POTENTIAL PROTEASOME-INHIBITORY EFFECTS OF COMMONLY USED DRUGS: AN *IN VITRO* REPURPOSING STUDY

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Identifying novel or repurposed proteasome inhibitors offers a promising strategy for cancer therapy, especially given the toxicity of bortezomib. This study screened fourteen commonly used drugs, including anticancer agents, plant extracts, and antifungals, in MCF7, MDA-MB-231, and MCF10A cell lines. Tamoxifen, docetaxel, and ketoconazole showed notable cytotoxicity, with tamoxifen exhibiting selective proteasome inhibition. EGCG confirmed assay reliability, while ATP measurements suggested metabolic interference may affect proteasome function. Interestingly, tamoxifen and cisplatin increased proteasome activity in specific contexts. These findings support drug repurposing as a route to safer, more targeted proteasome inhibitors in cancer treatment.

Keywords: ubiquitin-proteasome system, anticancer, estrogen, tamoxifen

1. Introduction

The ubiquitin–proteasome system (UPS) plays a central role in maintaining cellular protein homeostasis and regulating vital biological processes such as cell cycle progression, apoptosis, immune surveillance, and stress responses [1,2]. Damaged or misfolded proteins are selectively degraded through this molecular machinery, thereby preventing cytotoxicity due to their accumulation. Otherwise, accumulation of these aberrant proteins within the cell triggers endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). If this stress cannot be resolved or persists, the UPR activates apoptotic signals, such as the CHOP and caspase pathways, ultimately leading to apoptosis. However, if ATP stores are depleted or antioxidant defenses collapse, necrotic cell death may occur. In cases of strong proteasome inhibition, such as with bortezomib, alternative cell death pathways like necroptosis may also be triggered [3,4].

This cascade of cellular events can be exploited for therapeutic purposes in tumor cells; inhibition of the proteasome system in cancerous cells induces cell

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death and tumor shrinkage, resulting in an anticancer effect. The development of proteasome inhibitors—such as bortezomib, carfilzomib, and others—has revolutionized the treatment of hematologic malignancies, including multiple myeloma and mantle cell lymphoma [5,6]. These inhibitors, particularly peptide boronates and epoxy ketones, exploit the proteasome's unique threonine-dependent catalytic mechanism to achieve selective and potent inhibition. Despite their clinical success, limitations such as resistance development and dose-limiting toxicities necessitate the discovery of novel compounds with proteasome-inhibitory potential and improved safety profiles [7].

Interestingly, some anticancer agents not primarily designed to target the proteasome have been found to interfere with the UPS, either directly or indirectly [8]. Tamoxifen, a selective estrogen receptor modulator (SERM) widely used in hormone receptor-positive (ER+) breast cancer, has been reported to exhibit off-target effects, including modulation of proteasomal activity [9–11]. Likewise, several cytotoxic and targeted anticancer drugs may impact protein degradation pathways either as part of their primary mechanism of action or through unintended interactions with cellular proteases [12–14]. These findings suggest that commonly used anticancer agents may contribute to proteasome inhibition, potentially enhancing therapeutic outcomes or revealing novel mechanisms of cytotoxicity.

Beyond its classical role as an ER modulator, tamoxifen is increasingly recognized for its ability to influence cellular processes beyond estrogen signaling, including proteostasis [15–17]. A key link in this broader activity is the behavior of tamoxifen's active metabolites—particularly endoxifen—which not only bind with high affinity to ERα but also promote its degradation via the UPS [18]. This process results in sustained downregulation of ERα protein levels, amplifying tamoxifen's antiestrogenic effects at a post-translational level. These findings suggest that tamoxifen's therapeutic efficacy may, in part, depend on proteasome-mediated receptor turnover, highlighting a mechanistic intersection between endocrine modulation and proteasome inhibition. This dual mechanism broadens the scope of tamoxifen's anticancer activity and underscores the potential of targeting proteasomal degradation as a complementary strategy in hormone receptor-positive breast cancer therapy.

