (12, 24 and 48 h) of nortriptyline treatment at 5, 15 and 30 µM. Asterisks denote statistical significance at $p<0.001$ (n=2).

3.2 Nortriptyline-Cisplatin Combination is Antagonistic

Cancer treatment efficiency significantly increases when drugs are used in combination. Based on promising anticancer potential of NTP shown in the previous section, we decided to test cis-NTP combination on MM cell viability. Molar ratio of the mixed drugs is a critical factor determining the type (synergestic, antagonist oradditive) and strength of the combination effect [14]. In this preliminary study, we used a simple approach with only four combinations as listed in Table 3.

Table 3. Combination Effect of cisplatin and nortriptyline.

NTP (μM)	Cis (μM)	Combination Cytotoxicity (%)	Combination Index (CI)
6	6	8.1 ± 0.3	1.8 ± 0.2
15	15	26.9 ± 1.6	2.0 ± 0.0
30	30	65.5 ± 1.7	1.7 ± 0.3
45	45	84.7 ± 3.0	1.5 ± 0.2

Inhibitory effect of NTP, cis and cis-NTP combination at 24 h was determined by cell viability assay (n=2). Combination Index (CI) value was calculated from drug cytotoxicity or growth inhibition curves using the computer software CompuSyn. CI < 1 (synergy), CI = 1 (additivity), CI > 1 (antagonism).

Cell viability was measured using CellTiter-Blue assay and results were analyzed on CompuSyn software to determine the Combination Index (CI) values. Interestingly, all four cis-NTP combinations resulted in strong antagonism as indicated by the corresponding CI values.

3.3 Nortriptyline Arrests U266 Cell Cycle at G2/M Phase

Inhibitory effect of NTP on U266 cells may arise from anti-proliferative and/or cytotoxic mechanisms. We first investigated anti-proliferative effect by analyzing the progression of cell cycle with propidium iodide (PI) staining and flow cytometry after NTP treatment (15 μM, 24 h). Distribution of untreated and NTP treated cells at G1 (% 44.9 vs %38.7), S (%25.5 vs %20.5) and G2/M (29.1 vs 40.2) phases of cell cycle are provided in Fig. 6. According to these results, there is a statistically significant difference in G2/M population of untreated and NTP-treated cells, indicating a drug induced cell cycle arrest.

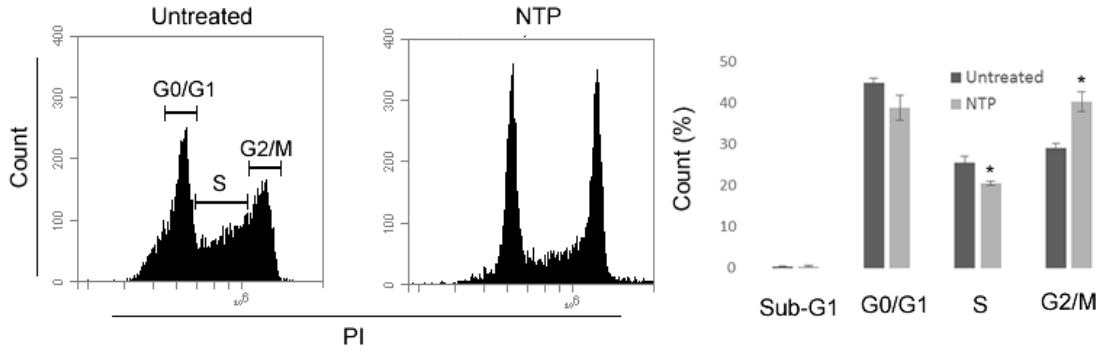

Figure 6. Effect of nortriptyline treatment (15 μM, 24 h) on U266 cell cycle. A) Representative flow cytometry fluorescence intensity histograms of cells stained with propidium iodide. Intensity ranges for corresponding cell-cycle phases (G0/G1, S and G2/M) were labeled. B) Bar plots of normalized count values of each phase for untreated and nortriptyline-treated cells (n=3). Error bars (1-3%) indicate standard error of mean. Asterisks denote statistical significance at $p<0.05$.

