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Selective anastasis induction by bee venom in normal cells: a promising strategy for breast cancer therapy with minimal impact on cell viability

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Anastasis is a phenomenon described as a cellular escape from ethanol-induced cell death. Although the relevant mechanism has not yet been fully elucidated, anastasis is thought to play a role in drug resistance in cancer cells. To date, the regulation of anastasis in normal and cancerous cells has not been clarified. The current cancer treatment strategies are expected to selectively attack cancer cells without negatively affecting normal cell proliferation. Inspired by the anti-cancer potential of bee venom, this study is the first to evaluate whether bee venom has similar selectivity in producing an anastatic effect. The results indicated that bee venom induces anastasis in normal cells (MCF10A, ARPE19, and NIH3T3) but causes irreversible cell death in breast cancer cells (MDA-MB-231 and MCF7). Liver cancer cells (HEPG2) were moderately more resistant to permanent cell death after bee venom treatment compared to breast cancer cells. However, cisplatin caused permanent non-selective cell death in both normal and cancerous cells. The selectivity indices after bee venom treatment were higher compared to cisplatin. Taken together, bee venom was shown to induce selective anastasis only in normal cells not in cancer cells, which suggests that bee venom has significant potential in selective cancer therapy, especially breast cancer, via promoting the recovery and maintenance of viability of normal cells.

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HeLa cells undergo anastasis after apoptosis in response to a chemotherapy drug, jasplakinolide (Tang et al., 2009). Ethanol-induced anastasis was previously shown in MDA-MB-231 and MCF7 cells as well as normal breast (MCF10A) cells (Göker Bağca, 2022). The molecular mechanism for ethanol-induced anastasis was revealed by the time-dependent mRNA expression profiles. The expression of specific genes involved in the survival pathway was found to increase (Tang et al., 2012; Tang et al., 2022).

However, no study has assessed the selective induction of anastasis in normal cells over cancer cells. Bee venom is a promising natural product to induce selective cytotoxicity in cancer cells (Tu et al., 2008; Sharkawi et al., 2015). Although bee venom has a strong cytotoxic potential, its cytotoxic specificity regarding the discrimination between anastatic and non-anastatic profiles in cancer and normal cells has not yet been revealed. This study showed that bee venom significantly induces anastasis in normal cells while triggering irreversible cell death in breast cancer cells. The detailed methodology is given in the supplementary material.

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MALDI-TOF MS analysis of the bee venom determined the molecular weight as about 16 kDa of PLA2 homologs with different N-linked carbohydrate chains (Kubelka et al., 1993; Hollander et al., 1993). Additionally, LC-MS analysis of bee venom obtained the molecular weights of seven different peptide biomolecules that are the main peptide components of bee venom, as reported in previous studies (Baracchi and Turillazzi 2010; van Vaerenbergh et al. 2014) **(Table 1)**. The MALDI-TOF and LC-MS q-TOF spectra were given in the supplementary material **(Figs. S1-S8)**. Melittin was the most abundant component (50%-60%) of bee venom. It was eluted at about 20 min and its monoisotopic m/z value was observed as 2844.806 Da. The other abundant components were melittin-F, apamin, mast cell-degranulating (MCD) peptide, secapin, secapin-1, and tertiapin. These results confirm that the crude bee venom samples used in this study were genuine.

Table 1. Most abundant components found in the bee venom are characterized by LC-MS qTOF

Bee venom constituent	Number of amino acids	Calculated monoisotopic molecular weight (Da)	Observed monoisotopic molecular weight (Da) - Deconvoluted
Melittin	26	2844.744	2844.806
Melittin-F	19	2206.332	2206.398
Apamin	18	2025.907	2025.923
MCD Peptide	22	2585.412	2585.447
Secapin	25	2864.572	2864.615
Secapin-1	25	2821.566	2821.609
Tertiapin	21	2453.246	2453.262

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Next, to reveal the cytotoxic doses for both bee venom and cisplatin, half maximal inhibitory concentration (IC_{50}) values were determined by MTT assay for each cell line after bee venom or cisplatin. Cancer cells were more sensitive to bee venom with IC_{50} values of 8 µg/mL, 8 µg/mL and 12 µg/mL in MDA-MB-231 (human metastatic breast cancer), MCF7 (human breast cancer) and HEPG2 (human liver cancer) cells, respectively **(Figs. 1a-1c)**; however, normal cells were more resistant to higher doses of bee venom with IC_{50} values as 36 µg/mL, 38 µg/mL and 50 µg/mL in MCF10A (human normal breast cells), ARPE-19 (human normal retinal epithelium cells) and NIH3T3 cells (mouse embryonic fibroblast), respectively **(Figs. 1d-1f).** Normal cells (except ARPE-19 cells) had a greater tendency towards cell death after treatment with low doses of cisplatin compared to bee venom. The IC_{50} values of MDA-MB-231, MCF7, and HEPG2 cells were 12 µg/mL, 20 µg/mL and 12 µg/mL, respectively **(Figs. 1h-1j)**, and those of MCF10A, ARPE-19, and NIH3T3 cells were 25 µg/mL, 70 µg/mL and 8 µg/mL, respectively **(Figs. 1k-1m).** The cell morphologies and population density of culture were shown by representative microscopy images after 24 h incubation of bee venom or cisplatin at different doses **(Figs. 1g and 1n).** Bee venom treatment resulted in higher selective indices (SI) than SI values detected after cisplatin **(Table 2).** This suggests a higher selectivity of bee venom for triggering cell death in cancer cells with a minimal cytotoxic effect on normal cells.

