**Title:** Mitragyna alkaloids derived from the medicinal plant *Mitragyna speciosa* Korth. and cisplatin synergistically inhibit cell proliferation of Nasopharyngeal Carcinoma (NPC) cell lines

Gregory Domnic[1], Siti Fairus Abdul Rahman[1], Nelson Jeng Yeou Chear[2], Darshan Singh[2], Nethia Mohana-Kumaran[1*]

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

[2]Centre for Drug Research, Universiti Sains Malaysia, 11800 Penang, Malaysia

***Corresponding author details:**
Dr. Nethia Mohana-Kumaran
School of Biological Sciences
Universiti Sains Malaysia
11800
Pulau Pinang, Malaysia
Tel: +604-6534016
Fax: +604-6535125
Email: nethiakumaran@usm.my
ORCID ID: orcid.org/0000-0002-2162-8409

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**Email addresses of all authors:**

**Gregory Domnic:** gregoryunited@yahoo.com

**Siti Fairus Abdul Rahman:** fairus_rahman90@yahoo.com

**Nelson Jeng Yeou Chear:** jychear@gmail.com

**Kwok-Wai Lo:** kwlo@cuhk.edu.hk

**Darshan Singh:** darshan@usm.my

**Nethia Mohana-Kumaran:** nethiakumaran@usm.my; nethia82@gmail.com

**Abbreviations**

**NPC:** Nasopharyngeal carcinoma

**2D:** 2-dimensional

**NMR:** Nuclear Magnetic Resonance

**GC-MS:** Gas Chromatography-Mass Spectrometry

HPLC-PDA: High-Performance Liquid Chromatography–Photodiode Array

**Abstract**

*Ethnopharmacological relevance*

Cisplatin is the standard of care treatment for patients diagnosed with nasopharyngeal carcinoma, where it is administered concurrently with radiotherapy in patients diagnosed with primary NPC or locoregionally advanced NPC. Patients respond to cisplatin well but eventually develop resistance to the drug making subsequent treatment with cisplatin difficult. *Mitragynine* is one of the most abundant mitragyna alkaloid purified from the leaves of *Mitragyna speciosa* (Korth.). A number of cancer cell lines have been reported to be sensitive to *Mitragynine* at high doses. Here we evaluated, the potential of *Mitragynine* and other mitragyna alkaloids namely *Speciociliatine* and *Paynanthiene* as chemosensitizers for cisplatin.

*Aim of the study*

To assess the potential of mitragyna alkaloids (*mitragynine, speciociliatine* or *paynantheine*) as chemosensitizers for cisplatin in NPC cancer cell lines.

*Materials & Methods*

NPC cell lines HK-1 and C666-1 were treated with combinations of cisplatin and mitragyna alkaloids in 2D monolayer culture and in spheroid model.

*Results*

*Mitragynine* and *Speciociliatine* sensitised the HK-1 and C666-1 to cisplatin ~4- and >5-fold, respectively in 2D monolayer culture. Similarly, combination of *Mitragynine* and cisplatin inhibited growth and invasion of HK-1 spheroids in a dose-dependent manner. Moreover, the spheroids did not rapidly develop resistance to the drug combination over 10 days.

*Conclusion*

Collectively, data shows that both *Mitragynine* and *Speciociliatine* could act as sensitizers for cisplatin. Moving forward, extensive drug mechanistic studies and investigations in animal models are necessary to unleash the prospect of this combinations for NPC therapy.

## 1. Introduction

Nasopharyngeal carcinoma (NPC), a malignancy manifesting in the nasopharynx, specifically from within the pharyngeal recess, known as the Fossa of Rosenmüller (Wei and Sham, 2005). NPC is responsible for 129,079 new cases and 72,987 deaths in 2018 (Bray et al., 2018). Concurrent cisplatin-based chemoradiotherapy is the standard of care treatment for NPC. Patients usually respond well in the initial stages of cisplatin treatment but gradually develop resistance to the drug, severely limiting its use in subsequent treatments (Köberle et al., 2010). Combining cisplatin with compounds purified from natural resources is one approach to resensitize cancer cells to cisplatin. Compounds from natural resources are generally less toxic and may be more affordable and accessible to patients.