3.4 Apoptosis Assays

Based on previous TCA studies, we expected NTP to also induce apoptosis. In the following part, three common apoptotic biomarkers were probed to test this strong possibility.

3.4.1 Nortriptyline Causes Mitochondrial Membrane Depolarization

Mitochondria play a critical role in the programmed cell death and depolarization of the mitochondrial membrane has been shown to be one of the early events of apoptosis in some of the earlier cases [15]. To investigate the effect of NTP (30 μM) on mitochondrial membrane potential, we used JC-1 staining and analyzed the treated cells at 12, 24 and 48 h time points with flow cytometry. Red to green-shift of the JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide) fluorescence in the flow cytometry histograms (Fig. 7) was used as an indicator of the mitochondrial health.

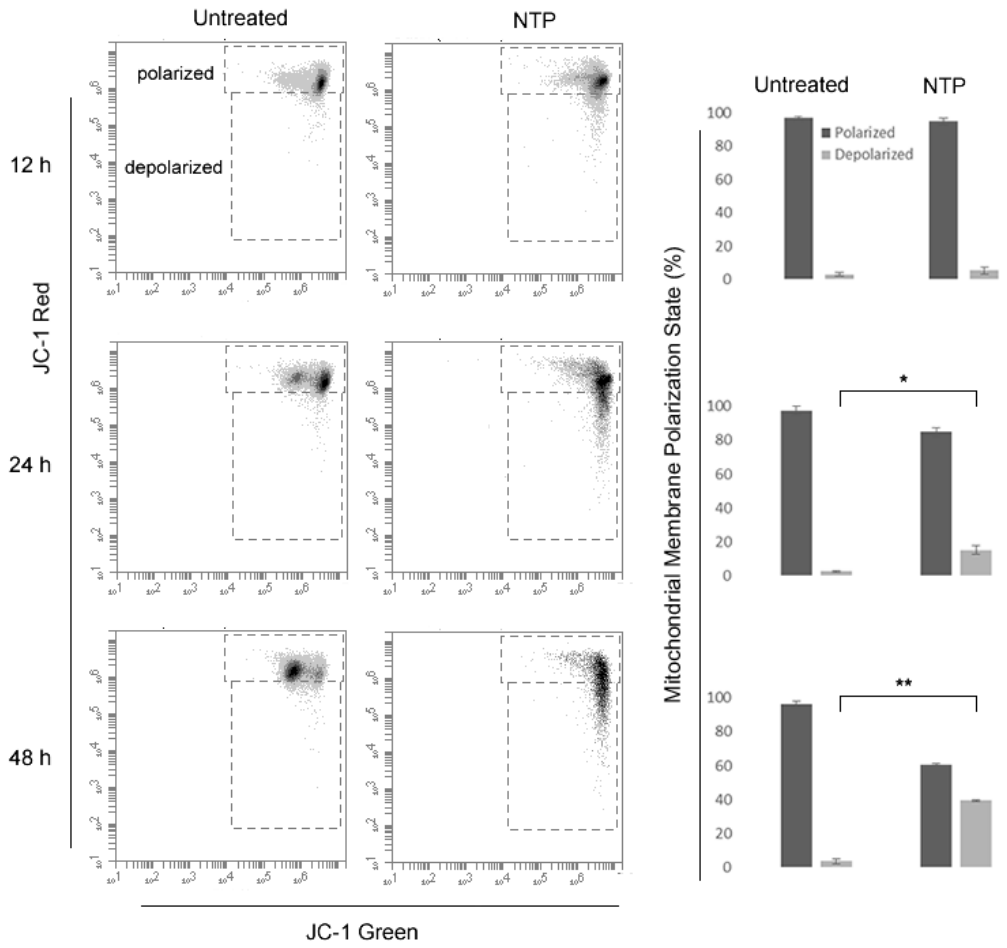

Figure 7. Effect of nortriptyline (30 μM) on mitochondrial membrane potential as a function of treatment time (12, 24 and 48 h). A) Representative flow cytometry fluorescence intensity dot plots of cells stained with JC-1. Gated fluorescence intensity values for polarized and depolarized states were labeled. B) Bar plots of normalized mitochondrial membrane polarization state values for untreated and nortriptyline-treated cells (n=3). Error bars (1-3%) indicate standard error of mean. Asterisks * and ** denote statistical significance at $p<0.05$ and $p<0.01$, respectively.