Cisplatin, as one of the most common chemotherapeutics clinically used in various cancers among the most preferred chemotherapeutic agents for comparison with new compounds/treatments in *in vitro* and *in vivo* cancer research. The IC_{50} values in MDA-MB-231 and MCF7 cells after cisplatin treatment for 24 h were found to be 11.08 µg and 21.08 µg (Kashyap et al. 2024), respectively, which are similar to our results. However, cisplatin (>50 µmol/L) treatment for 24 h was found cytotoxic for MCF7 and MDA-MB-231 cells (Mizielska et al. 2023). Another study reported that extended incubation with cisplatin (48 h) resulted in higher IC_{50} values than 24 h incubation for MDA-MB-231 and MCF7 at 38.6 µmol/L and 83.1 µmol/L, respectively (Ben Hassen et al. 2023). HePG2 cells showed a variety of cytotoxic behaviors as IC_{50} values after 24 h cisplatin detected at 7.5 µmol/L (Kntayya et al. 2018) and 26.71 µmol/L (Brito et al. 2012). Our study showed that ARPE-19 cells were more resistant to cisplatin-induced cytotoxicity with an IC_{50} value at 70 µg/mL. Consistently, a study

suggested that the IC_{50} concentration of cisplatin in ARPE-19 cells was more than 30 μmol/L after 24 h of incubation (Sevim Nalkiran et al. 2023). However, prolonged incubation resulted in lower IC_{50} values such as 25 μmol/L after 48 h (Saydam and Nalkiran 2021) and 6 μmol/L after 96 h (Allison et al. 2017). NIH3T3 showed a time-dependent decrease in IC_{50} values such as 98 μmol/L after 24 h (Demirbağ et al. 2024) and 3.42 after 72 h (Zhang et al. 2024). Research indicates that the cytotoxic response of cells against cisplatin may show variation, which may depend on different parameters such as the passage number of cells, the stage of confluency when treated, the total days of pre-culture/passage before treatment, *etc*. For this reason, cisplatin should be included in each experiment during an MTT assay with the target compounds.

Table 2 Selective index values (SI) after bee venom or cisplatin treatment

BEE VENOM				
	Selective indices (SI) *(SI = Normal cell IC_{50} / Cancer cell IC_{50})*			
Cells	**MCF10A**	**ARPE19**	**NIH3T3**	**Average SI**
MDA-MB-231	4.5 (36/8)	4.75 (38/8)	6.25 (50/8)	5.17
MCF7	4.5 (36/8)	4.75 (38/8)	6.25 (50/8)	5.17
HEPG2	3 (36/12)	3.17 (38/12)	4.17 (50/12)	3.45
CISPLATIN				
	Selective indices (SI) *(SI = Normal cell IC_{50} / Cancer cell IC_{50})*			
Cells	**MCF10A**	**ARPE19**	**NIH3T3**	**Average SI**
MDA-MB-231	2.1 (25/12)	5.83 (70/12)	0.67 (8/12)	2.87
MCF7	1.25 (25/20)	3.5 (70/20)	0.4 (8/20)	1.72
HEPG2	2.1 (25/12)	5.83 (70/12)	0.67 (8/12)	2.87