*Mitragyna speciosa* (Korth.) is locally known as '*biak'* or '*ketum'* in Malaysia, or *kratom* in Thailand and U.S., is a medicinal plant, indigenous to Malaysia and Thailand (Hassan et al., 2013; Singh et al., 2016). *Mitragynine, Speciociliatine* and *Paynanthiene* are the major mitragyna alkaloids purified from the leaves of *M. speciosa*. Thus far, there is only one study which reports sensitivity of cancer cell lines to *Mitragynine* (Goh et al., 2014). The broad aim of the study is to investigate the sensitivity of NPC cancer cell lines to combination of cisplatin and mitragyna alkaloids, in 2D monolayer culture and in 3-dimensional spheroids, which resemble a microenvironment closer to tumours *in vivo* (Sankar et al., 2017).

## 2. Materials & methods

### *2.1 Plant material*

The leaves of *Mitragyna speciosa* (Korth.) (Rubiaceae) were collected from Permatang Rawa, Penang, Malaysia. The taxonomic identity of the plant was authenticated by Dr. Rosazlina Rusly, a botanist from School of Biological Sciences, Universiti Sains Malaysia. A voucher specimen (NEL-(K)-2019(01)) was deposited at the Herbarium Unit of the premise.

*2.2 Extraction and purification of Mitragyna alkaloids*

The tested mitragyna alkaloids: *mitragynine* (purity > 98%), *speciociliatine* (purity > 95%) and *paynantheine* (purity > 95%) were extracted and purified from the leaves of *M. speciosa* according to methods described by (Shellard, 1974; Sharma et al., 2019). The chemical structure of each alkaloids were confirmed using 1H & 13C NMR and GC-MS, and the spectroscopic data was compared with those reported in literatures (Singh et al., 2018a; Singh et al., 2018b; Sharma et al., 2019; Singh et al., 2020). The purity level of each alkaloid was evaluated using HPLC-PDA according the method described by (Saref et al., 2019).

*2.3 Drug sensitivity assay*

NPC cell lines HK-1 and C666-1 were authenticated using the AmpFISTR profiling as described previously (Daker et al., 2012). Drug sensitivity assays were performed as described previously (Allen et al., 2002). Sensitivity of the HK-1 cells to drug combinations was measured by testing a fixed concentration of either *mitragynine*, *speciociliatine* or *paynantheine* at 8, 16 or 32 μM with increasing concentrations of cisplatin (MedChemExpress, NJ, USA) diluted in two-fold steps (0.25, 0.5, 1, 2, 4, 8, 16 and 32 μM). The C666-1 were treated similarly except that a fixed concentration of either *mitragynine*, *speciociliatine* or *paynantheine* at 25, 35 or 45 μM was employed. The drug sensitivity assays were performed four times (n=4) and mean $IC_{50}$ values were calculated from the experimental data. The dose curves were generated as described previously (Abdul Rahman et al., 2020). The CalcuSyn 2.11 program (Biosoft, Cambridge, UK) was used to access drug interactions.

*2.4 Three-dimensional spheroids*

Spheroids were generated from HK-1 cells and embedded in collagen matrix as previously described (Smalley et al., 2008; Abdul Rahman et al., 2020). Spheroids were treated with

cisplatin and *Mitragynine* alone and in combination. Drug/compound and media were replenished every 72 hours for experiments conducted over 10 days. Spheroids growth and invasion were photographed using the Nikon C2+ inverted confocal microscope. Images were processed using ImageJ (v1.51s, NIH) (Schindelin et al., 2012).