Majority of the control and NTP-treated cell populations have healthy mitochondria (polarized membrane) at 12 h. However, depolarization signal of the drug-treated cells starts to increase at 24 h (3% control vs 15% NTP) and becomes almost ten-fold higher at 48 h (4% control vs 39% NTP).

3.4.2 Nortriptyline Increases Caspase-3 Activity

Caspase-3 is a key protease activated in both intrinsic and extrinsic apoptotic pathways at an early stage. We used an immunofluorescence-based caspase-3 assay to specifically detect the active form of the protease as a second apoptotic biomarker. NTP treatment has no significant effect on active caspase-3 level at 12 h (Fig. 8). However, drug induced significant rises are observed at 24 h (5% control vs 28% NTP) and 48 h (6% control vs 35% NTP).

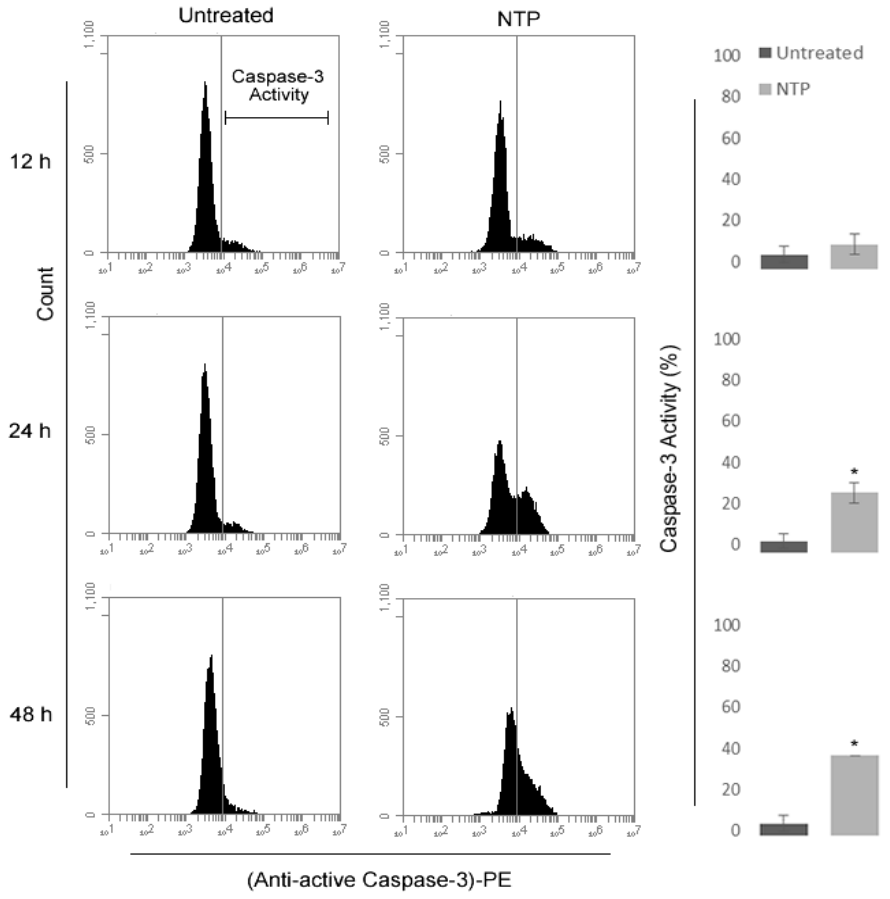

Figure 8. Caspase-3 activity of nortriptyline treated (30 μM) U266 cells at 12, 24 and 48 h. A) Representative flow cytometry fluorescence intensity histograms of cells stained with anti-active caspase-3 PE. Intensity threshold for caspase-3 activity is indicated in the upper-left panel. B) Bar plots of corresponding histograms (n=3). Error bars (4-5%) indicate standard error of mean. Asterisks denote statistical significance at $p<0.05$.