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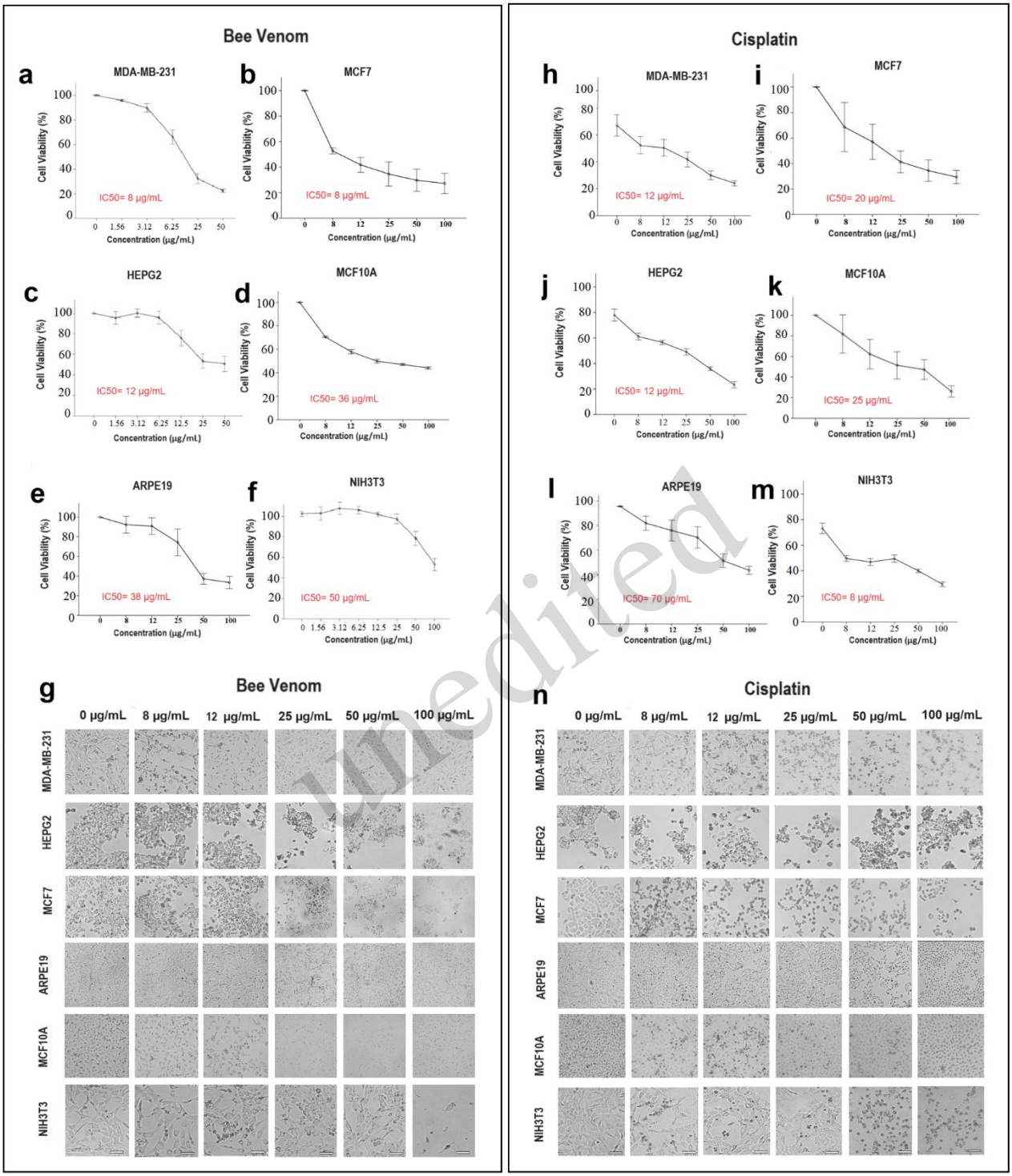

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Figure 1. Cytotoxicity profiles of bee venom (a-g) and cisplatin (h-n) in MDA-MB-231 (a, h), MCF7 (b, i), HEPG2 (c, j), MCF10A (d, k), ARPE19 (e, l), and NIH3T3 (f, m) cell lines. Representative microscopy images are given after bee venom and cisplatin treatment (g, n).

The anastatic response of cells against bee venom and cisplatin was next assessed. All cells were treated with bee venom or cisplatin to determine the number of living cells. A group of each cell line was left untreated. Cells

were washed after 24 h treatment and left to recover for up to 72 h **(Fig. S9)**. MDA-MB-231 cells continued to die even if bee venom (≥25 μg/mL) was removed, and they entered anti-anastasis after 12 μg/mL of bee venom **(Fig. 2a)**. Another breast cancer cell line, MCF7, was sensitive to bee venom at each concentration after removal and each condition was anti-anastatic for MCF7 **(Fig. 2b)**. However, HEPG2 tended to enter anastasis at up to 25 μg/mL bee venom, and the minimum anti-anastatic dose was 50 μg/mL in liver cancer cells (HEPG2) **(Fig. 2c)**. MCF10A cells recovered at up to 12 μg/mL bee venom **(Fig. 2d)**. NIH3T3 cells were anastatic at each concentration including 100 μg/mL **(Fig. 2e)**. ARPE-19 cells showed an anastatic profile at up to 25 μg/mL bee venom **(Fig. 2f)**. Statistical comparisons indicated that 12 μg/mL of bee venom had an anastatic effect in normal cells **(Fig. 3d-4f)**, but this was anti-anastatic in both breast cancer cells (MDA-MB-231 and MCF7) **(Fig. 3a and 4c)**. HEPG2 cells seemed more resistant to anti-anastasis since they entered permanent cell death after 50 μg/mL bee venom **(Figs 2 and 3b)**. These results suggest that bee venom significantly induced selective anastasis in normal breast cancer cells compared to two breast cancer cell lines. However, none of the cells entered the anastasis process after cisplatin treatment **(Figs. 4a-4f and 5a-5f)**. Cisplatin was found to be selectively cytotoxic on HEPG2 cells (compared to non-cancerous epithelial cells, ARPE-19) with a 5.83 SI value **(Fig. 1, Table 2)**; however, it did not change the anastatic response of ARPE19 cells compared to HEPG2 cells. This indicates that cisplatin also harms normal cells in the long term, which cannot be recovered via induced anastasis. The development of resistance of cancer cells in chemotherapy treatment processes suggests that cancer cells can escape apoptosis by activating anastasis (Seervi et al. 2019; Y. Wang et al. 2023). From this perspective, the anastasis event can well explain the developmental mechanisms that play a role in cancer recurrence. However, the potential of anti-cancer agents to trigger anastasis in normal cells while inducing cell death in cancer cells remains unrevealed. Therapeutic agents for cancer treatments are expected to have a highly cytotoxic effect on cancer cells while showing less cytotoxicity to healthy cells.