## 3. Results and Discussion

The NPC cell lines were first tested to single agent treatment of all three mitragyna alkaloids and cisplatin. Both NPC cell lines were resistant to single agent treatment of all three mitragyna alkaloids and cisplatin except at high doses (Supplementary table 1). Similar observations were reported in another study which investigated the sensitivity of HCT 116 (colon carcinoma cell line) and K562 (leukemia cell line) to single agent *mitragynine*. Both cell lines were sensitive to *mitragynine* at concentrations > 100 μM (Goh et al., 2014). The sensitivity of cancer cells to other mitragyna alkaloids has to our knowledge not yet been reported. Given that the NPC cell lines were insensitive to single agent treatment of the mitragyna alkaloids, we investigated the potential of these compounds as chemosensitizers for cisplatin. NPC cells were treated with a fixed concentration of either *mitragynine*, *speciociliatine* or *paynantheine* with increasing concentrations of cisplatin.

At 8 μM of *mitragynine*, HK-1 cells were sensitized to cisplatin by 2.2-fold. The sensitization increased to 3.5-fold and 4-fold at a concentration of 16 μM and 32 μM *mitragynine* (Fig. 1A and Supplementary table 2). In C666-1 cells, *mitragynine* at 25 μM and 35 μM only sensitized C666-1 cells to *mitragynine* by 2-fold. The sensitization increased to > 5-fold when the concentration of *mitragynine* was increased to 45 μM (Fig. 1B and Supplementary table 3).

Combination with 8 μM of *speciociliatine* sensitized the HK-1 cells to cisplatin by ~ 2-fold. The sensitization increased to 3-fold and 4-fold when the concentration of *speciociliatine* was increased to 16 μM and 32 μM (Fig. 1C and Supplementary table 2), respectively. In C666-

1 cells, *speciociliatine* at 25 μM sensitized the C666-1 cells to cisplatin by ~ 2-fold (Fig. 1D and Table x). The sensitization increased by 4-fold and by > 5-fold at 35 μM and 45 μM (Fig. 1D and Supplementary table 3), respectively.

In HK-1 cells, the presence of either 8 μM or 16 μM or 32 μM *paynantheine*, sensitized the cells to cisplatin only by ~ 2-fold (Fig. 1E and Supplementary table 2). In C666-1 cells *paynantheine* at 25 μM sensitized the C666-1 cells to cisplatin by ~ 2-fold. The sensitization increased to by 4-fold and > 5-fold when the concentration of *paynantheine* was increased to 35 μM and 45 μM (Fig. 1F and Supplementary table 3), respectively. Drug interaction analyses indicated that combination of the mitragyna alkaloids *(mitragynine, speciociliatine or paynanthiene)* and cisplatin demonstrated synergism at several concentrations in both cell lines (Supplementary Fig. 1 & 2).

Next, the combination of *mitragynine* and cisplatin were tested in spheroid model. The effect of combination of cisplatin and *mitragynine* on the growth and invasion of the spheroids were first studied over 3 days of treatment. Combination of 16 μM of *mitragynine* and 4 μM of cisplatin reduced spheroid growth and invasion and the reduction was more significant in the presence of 16 μM of *mitragynine* and 8 μM of cisplatin (Fig. 2). The spheroids were monitored till day 10 for emergence of resistant cells. Combination of 16 μM of *mitragynine* and 2 μM of cisplatin reduced spheroid growth and invasion and the reduction was more significant in the presence of 16 μM of *mitragynine* and 4 μM of cisplatin there was a further reduction in spheroid growth and invasion (Supplementary Fig. 3). Moreover, the spheroids did not rapidly develop resistance to the drug combination over 10 days.

The mechanism of action of the mitragyna alkaloids in combination of cisplatin is currently unknown. High expression of COX-2 is associated with resistance of NPC cells to cisplatin (Chen et al., 2015). *Mitragynine* is reported to inhibit COX-2 mRNA and protein expression, and thus inhibit prostaglandin E2 (PGE2) formation (Utar et al., 2011). We

speculate that one possible mechanism could be that the mitragyna alkaloids down-regulate the expression of COX-2 and sensitize NPC cells to cisplatin.