3.4.3 Annexin-V Assay Also Indicates to Nortriptyline-Induced Apoptosis

As the third and last apoptotic indicator, we employed a commonly-used assay, Annexin-V, which is based on the loss of cell membrane phospholipid asymmetry. As labeled in the top-left flow cytometry dot plot (Fig. 9), healthy cells are PE Annexin V and 7-AAD negative, early apoptotic cells are PE Annexin V positive and 7-AAD negative while late apoptotic/dead cells were considered PE Annexin V and 7-AAD positive.

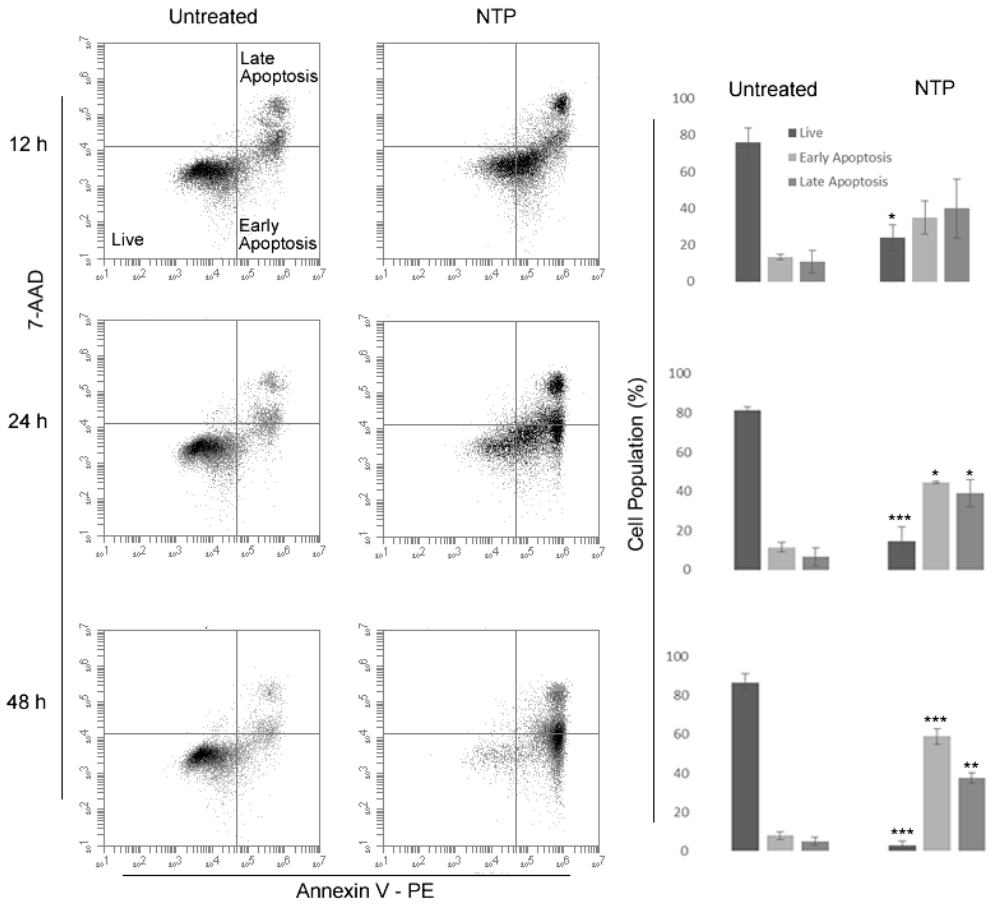

Figure 9. Flow cytometry analysis (12, 24 and 48 h) of Annexin V-PE/7-AAD stained U266 cells treated with 30 μM nortriptyline. A) Representative dot plots of Annexin V-PE vs 7-AAD signals gated as live, early apoptotic and late apoptotic quadrants B) Cell population bar graphs of corresponding dot plot quadrants (n=3). Asterisks *, ** and *** denote statistical significance between control and treatment populations at $p<0.05$, $p<0.01$ and $p< 0.001$, respectively.

