Brief Report

XMU-MP-1, the Hippo Signaling Pathway MST-1 Kinase Inhibitor, Prevents the Development of Drug Resistance to Doxorubicin in Hematological Tumor Cells

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XMU-MP-1, the Hippo Signaling Pathway MST-1 Kinase Inhibitor, Prevents the Development of Drug Resistance to Doxorubicin in Hematological Tumor Cells

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Abstract

Background/Objectives: Search for the new drugs capable of suppressing the development of drug resistance in tumor cells is extremely important for clinical practice. Cell signaling pathway inhibitors that control cell proliferation and death can be used in the complex therapy of malignant tumors. **Methods**: *Cell cycle assay by flow cytometry*, In Vitro *Cell Viability Assay* Cells chemosensitivity was analyzed by direct cell counting after trypan blue staining using microscope. **Results**: In the present work, we have shown that the combined action of doxorubicin and XMU-MP-1, the inhibitor of the MST1/2 kinase in the Hippo signaling pathway, prevents the development of drug resistance in Namalwa cells and significantly slows it down in K562 cells. and restores the sensitivity of resistant K562 cells to doxorubicin. We have shown that the combined action of doxorubicin and XMU-MP-1, causes a significant decrease in cell division rate and leads to the death of hematological tumor cells the Burkitt's lymphoma Namalwa, and myeloma K562 cells compared to monotherapy. Cell cycle analysis has demonstrated that the combined action of XMU-MP-1 and doxorubicin results in a catastrophic disruption of the cell cycle, and a significant increase in the number of cells undergoing apoptosis containing fragmented DNA. **Conclusions**: Thus, XMU-MP-1 can potentially be used in combination with anthracyclines for the treatment of hematological malignancies and, in particular, the drug-resistant forms of cancer.

Keywords: drug resistance; hippo pathway; XMU-MP-1; cell cycle; anthracycline

1. Introduction

Malignant tumors of hematopoietic and lymphoid tissue are divided into two large groups – lymphomas and leukemias. Many of them are characterized by unfavorable prognosis and low survival rate.

Doxorubicin is used to treat many different malignancies including lymphoblastic leukemias and non-Hodgkin's lymphomas and is often considered as the first-line drug. Its activities include DNA intercalation, topoisomerase II inhibition, and reactive oxygen species formation, all leading to DNA damage, cell cycle disturbance, apoptosis, and cell death. However, this drug being highly efficient, its administration often leads to significant side effects, such as high cardiotoxicity, necrotizing colitis, and reduced blood cell count, as well as acquired drug resistance. Therefore, an important clinical challenge is to develop ways to reduce the effective therapeutic dose of doxorubicin and prevent drug resistance development.

Higher efficiency and selectivity of chemotherapy drugs can be achieved by combining them with the compounds that modulate the work of cell signaling pathways. Drugs that modulate the Hippo signaling pathway are considered among the potential drug groups for the combination therapy of cancer [1].

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The Hippo signaling pathway is an evolutionary conserved signaling cascade that regulates many biological processes. The Hippo signaling pathway core in mammals consists of the kinase cascade including the MST1 (serine-threonine kinase 4 (STK4)) and MST2 (serine-threonine kinase 3 (STK3)) kinases and the LATS1 and LATS2 kinases, as well as the downstream effectors – the transcription coactivators YAP and TAZ. These key components of the Hippo pathway control the transcriptional programs involved in cell proliferation, survival, motility, stem cell maintenance and cell differentiation, as well as tissue regeneration and organ growth, performing complex regulation through the formation of biomolecular condensates [2].

Tumor cells with impaired Hippo signaling regulation not just overcome the internal cell death mechanisms, but also show resistance to chemotherapeutic drugs or targeted molecular therapy, which is another factor contributing to cancer recurrence [3]. Genetic inactivation of MST1 or high YAP expression in hematological tumors, in contrast to epithelial tumors, leads to the inhibition of hematological tumor growth and activation of apoptosis [4].