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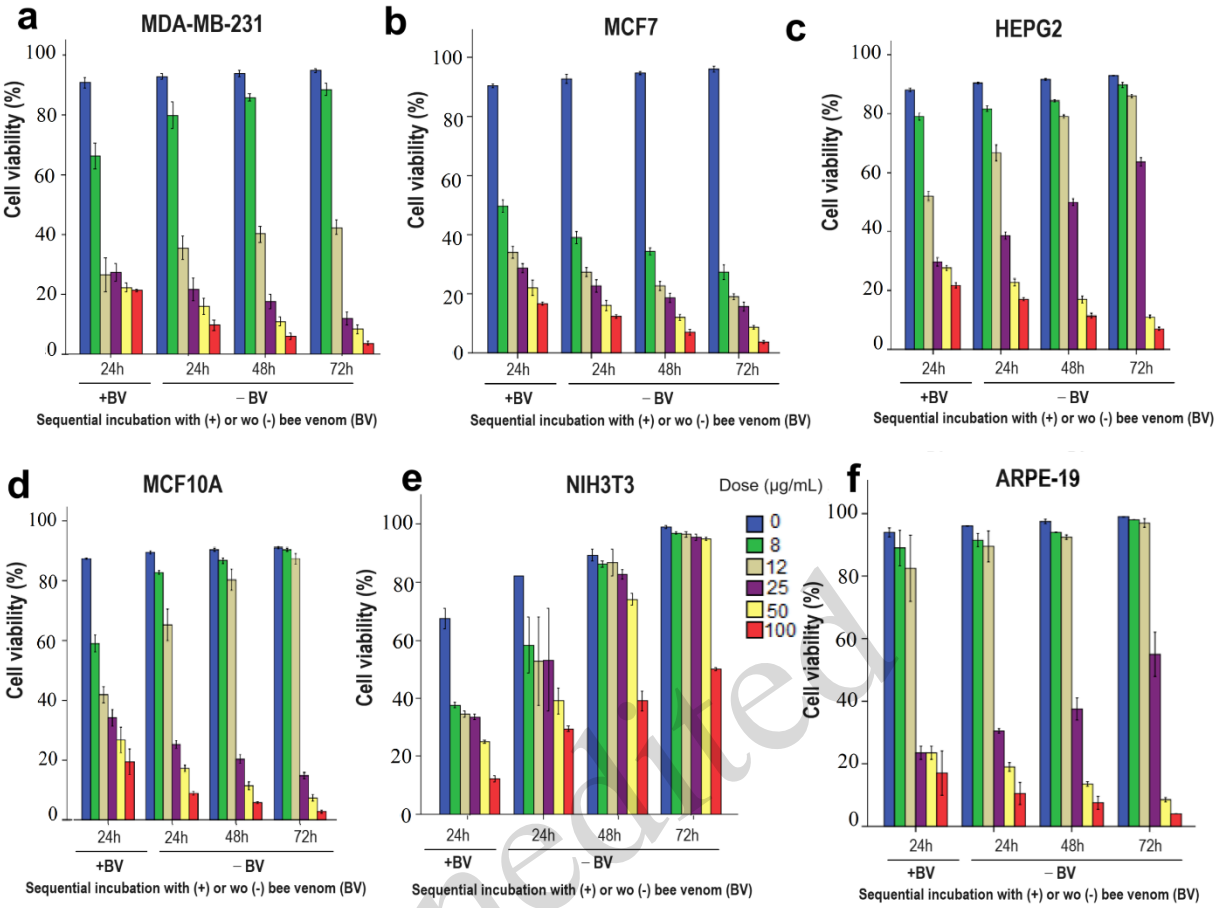

Fig. 2 Bee venom (BV)-induced cell viability (%) in cancer (a-c) and normal (d-f) cells during anastasis. +BV; treatment with bee venom, -BV; post-treatment after bee venom.