Starting at 12 h, NTP treatment caused a significant decrease in the healthy cell population (76% control vs 24% treatment). As the treatment time extended to 24 and 48 h, increase in the percentage of early and late apoptotic cells (~4 and 6-fold in 24 h, ~7 and ~8-fold in 48 h, respectively) also became significant. Collectively, all three assay results show that NTP induces apoptosis in U266 cells**.**

CHAPTER 4

DISCUSSION

Results on the inhibitory effect and mechanism of NTP reported in this work agree with previous studies which strongly support the anticancer effect of antidepressants [7].

NTP has dose and time-dependent inhibitory effect on U266 cells. Similar results were obtained by others on osteosarcoma, prostate, melanoma and bladder cancer cells [9, 10, 12, 16]. IC_{50} values of NTP determined by us 26 μM on MM cells and Yuan et al (40 μM) on bladder cancer cells are similar. Within 24 h, NTP arrested cell cycle, caused mitochondrial membrane depolarization, increased active caspase 3 levels and induced loss of cell membrane asymmetry. These observations confirm both anti-proliferative and apoptotic effects of the drug on U266 cells.

Is NTP toxic to normal blood cells? Yuan et al [12] found less than 20% inhibitory effect on peripheral blood mononuclear cells at a much higher concentration (100 μM) than we used in this study. In addition, Mao et al [11] reported that amitriptyline, methylated form of NTP (Fig.4), was not cytotoxic to normal blood cells. Comparison of *in vitro* potency and *in vivo* serum concentration is another important point that needs addressing. Approximately 10 μM NTP was detected in the serum after daily administration at typical doses up to 75 mg [17]. This concentration is about one-third of the calculated IC_{50} value. At this point, it is quite difficult to judge the clinical potential of NTP for MM treatment which requires additional *in vivo* experiments.

NTP combination with cis was shown to be antagonistic within the tested dose regime. This is a clinically relevant finding since adjuvant therapy with antidepressants is commonly used for cancer patients [18]. In recent years, some other antidepressants (paroxetine, fluoxetine, and bupropion) were also shown to have negative effect on a commonly used cancer drug, tamoxifen, in breast cancer patients by inhibiting the metabolic conversion of the drug into its active form [19, 20]. Based on our results and tamoxifen example, we suggest that NTP may also have a negative impact on the clinical effectiveness of cis involving chemotherapy regimens.On the other hand, there are also examples revealing synergestic effect of TCAs in combination with other drugs. Amitriptyline combinations with dexamethasone and bortezomib were shown to have synergy on myeloma and MM cells [11, 21]. Similary, combining clomipramine with LiCl or imatinib also resulted in a synergism on neuroblastoma and glioma cells [21-23].

Are there other antagonism examples involving platinum-based drugs? There are at least two reported cases in which cis combinations of gefitinib and fingolimod were antagonistic [24, 25]. To our knowledge, cis-NTP combination was not previously investigated, however, we found a study by Kabolizadeh et al. [26] which involves desipramine, a structural analog of NTP (Fig 4). In this work, desipramine (5 to 50 μM) highly enhanced the cytotoxicity of cis
(1 to 15 μM) on colorectal carcinoma cell lines with CI values reported in 0.174 to 0.922 range.

Antagonistic effect of cis-NTP combination may have more than one explanation. Direct drug-drug interaction may be one of these although such an interaction was not evident in the cis-desipramine case as shown by Nuclear Magnetic Resonance experiments [27]. NTP might have caused an increase in DNA repair mechanism which would diminish the susceptibility of cis-induced DNA damage. NTP may also reduce the cellular accumulation of cis as in the case of cis-Raf kinase inhibitor BAY 43-9006 combination [28]. In this scenario, most likely NTP target would be organic cation transport machinery which was previosly proposed for cellular cis uptake [27, 29]. Another possibility is the activation of conflicting signaling pathways by cis and

NTP. A related TCA, imipramine, has been previously shown to induce autophagy in glioma cells [30]. Induction of autophagic machinery by NTP might have interfered with the apoptotic pathway of cis-induced cell death.