When considered with straightforward logic, inhibition of the proteasome by tamoxifen and/or its metabolites would appear contrary to its anticancer effect, as proteasome inhibition would prevent the reduction of ERα protein levels—which constitutes a key therapeutic mechanism of the drug. From this perspective, investigating both the cytotoxic and potential proteasome-inhibitory effects of tamoxifen in ER-positive (MCF-7) and ER-negative (MDA-MB-231) cancer cells may help clarify this apparent paradox.

In this study, in addition to evaluating the potential proteasome-inhibitory effects of tamoxifen, a group of commonly used drugs with various indications were

systematically screened for proteasome-inhibitory activity as part of a drug repurposing approach. Although bortezomib is a clinically approved and highly effective proteasome inhibitor, its use is often limited by off-target toxicities—such as peripheral neuropathy or bone marrow suppression [1,6,19]. These challenges underscore the need for alternative proteasome-targeting compounds with improved safety profiles, selectivity, and pharmacokinetic properties. Several improved alternatives have been developed, such as carfilzomib; however, these agents may exhibit different systemic toxicities, including cardiotoxicity [20].

The present study aims to explore the proteasome-inhibitory potential of over fourteen drugs belonging to various pharmacological classes (e.g., anticancer agents, plant extracts, and antifungals). Following cytotoxicity assessments, compounds demonstrating the highest cytotoxicity (i.e., lowest IC_{50} values) were further evaluated for proteasome inhibition.

The novelty of this study lies in investigating the relationship between the ERα antagonistic mechanism and proteasome inhibition in tamoxifen's anticancer action, while contributing to the identification of repurposed drugs with potential proteasome-inhibitory effects. Moreover, evaluating proteasome inhibition in both ER+ and ER– cancerous and healthy cells is expected to provide deeper insights into its possible mechanisms.

2. Materials and Methods

2.1. Materials

A group of 14 drugs belonging to various classes such as anticancer drugs, antifungals, plants' extracts were tested in terms of their cytotoxicity and proteasome-inhibitory effect in both human breast cancer and non-tumorogenic breast epithelial cells. The active substances purchased from Sigma-Aldrich and Fluka were: DTPA (Diethylenetriamine pentaacetate, a medicine that helps reduce radioactive material in the body), aprotinin (a small protein bovine pancreatic trypsin inhibitor (BPTI)), tamoxifen (selective estrogen receptor modulator used to prevent breast cancer in women and men), quercetin (a plant flavanol from the flavonoid group of polyphenols), deferoxamine (DFOA, known as desferrioxamine and sold under the brand name desferal, is a medication that binds iron and aluminum), glycitein (an O-methylated isoflavone and phytoestrogen with weak estrogenic activity); ketoconazole (antifungal), daidzein (a natural occurring soy isoflavone); letrozole (aromatase inhibitor medication that is used in the treatment of breast cancer for postmenopausal women), pepstatin A (a potent inhibitor of aspartyl proteases), cisplatin (a coordination complex of platinum that is used as a chemotherapy medication), metformin (first-line medication for the treatment of type 2 diabetes); naproxen (a nonsteroidal anti-inflammatory drug used to treat pain, menstrual cramps, and inflammatory diseases such as rheumatoid arthritis, gout and fever); and docetaxel (antimitotic chemotherapy medication).

2.2. Cell culture and cytotoxicity assay

MDA-MB-231 (ATCC) and MCF-7 (ATCC) cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C. MCF10A (ATCC) cells were cultured in DMEM:F12 medium supplemented with 5% horse serum (HS), 1% penicillin-streptomycin (Pen-Strep), 20 ng/mL epidermal growth factor (EGF), 0.5 µg/mL hydrocortisone, and 10 µg/mL insulin under standard cell culture conditions (37 °C, 5% CO_2). The cytotoxic potential of the active substance was tested by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. MTT reagent was reduced to purple formazan crystal form when incubated with viable cells.

MDA-MB-231, MCF-7 and MCF10A cells were seeded into 96-well microplates at a density of approximately 5,000–10,000 cells per well in 100 µL of complete growth medium and incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2 to allow cell attachment. Each tested active substance's stock solution was prepared by dissolving the required amount of each of the chemicals in 1 mL of DMSO to obtain a 20 mM solution, which was stored at -20 °C.