## 4. Conclusion

*Mitragynine* and *speciociliatine* sensitized the NPC cells to cisplatin > 4-fold at concentrations close to its $IC_{50}$ values. These compounds might thus be effective sensitizers to cisplatin. The C666-1 cells were more sensitive to combination of *paynanthiene* and cisplatin compared to HK-1 indicating that the effect of paynantheine is more cell-type specific. Combination of mitragynine and cisplatin reduced spheroid growth and invasion in a dose-dependent manner suggesting that the combination may be effective in *vivo*. Elucidating the mechanism of action of the mitragyna alkaloids and testing the combination in animal models are necessary to unleash the prospect of this combination for cancer therapy.

## Author contributions

**Gregory Domnic:** Methodology, Formal analysis; **Siti Fairus Abdul Rahman:** Methodology, Formal analysis; **Nelson Jeng Yeou Chear:** Methodology, Formal analysis; **Darshan Singh:** Resources, Funding acquisition, Writing – Review & Editing; **Nethia Mohana-Kumaran:** Conceptualization, Resources, Writing – Original Draft, Funding acquisition

## Declaration of competing interest

The authors declare that there are no conflicts of interest

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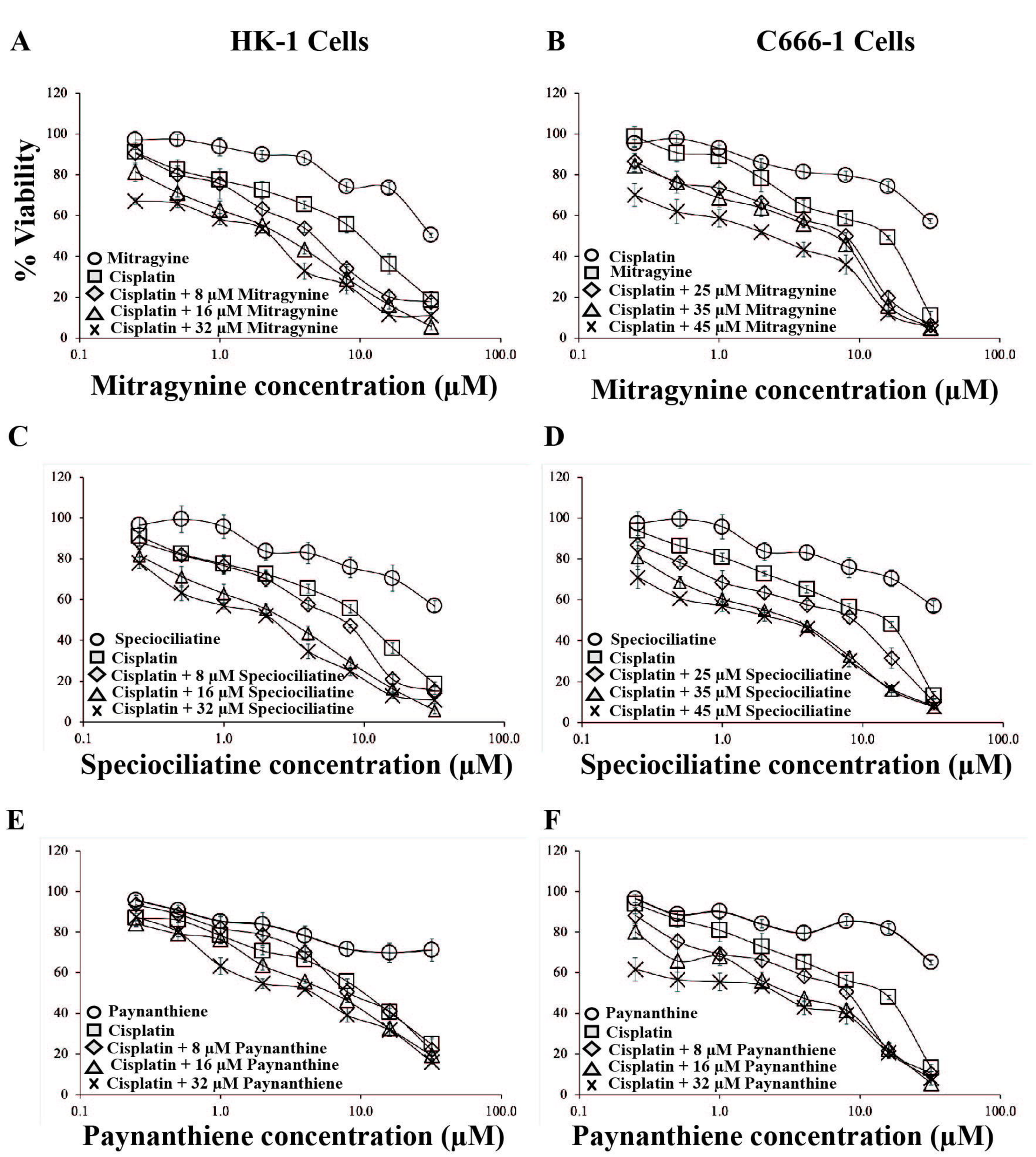
**Figure legends:**