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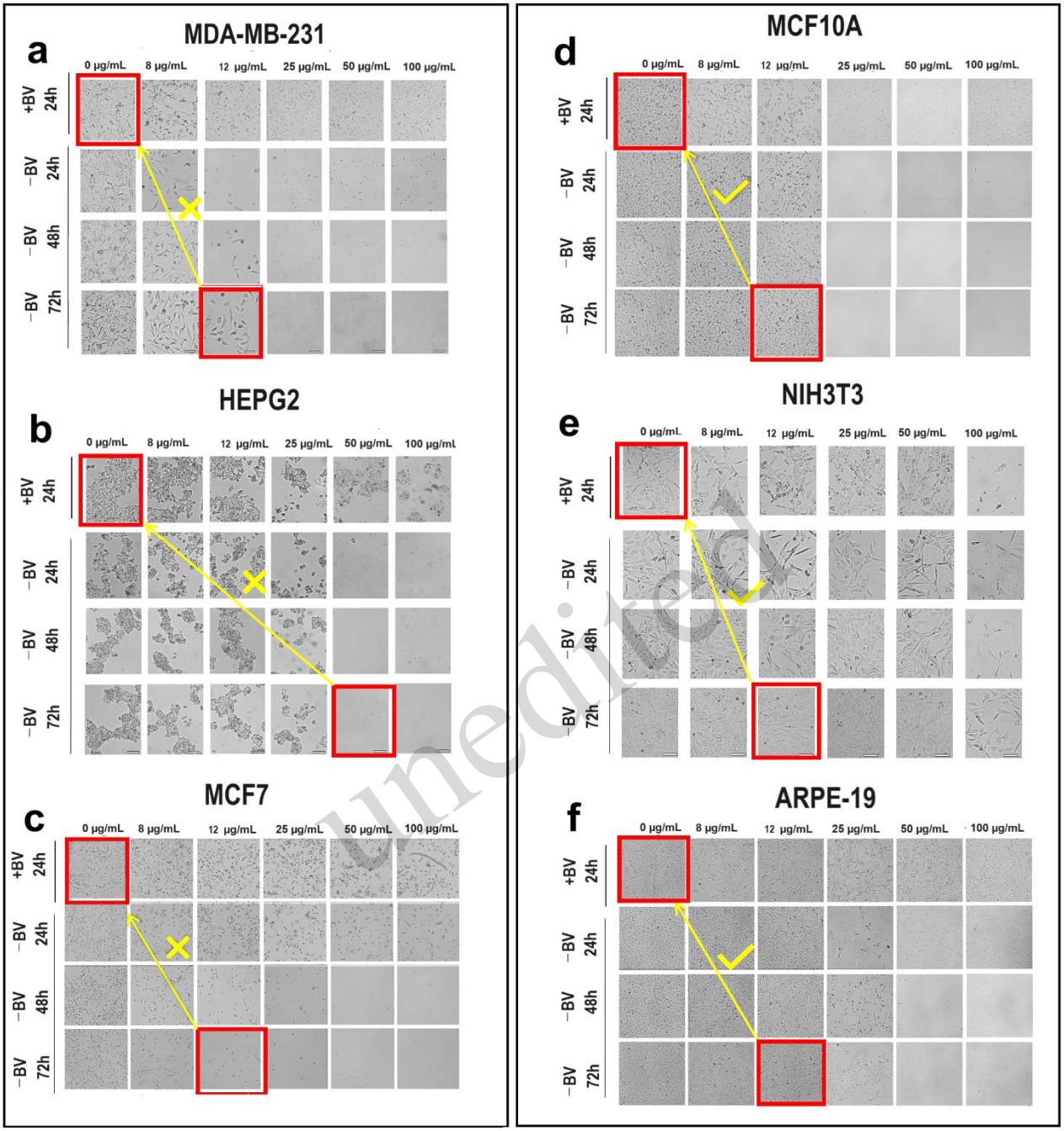

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Fig. 3. Bee venom-induced anti-anastatic profile in cancer (a-c) and anastatic profile in normal (d-f) cells. The red squared images represent the possible reversal of cell death to viable cells. X, no reversal, tick means reversal.