Working concentrations were obtained by diluting stock solutions in culture medium, ensuring that the final DMSO concentration did not exceed 0.1%. Cells were treated with test compounds or vehicle control for 48 hours, after which 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. Plates were then incubated at 37 °C for 4 hours. Following incubation, the medium was gently removed, and 100 µL of DMSO was added to dissolve the purple formazan crystals formed by metabolically active cells. Absorbance was measured at 540 nm using a Varioskan Flash plate reader (Thermo). Cell viability was calculated as the percentage of absorbance relative to control wells (considered 100%). Data are presented as mean ± standard deviation (SD) from at least three independent experiments, each performed in triplicate. Statistical significance was assessed using ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. ($p < 0.05$). Cell viability was calculated relative to control wells using equation 1:

$$\%Viability = (A_{sample}/A_{control}) \times 100 \quad (1)$$

IC_{50} value of each compound was obtained by using GraphPad Prism5 software.

2.3. Proteasome activity

Proteasome inhibition was assessed using the Cayman Chemical 20S Proteasome Assay Kit (Item No: 10008041), which utilizes a fluorogenic substrate to measure the proteasome assay, compounds were screened for cytotoxicity using the MTT assay at a fixed concentration of 100 µM for 48 hours.

Compounds that caused more than 50% inhibition in cell viability were further analyzed to determine their half maximal inhibitory concentration (IC_{50}) values through the dose-response MTT assay ranging from 3.125 to 100 µM. These IC_{50} concentrations were subsequently used in the proteasome activity assay. For the proteasome assay, 96-well black plates were prepared by adding 10 µL of cell lysate, 10 µL of each test compound at its IC_{50} concentration, 70 µL of assay buffer, and 10 µL of the fluorogenic substrate solution, making a total volume of 100 µL per well. The reaction mixture was incubated at 37°C for 30–60 minutes, and fluorescence was measured at an excitation wavelength of 360 nm and emission at 460 nm.

The degree of proteasome inhibition was determined by comparing the relative fluorescence units (RFU) of compound-treated samples to the untreated control, using equation 2:

$$\%Inhibition = \left[\left({RFU_{control}}/{RFU_{sample}}\right)\right] \times 100 \quad (2)$$

All experiments were conducted in triplicate, and care was taken to keep DMSO concentrations below 1% to avoid interference with enzymatic activity.

3. Results and discussions

3.1. Cytotoxicity evaluation

The cytotoxic effects of the screened drugs were evaluated by using the MTT assay that measures cellular metabolic activity by assessing the reduction of MTT to purple formazan crystals primarily by mitochondrial dehydrogenases, thus providing insights into cytotoxicity levels in cells. Fig. 1A and B described the cell viability trendlines for the screened drugs applied for the three types of cell lines: MDA-MB-231 (a highly aggressive, invasive and poorly differentiated triple-negative breast cancer), MCF-7 (human breast cancer cell possessing functional estrogen and EGF receptors) and MCF 10A (a non-tumorigenic epithelial cell line). By treating the cell lines with one dose of each drug of 100 µM.

As observed within Fig. 1A and B, tamoxifen, ketoconazole, cisplatin and docetaxel as expected, exhibited the most significant decrease in viability, suggesting a strong antiproliferative effect, while quercetin, deferoxamine, glycitein and pepstatin showed moderate cytotoxicity and possess promising anticancer potential and warrant further investigation to elucidate their mechanisms of action.

The remaining drugs—daidzein, naproxen, metformin, letrozole, aprotinin, and DTPA—showed a weaker cytotoxic effect compared to the first group of drugs, with decreasing potency. The four most cytotoxic drugs—tamoxifen, ketoconazole, cisplatin, and docetaxel—exhibited significantly greater

cytotoxicity in the cancer cell lines MCF-7 and MDA-MB-231 compared to the healthy MCF-10A cells.