**Fig 1: Sensitization of the NPC cell lines to cisplatin by the mitragyna alkaloids.** NPC cell lines HK-1 and C666-1 were treated with increasing concentrations of cisplatin (0-32 μM) in the presence and absence of **(A-B)** *Mitragynine*; **(C-D)** *Speciociliatine* and **(E-F)** *Paynanthiene*. Sensitivity of the NPC cell lines to single agent treatment of either *Mitragynine, Speciociliatine* or *Paynanthiene* are also shown in each dose-response graph. Both NPC cell lines were resistant to single agent treatment of all three mitragyna alkaloids. Points represent mean ± SEM of four experiments.

**Fig 2: The effect of combination of *Mitragynine* and Cisplatin on the growth and invasion of HK-1 spheroids over three days.** The spheroids were treated with single agents *Mitragynine* and Cisplatin and combination of both over three days at the indicated concentrations, $n$ = 2-3 spheroids per combination. Size bar: 200 μm.


A
HK-1 Cells
% Viability
Mitragyine
Cisplatin
Cisplatin + 8 µM Mitragynine
Cisplatin + 16 µM Mitragynine
Cisplatin + 32 µM Mitragynine
Mitragynine concentration (µM)
B
C666-1 Cells
Cisplatin
Mitragyine
Cisplatin + 25 µM Mitragynine
Cisplatin + 35 µM Mitragynine
Cisplatin + 45 µM Mitragynine
Mitragynine concentration (µM)
C
Speciociliatine
Cisplatin
Cisplatin + 8 µM Speciociliatine
Cisplatin + 16 µM Speciociliatine
Cisplatin + 32 µM Speciociliatine
Speciociliatine concentration (µM)
D
Speciociliatine
Cisplatin
Cisplatin + 25 µM Speciociliatine
Cisplatin + 35 µM Speciociliatine
Cisplatin + 45 µM Speciociliatine
Speciociliatine concentration (µM)
E
Paynanthiene
Cisplatin
Cisplatin + 8 µM Paynanthine
Cisplatin + 16 µM Paynanthine
Cisplatin + 32 µM Paynanthiene
Paynanthiene concentration (µM)
F
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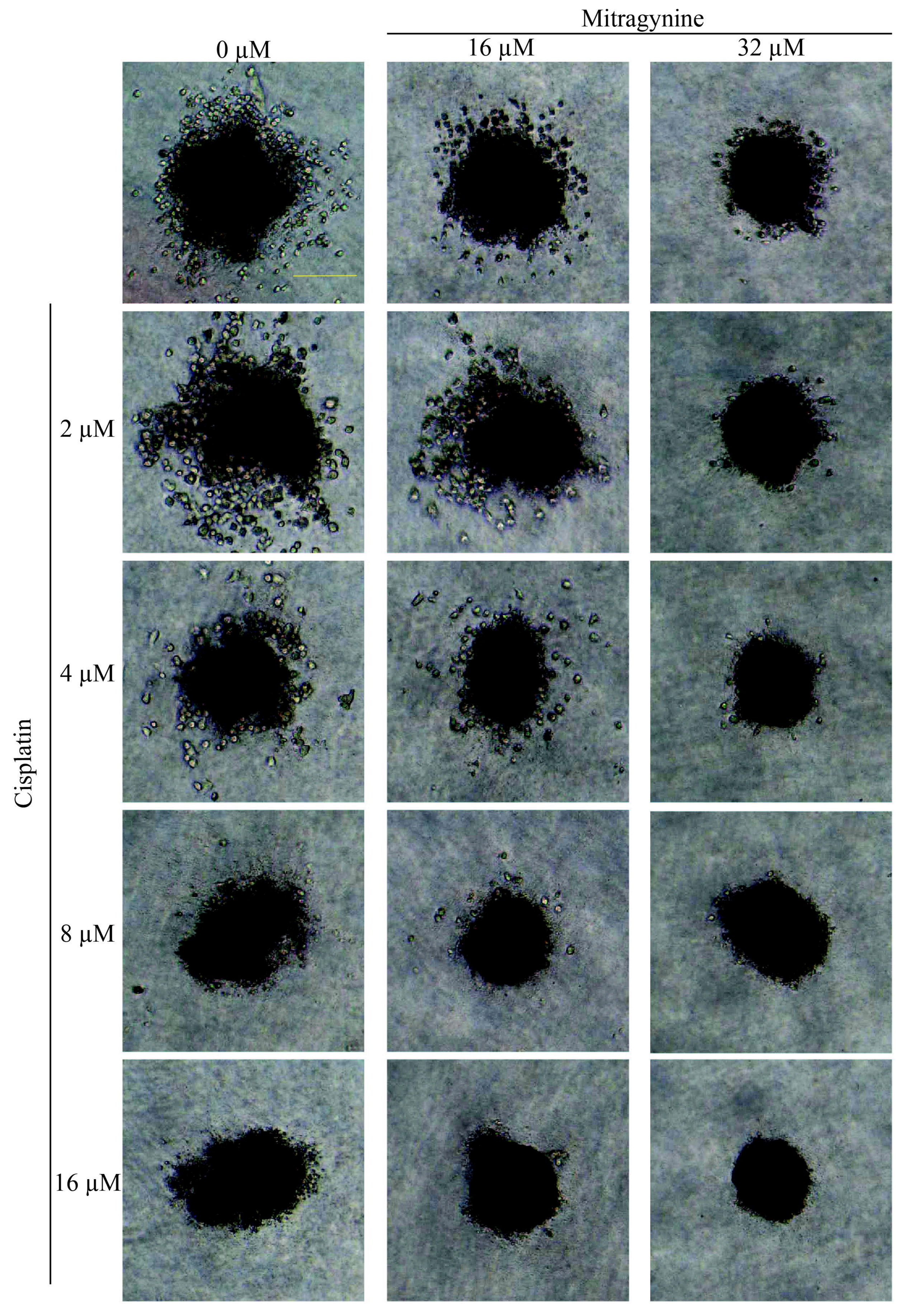
Cisplatin + 16 µM Paynanthine
Cisplatin + 32 µM Paynanthine
Paynanthiene concentration (µM)


Mitragynine
0 μM
16 μM
32 μM
Cisplatin
2 μM
4 μM
8 μM
16 μM