NTP arrested U266 cells at G2/M phase of cell cycle. Yuan et al. also reported cell cycle arrest at G0/G1 and G2/M phases in NTP-treated human and mouse bladder cancer cells [12]. Amitriptyline (20 μM, 24 h) arrests KMS11 and LP1 MM cells at G0/G1 by down-regulating cyclin D expression and increasing cyclin-dependent kinase inhibitors p27 and p21 expression [11]. Over-expression of p21 and p27 and cell cycle arrest was also reported in skin squamous carcinoma Ca3/7 cells treated with desipramine [31]. Finally, fluoxetine-induced G0/G1 arrest in lung and colon tumor cells was shown to involve cyclin A, cyclin D1, p21 and p53 [32]. As summarized above, all TCAs seem to have cell cycle arrest effect on cancer cells. Is there a significance of the phase in which the cells are arrested? Ruetz and DiPaola both reached the conclusion that G2/M arrest is less well tolerated by the cells, leading to increased apoptotic outcome [33, 34]. This evaluation provides a therapeutic advantage for NTP which causes G2/M arrest in U266 cells.

We also showed that NTP induces apoptosis in MM cells. Various other TCAs were all reported to have the same effect on tumor cell lines [7]. In particular, NTP induced caspase-dependent apoptosis in bladder cancer cells by both mitochondria and death receptor-mediated pathways [12]. Zhang et al. studied amitriptyline triggered apoptosis in MM xenograft models [21].

CHAPTER 5

CONCLUSION

In this work, we showed dose and time-dependent inhibitory effect of antidepressant drug nortriptyline on U266 MM cell line. It was also demonstrated that *in vitro* potency of NTP was greater than that of cisplatin, a well-known cancer drug. Previous studies on NTP showed minimal or no toxicity to normal blood cells and *in vivo* anti-tumor effect. Our study provides the first *in vitro* evidence of NTP induced cell cycle arrest and apoptosis in U266 cells. Molecular mechanism of both outcomes should be elucidated and compared to those of other antidepressants. Also, based on the reported results, NTP warrants further investigation using *in vivo* MM models.

Antidepressants are commonly used in adjuvant therapy for depression and pain relief. Therefore, antagonism of cis-NTP combination was another clinically significant finding in this work. In future studies, additional dose combinations can be tested to investigate the changes in the type and strength of the combination effect.

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APPENDIX A

SOLUTIONS

Table 4. Compound preparation

Name	Stock Concentration	Solvent	Storage Conditions
* Tricyclic Antidepressants	10 mM	dH_2O	-20ºC / 1 month
** Tetracyclic Antidepressants	10 mM	dH_2O	-20ºC / 1 month
Cisplatin	1 mM	0.9 % NaCl	Room Temperature/ 1 month

* imipramine, clomipramine, amitriptyline, opipramol, protriptyline, nortriptyline, desipramine

** maprotiline, mianserin

APPENDIX B

MEDIUM SPECIFICATIONS

Table 5. Composition of the RPMI 1640 medium

Substance	Concentration (mg/L)
NaCl	6000
KCl	400
$Na_2HPO_4.7H_2O$	1512
$MgSO_47H_2O$	100
$Ca(NO_3).4H_2O$	100
D-glucose	2000
Phenol red*	5
$NaHCO_3$	2000
L-arginine	200
L-asparagine	50
L-aspartic acid	20
L-cysteine	50
L-glutamine	300
L-glutamic acid	20
Glycine	10
L-histidine	15
L-hydroxiproline	20
L-isoleucine	50
L-leucine	50
L-lysine-HCl	40
L-methionine	15
L-phenylalanine	15
L-proline	20
L-serine	30
L-threonine	20
L-tryptophan	5
L-tyrosine	20
L-valine	20
Glutathione	1

(Table 5. continued)	0.005
Vitamin B12	
D-Ca-pantothenate	0.25
Cholin chloride	3
Folic acid	1
Myo-inositol	35
Nictoninamid	1
p-amino benzoic acid	1
Pyridoxin·HCl	1
Riboflavin	0.2
Thiamine-HCl	1
Biotin	0.2

* (1x) liquid medium contains 10 mg/l phenol red.