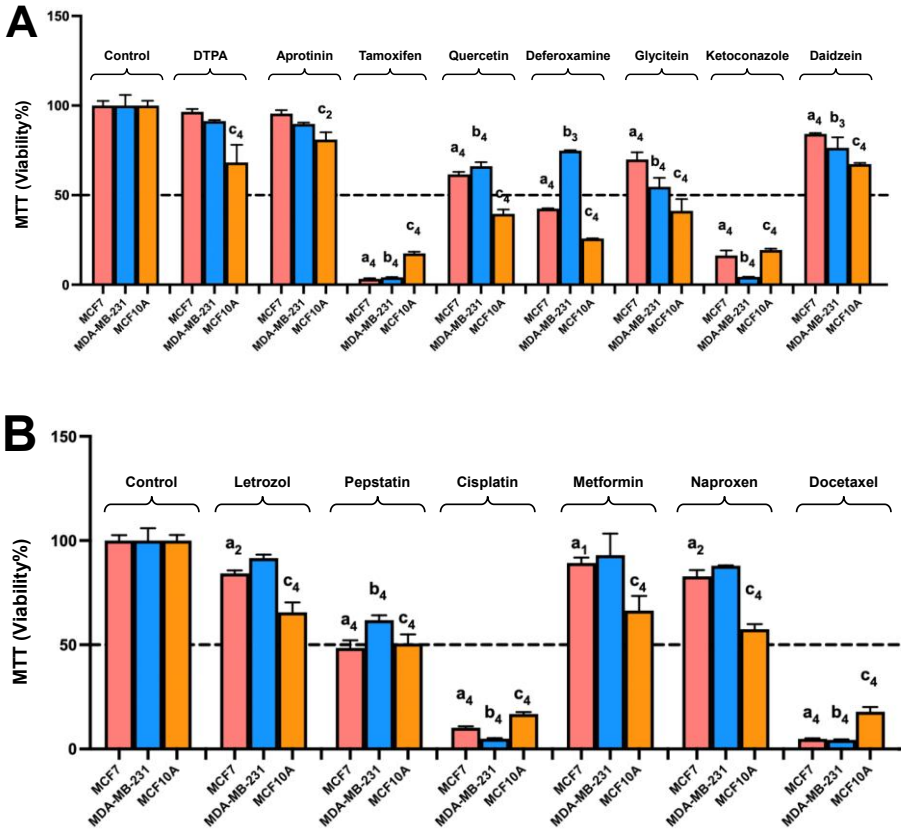

Fig. 1. Cell viability (MTT) percentages of the test drugs. The results were divided into graphs A and B due to space limitations, as the entire dataset could not be displayed in a single graph. DTPA: diethylenetriamine pentaacetate. a, b, c: Significantly different from their respective controls. Superscript indices 1, 2, 3, and 4 indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

This forms the basis of the selectivity index, which is essential for a compound to be considered anticancer: namely, its cytotoxic effect must be significantly lower in healthy cells than in cancer cells. Our results once again confirm this property of these four anticancer-antifungal drugs. In contrast, for the remaining drugs from different pharmacological classes that exhibited lower cytotoxicity, the cytotoxic effect was significantly higher in the healthy MCF-10A cells. This indicates that these drugs do not possess the potential to be developed as alternative anticancer agents.

The study proceeded with the four most cytotoxic anticancer–antifungal drugs, including pepstatin A, to test their potential effects on the proteasome. As a

standard measure of cytotoxic potency, the half-maximal inhibitory concentration (IC_{50}) reflects the concentration of a compound required to reduce cell viability by 50%. To further evaluate the therapeutic potential of the test compounds, the selectivity index (SI) was calculated as the ratio of IC_{50} in non-malignant (MCF-10A) to malignant (MCF-7 or MDA-MB-231) cell lines. A higher SI value indicates greater specificity for cancer cells over normal cells, with an SI ≥ 10 generally regarded as the threshold for promising candidates [21].

In MCF-7 cells, the highest selectivity was observed with tamoxifen. indicating strong cytotoxic activity across both estrogen receptor-positive and triple-negative breast cancer lines. In non-malignant MCF-10A cells, its IC_{50} was significantly higher at 58.91 μM, resulting in SI values of 4.7 (MCF-7) and 4.1 (MDA-MB-231), which supports its preferential toxicity toward cancerous cells (Table 1). These findings are consistent with previously reported data by Suicmez et al [22] who documented an IC_{50} of 17.26 μM for Tamoxifen in MCF-7 cells. While not exceeding the ideal SI threshold of 10, the values observed still highlight Tamoxifen's relative selectivity and support its continued use as a chemotherapeutic benchmark. The second highest SI was observed with ketoconazole (Table 1). As being primarily an antifungal agent, demonstrated moderate cytotoxic effects with an SI of 3.1 against MCF-7 cells, ketoconazole raised questions about its off-target effects or alternative mechanisms of action in cancer cells. Similarly, Cisplatin and Pepstatin A showed modest anticancer activity but low SI values (~1.5–2), suggesting poor selectivity.