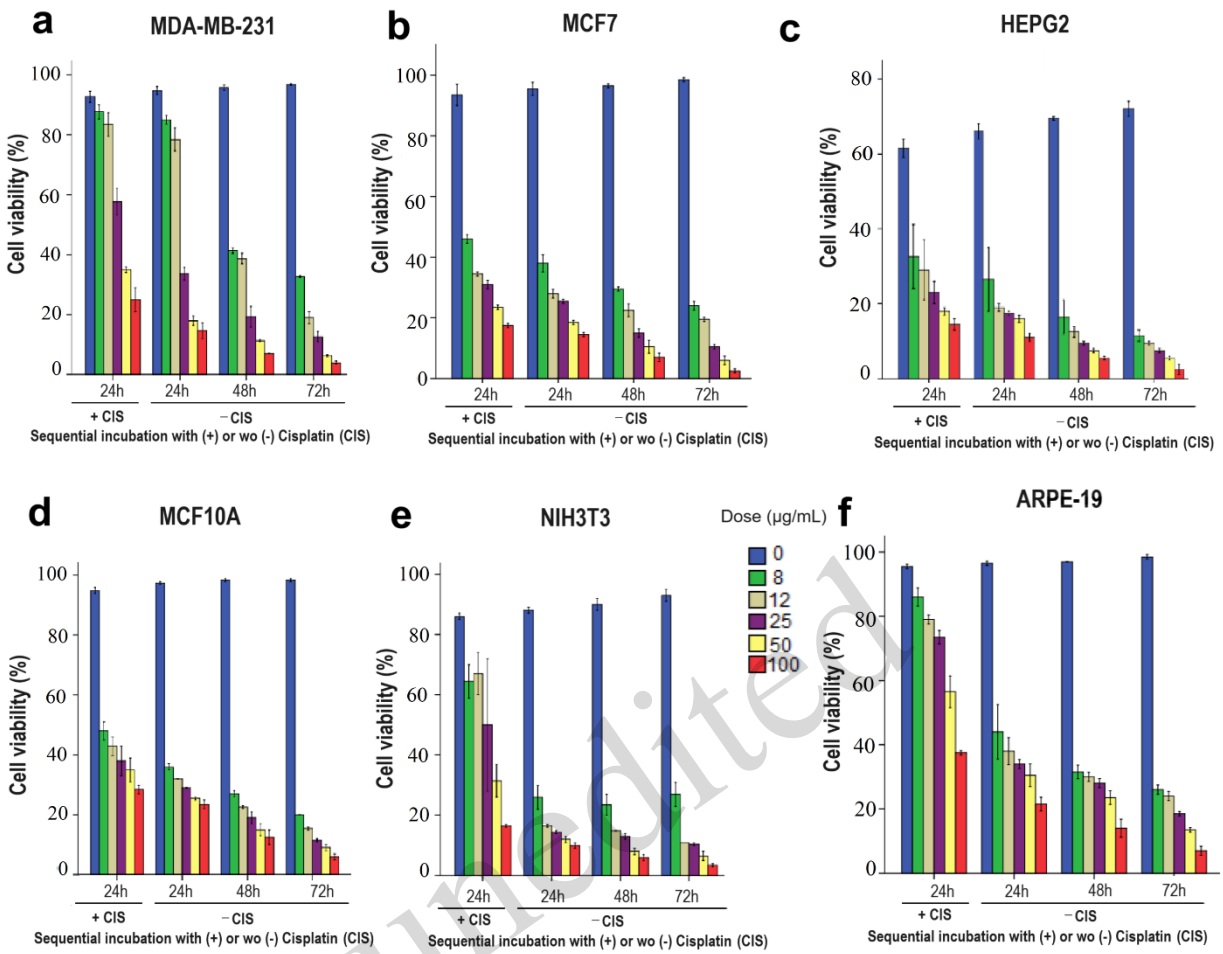

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Fig.4. Cisplatin (CIS)-induced cell viabilities (%) in cancer (a-c) and normal (d-f) cells in terms of anastatic expectation. +CIS; treatment with cisplatin, -BV; post-treatment with cisplatin.

The significant dose intervals for anastasis are summarised in **Table 3** for each cell line-agent combination. MCF7 was the most sensitive cancer cell line to both agents with no anastatic trend under all conditions. The MDA-MB-231 cell line appeared to induce moderate anastasis only at the lowest dose of bee venom (8 µg/mL). MCF10A cells were more anastatic after bee venom at higher doses than two breast cancers. However, HEPG2 cells were the most resistant cancer cells to bee venom with anastasis up to 25 µg/mL which was similarly observed in normal epithelial ARPE-19 cells. The highest anastasis was observed in NIH3T3 cell lines at up to 100 µg/mL. The trypan blue method and MTT method do not discriminate the types of cell death; therefore, at this stage, these results cannot indicate any specific type of cell death for reversal.

Table 3. Significant anastatic dose interval in each cell line after bee venom or cisplatin (at 72h)

	Cancer cells			Non-cancerous cells		
Agent	**MDA-MB-231**	**MCF7**	**HEPG2**	**MCF10A**	**ARPE19**	**NIH3T3**
Bee venom	8 µg/mL	N/D	8-25 µg/mL	8-12 µg/mL	8-25 µg/mL	8-100 µg/mL
Cisplatin	N/D	N/D	N/D	N/D	N/D	N/D

N/D, not detectable

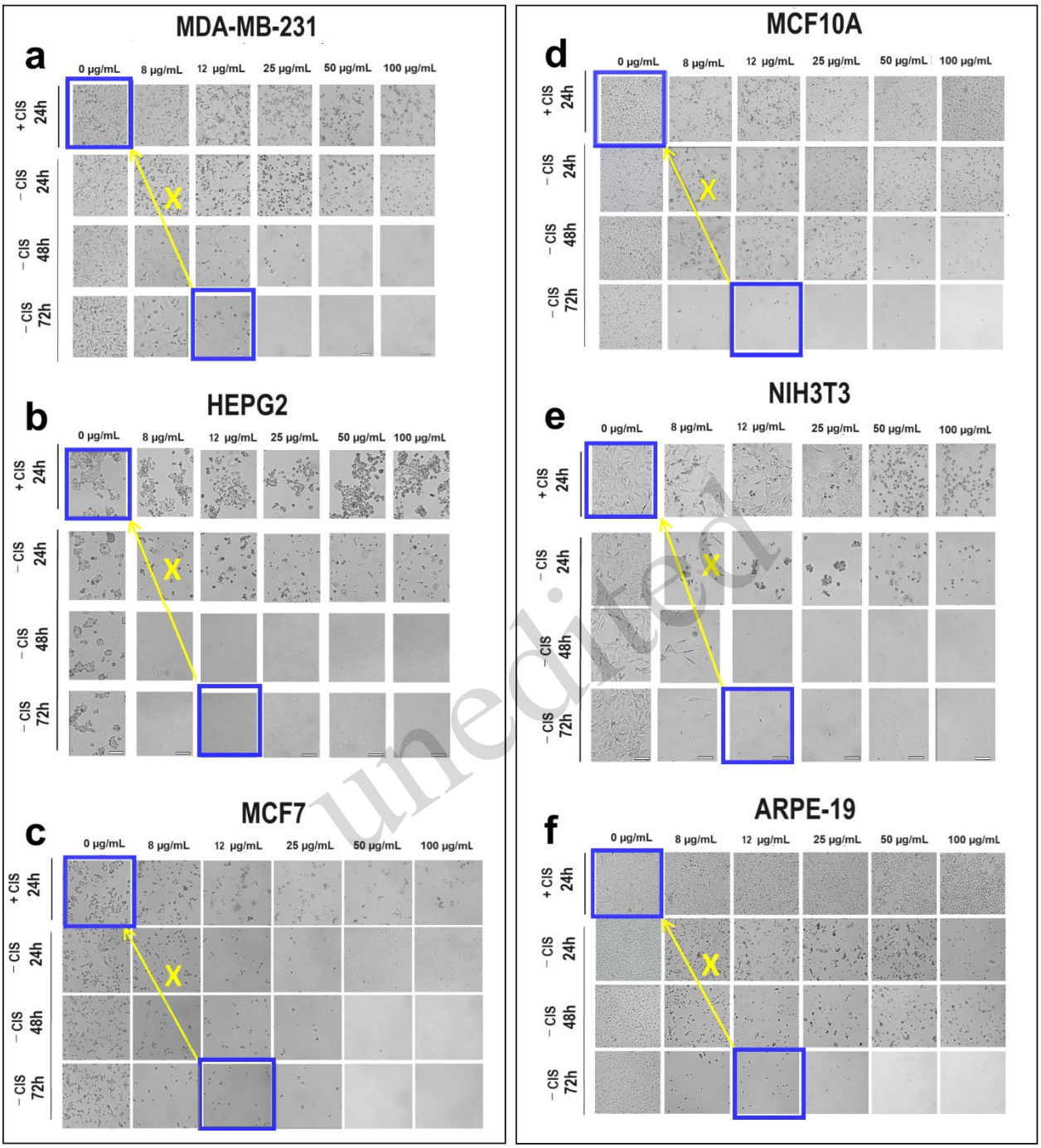

Fig. 5. Cisplain (CIS)-induced anti-anastatic profiles in cancer (a-c) and normal (d-f) cells, respectively. The blue squared images represent the possible reversal of cell death to viable cells. X, no reversal, tick means reversal.

We next analyzed the population doubling times for each cell after different doses/incubations with bee venom or cisplatin. Live cells were counted by trypan blue staining, and population doubling times (PDT) were calculated using a formula given in the supplementary material. **Fig. 6** shows each dose and incubation in cancerous and normal cells. **Table 4** indicates the average PDT between incubation with an agent for 24 h and

anastatic incubation for an extra 72 h.