In contrast to expectations, although the cytotoxicity of docetaxel at 100 μM was nearly 6 times greater in MCF-7 cells, the SI calculated from the sigmoid dose–response curve using six different concentrations yielded a value of 0.17 (Table 1).

Table 1.

The IC_{50} values of the test drugs and determined selectivity index (SI)

IC_{50} (μM)	Ketoconazole	Tamoxifen	Cisplatin	Pepstatin A	Docetaxel
MCF-7	25.9	12.6	24.3	326.9	12.0
MCF-10A	81.0	58.9	37.4	495.7	2.0
SI	**3.1**	**4.7**	**1.5**	**1.5**	**0.2**
MDA-MB-231	38.7	14.5	18.4	299.4	34.6
MCF-10A	81.0	58.91	37.36	495.7	2.0
SI	**2.1**	**4.1**	**2.0**	**1.7**	**0.1**

When the SI values of the other breast cancer cell line, MDA-MB-231, despite exhibiting strong activity against MDA-MB-231 cells (IC_{50} = 34.62 μM), showed extremely low selectivity (SI = 0.1), indicating a narrow therapeutic window and potential toxicity to normal cells. In this cell, the highest selectivity

was again observed with tamoxifen (4.1), followed by ketoconazole (2.1), cisplatin (2.0), and Pepstatin A (1.7). In the case of docetaxel, the selectivity was even lower than in MCF-7 cells, with a value of 0.1.
Overall, the data suggests that among the screened drugs, tamoxifen demonstrates the most favorable balance of efficacy and selectivity, making it a strong candidate for further exploration, especially in combination regimens or as a reference compound in a novel drug or formulation.

Proteasome inhibition occurs through the binding of inhibitory compounds to the catalytic sites of the 20S core particle, blocking the proteolytic degradation of ubiquitinated proteins and thereby disrupting cellular protein homeostasis. Proteasome inhibition typically targets the 26S proteasome, a large ATP-dependent complex composed of a 20S catalytic core and 19S regulatory particles. Inhibitory compounds may either block the proteolytic sites within the 20S core or interfere with the regulatory function of the 19S caps, thereby preventing the recognition, unfolding, and translocation of ubiquitinated proteins into the proteolytic chamber. This inhibition leads to the accumulation of undegraded proteins, triggering cellular stress responses such as apoptosis or cell cycle arrest [23].

Proteasome activity measured at one hour and again after 48 hours following the treatment with the selected drugs is shown in Fig. 2. The proteasome inhibition assay can be performed in two ways: With fast-acting and potent inhibitors, results are generally obtained quickly, so a cell lysate is prepared, and the proteasome apparatus within the lysate is directly exposed to the test compound and incubated for a short period (e.g., 1 hour). However, for slower-acting compounds, incubation is carried out with intact cells for a longer duration (e.g., 48 hours) to preserve compound integrity. In our study, when we initially conducted a 1-hour incubation using lysates from all three cell lines, we observed no effect at the IC_{50} doses of the test compounds—except for EPCG, a known proteasome inhibitor (Fig. 2A). In MCF-7 cells, EGCG as positive control, exhibited the most pronounced inhibitory effect on proteasome activity, significantly lower than the control and other compounds, suggesting its potential as a proteasome inhibitor [24].

In contrast, ketoconazole, cisplatin, tamoxifen, and docetaxel increased proteasome activity to varying degrees, possibly reflecting stress-induced compensatory proteolytic responses. Similar trends were observed in MDA-MB-231 cells, where EGCG again showed the strongest inhibition, while tamoxifen and cisplatin resulted in moderate activation. Interestingly, in the non-cancerous MCF-10A cells, EGCG continued to suppress proteasome activity, whereas other compounds—particularly docetaxel—led to notable activation.