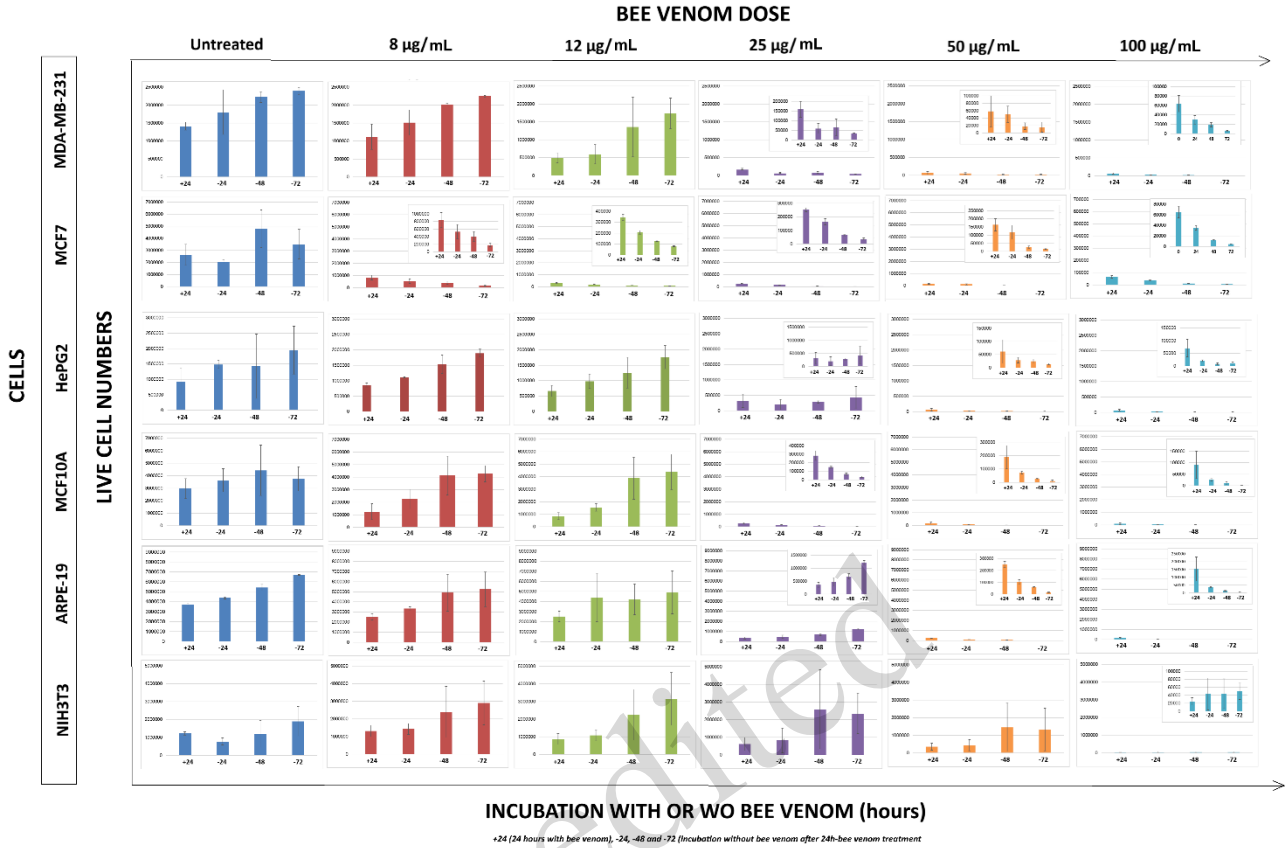

Fig. 6. Average live cell numbers in cancer cells and normal cells during anastasis-related response against bee venom

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The total live cell number increased at 8 µg/mL bee venom in MDA-MB-231 and 8-12 µg/mL in HEPG2, whereas the live cell number did not increase at any dose in MCF7 cells **(Fig. 6)**. HEPG2 cells are the only cancerous cells that tend to a degree of cell proliferation at 25 µg/mL. Most MCF10A progressively lived in a time-dependent manner at up to 12 µg/mL bee venom, but both ARPE-19 and NIH3T3 cells were further alive at 25 µg/mL **(Fig. 6)**. PDTs were consistent with the live cell trend **(Table 4)**. MCF7 was the most sensitive cancer cell line that could not recover via anastasis, with a constant decrease in live cell number at each dose **(Fig. 6 and Table 4)**. Bee venom resulted in higher PDTs in MDA-MB-231 and HEPG2 with higher doses; however, MCF10A and ARPE19 normal cells proliferated faster after bee venom than untreated counterparts **(Table 4)**. PDT of NIH3T3 cells fluctuated after 8 µg/mL and 100 µg/mL at a slow rate, but cells similarly proliferated at 12 µg/mL and 25 µg/mL as fast as their untreated counterparts **(Table 4)**.

Table 4. Average population doubling times (hour) (from the time for 24h incubation with bee venom to anastatic incubation for 72h) (N/D; not detectable)

BV dose (µg/mL)	Cell Line					
	MDA-MB-231	HEPG2	MCF7	MCF10A	ARPE19	NIH3T3
0	40,6676	37,5204	45,9007	65,2756	83,2831	38,6427
8	71,3494	63,1007	N/D	40,4933	68,4168	63,2968
12	N/D	51,4698	N/D	30,2763	74,8264	39,1825
25	N/D	162,155	N/D	N/D	40,6186	37,5684

50	N/D	N/D	N/D	N/D	N/D	38,4714
100	N/D	N/D	N/D	N/D	N/D	70,1518

Importantly, these results show that the anastasis mechanism can be activated in normal cells, but not in cancer cells, to escape the cytotoxic effect of treatment. Therefore, the anastasis mechanism can have a distinguishing function between normal and cancer cells and play a differential role in the response to treatment in favor of the survival of normal cells. Nonetheless, the prolonged effect of bee venom on the switch to anastasis from cell death should be tested in tumor tissues by *in vivo* models. Bee venom has been shown to inhibit the proliferation of different cancers such as cervical cancer for up to 4 weeks (Lee et al. 2015), breast cancer for 21 days (Oršolić et al. 2003), and melanoma for up to 12 days (Liu et al. 2002) in mouse models. *In vivo* experimental designs are considered advantageous to conclude the long-term treatment of bee venom.

Bee venom is composed of many biologically active compounds, with melittin being the major component exerting an anti-cancer effect (Wang et al. 2024; Honari et al. 2024; Cui et al. 2024). Phospholipase A is another important enzyme with an individual anti-cancer effect (Xie et al. 2024; Recuero et al. 2024). Therefore, to standardize the anastatic effect of bee venom, each component should be individually evaluated for breast cancer specificity. Our study showed that anastasis induced by bee venom plays a distinctive role in breast cancer treatment. However, the molecular mechanism of anastasis and the main responsible component of bee venom for anastasis remain to be elucidated.

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Data availability statement

All data about this work are included in the article and the supplementary data.

Acknowledgments

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Author contributions

Conceptualization; SCU, UU, Data curation; ST, MA, SCU, Funding acquisition; SCU, Investigation and Methodology; ST, MA, UU, SCU, Visualisation; ST, MA, SCU, Supervision; SCU, Resources; SCU, Writing – original draft; ST, SCU, MA, Writing – review & editing; UU, SCU.

Compliance with ethics guidelines

Sinan Tetikoglu, Muharrem Akcan, Ugur Uzuner ve Selcen Celik-Uzuner declare that they have no conflict of interest.

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Supplementary information

Figs. S1-S9

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