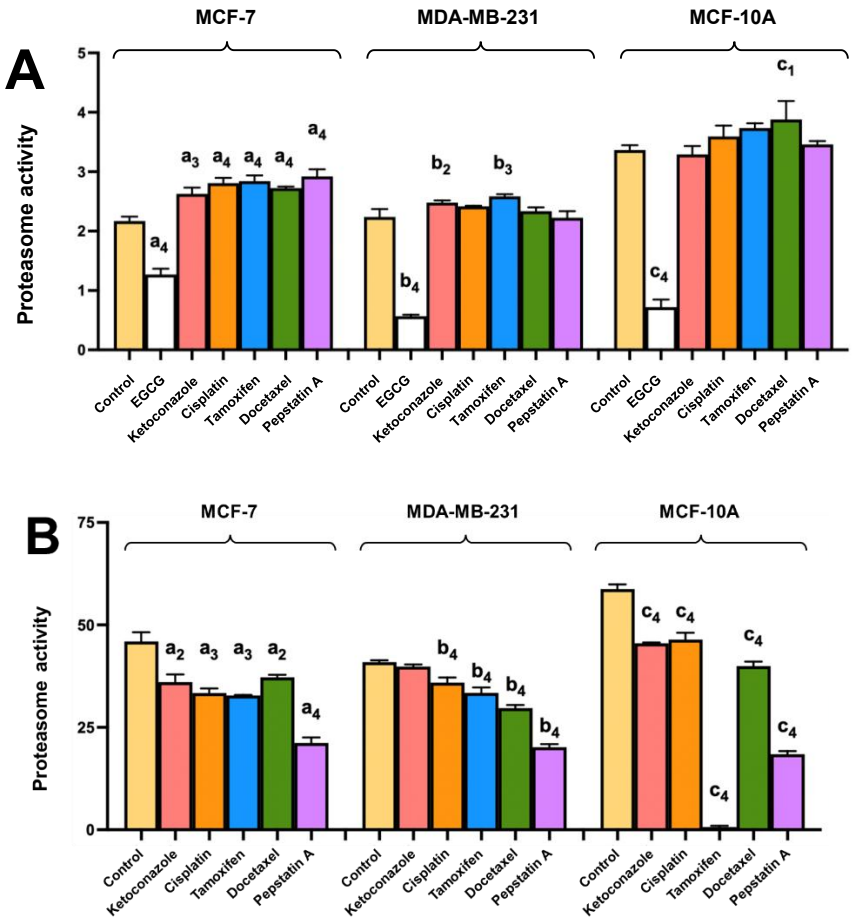

Fig. 2. The relative proteasome activities of three cells in the presence of test drugs. (A) Drugs were incubated with each cell lysate for 1 h, and (B) Drugs were incubated with each intact cell for 48h. EGCG, Epigallocatechin-3-gallate was used as positive control for proteasome inhibition. a, b, c: Significantly different from their respective controls. Superscript indices 1, 2, 3, and 4 indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

However, in subsequent 48-hour incubations with intact cells, significant proteasome inhibition was observed with all tested drugs across all three cell lines, with the strongest effect seen with Pepstatin A (Fig. 2B). The highest inhibition was noted for tamoxifen in healthy MCF-10A cells, where activity dropped nearly to zero after 48 hours. In contrast, tamoxifen caused only marginal inhibition in the two cancer cell lines, MCF-7 and MDA-MB-231 (Fig. 2B).

The activity of the 26S proteasome is tightly regulated by intracellular ATP levels, as ATP is required for proper assembly of the proteasome complex and for the unfolding and translocation of ubiquitinated proteins into the proteolytic core. Low ATP levels can impair the assembly of the 26S complex, thereby reducing

proteasome activity and leading to the accumulation of damaged or misfolded proteins. Therefore, compounds that affect mitochondrial function or cellular energy metabolism may indirectly modulate proteasome activity, making the simultaneous assessment of ATP levels a valuable indicator of both metabolic stress and proteolytic capacity [25]. Fig. 3 presents the ATP levels measured as result of the degradation of ubiquitinated proteins.

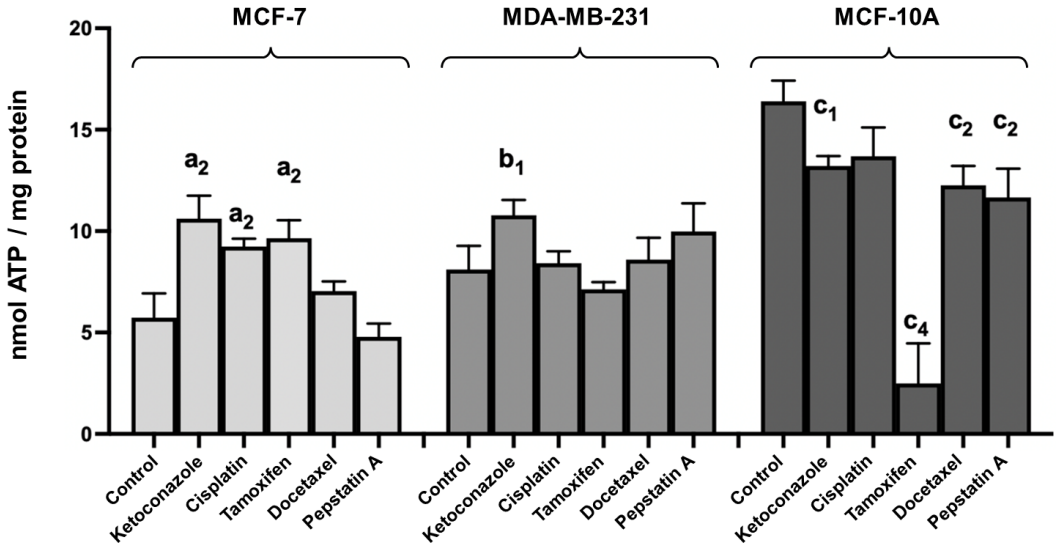

Fig. 3. The ATP levels determined as result of degradation of ubiquitinated proteins

As shown in Fig. 3, ATP levels varied notably among cell lines and treatments. In MCF-7 and MDA-MB-231 cells, ketoconazole and cisplatin induced an increase in ATP levels, suggesting maintained or enhanced metabolic activity. In contrast, tamoxifen significantly reduced ATP levels in MCF-10A cells, indicating potential mitochondrial dysfunction or energy stress, which may correlate with its proteasome inhibitory effect observed in the same cell line.

Similarly, it was reported [26-28] that the ATP level of breast cancer cells decreased after tamoxifen treatment. The use of tamoxifen seems to lead to the upregulation of the expression of metastasis-associated protein MTA1, which further destroys mitochondrial function, while drug-resistant cells meet their energy needs through enhanced autophagy and the enhancement of autophagy may be the result of the increased energy demand of tumor cells and the anti-stress response of tumor cells.

6. Conclusions

This study investigated the proteasome-inhibitory potential of various compounds, including tamoxifen and other established anticancer agents, using *in vitro* models of human breast cancer cells (MCF-7 and MDA-MB-231) as well as

a non-tumorigenic epithelial cell line (MCF-10A). Through cytotoxicity screening, tamoxifen, docetaxel and ketoconazole emerged as the most potent compounds, with tamoxifen exhibiting a favorable selectivity index and notable off-target effects on the proteasome. This repurposing approach highlights the therapeutic potential of clinically approved drugs beyond their primary indications, offering alternative strategies for targeting proteasomal function in cancer. Proteasome activity assays revealed compound- and cell line–specific effects; EGCG consistently inhibited proteasome function across all models, validating the assay and serving as a positive control. In contrast, tamoxifen and cisplatin were found to increase proteasome activity in certain contexts, possibly due to stress-induced compensatory mechanisms. Additionally, ATP measurements provided mechanistic insight, showing that compounds affecting mitochondrial function or energy metabolism can indirectly influence proteasomal efficiency. Tamoxifen, in particular, significantly reduced ATP levels in MCF-10A cells, further supporting its association with proteasome inhibition.

Ultimately, this study contributes valuable insight toward the rational design of selective, less toxic proteasome inhibitors for future oncological applications.

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