XMU-MP-1 (4-((5,10-dimethyl-6-oxo-6,10-dihydro-5H-pyrimido [5,4-b]thieno[3,2-e][1,4]diazepin-2-yl)amino)benzenesulfonamide), the potent reversible selective inhibitor of the mammalian sterile 20-like kinases 1 and 2 (MST1/2), the key molecules in the Hippo signaling pathway, has been described. XMU-MP-1 activates the downstream effector, the Yes-associated protein (YAP), by blocking the activity of the MST1 and MST2 kinases [5,6].

XMU-MP-1 has demonstrated very good pharmacokinetics in rats, with the half-life period of 1.2 hours and bioavailability of 39.5%. Maximum YAP inhibition is achieved between 1.5 and 6 hours after the intraperitoneal administration of the drug (1--3 mg/kg) [6]. XMU-MP-1 is pyrogen-free in a wide range of concentrations including potentially therapeutic. Preclinical studies of XMU-MP-1 have shown the absence of toxic effects, both when administered in the suggested therapeutic dose and in the four-times higher dose [6–9].

Therapeutic effects of MST1 kinase activity suppression have been described, in particular, the role of XMU-MP-1 in the treatment of diabetes, osteoarthritis, obesity, liver and intestine pathologies and improve the survival of cardiomyocytes, stimulates platelet recovery after immune thrombocytopenia [7—13]. Thus, XMU-MP-1 can be used in clinical practice given there being a reasonable mode of application for it.

However, we have found that XMU-MP-1 inhibits the growth of human hematological tumor cells by blocking the cell cycle and causing apoptosis [14].

In the present study, we used two human tumor cell lines, including the doxorubicin-sensitive/resistant K562 chronic myeloid leukemia cells and the Burkitt's B-cell lymphoma Namalwa cells, to investigate whether XMU-MP-1 can inhibit the development of drug resistance in tumor cells and reverse drug resistance. These cancer cell lines were used because chronic myeloid leukemia (CML) is one of the most common malignant hematological neoplasms, and Burkitt's lymphoma is one of the fastest growing tumors, both of them being difficult to treat using chemotherapy and known to develop drug resistance quite rapidly. In the present work, we have found that XMU-MP-1 can inhibit the development of doxorubicin drug resistance in Namalwa cells, impairs the induction of doxorubicin resistance in K562 line, and increase the sensitivity of cancer cells, in particular, resistant cancer cells to doxorubicin in vitro.

2. Results

2.1. XMU-MP-1 and Doxorubicin Arrested Cell Cycle and Induced Apoptosis in Hematological Tumor Cells

It is well-known that cell treatment with low doses of doxorubicin causes mitotic catastrophe [10,11,20]. We found that XMU-MP-1 also causes mitotic catastrophe, which is characterized by cell cycle arrest and a unique nuclear morphology, including the presence of micro- and/or multi-nuclei. Microscopic examination can also reveal many apoptotic changes, accompanied by nuclei fragmentation, cell swelling, and noticeable changes in the cell size - from cell fragments to large swollen cells. Co-treatment of cells with the MST-1 kinase inhibitor XMU-MP-1 and doxorubicin

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further increased the number of cells with mitotic catastrophe morphology. The normal appearance of these cells could not be restored when they were transferred into the fresh medium, and they continued to die. This motivated us to investigate how these two drugs affect the cell cycle separately and in combination. We have previously shown that in the Namalwa cells, XMU-MP-1 induces cell cycle arrest at the G2/M phase and activates genes that regulate the programmed cell death [14].

We estimated the effects of XMU-MP-1 and doxorubicin on the cell cycles of the Namalwa, K562, and Jurkat lines using flow cytometry. The flow cytometry results showed that the treatment with XMU-MP-1 or doxorubicin individually arrested the cell cycle in these cells at the G2/M and G1/S phases, respectively (Figure 1A). We demonstrated that the combined treatment with XMU-MP-1 and doxorubicin further enhances the pathological cell cycle changes in all three cell lines. Moreover, the treatment of the K562, Namalwa, and Jurkat cells with XMU-MP-1 in combination with Dox for 24 or 48 hours led to an increased subG1 peak, which means that it induced stronger apoptosis than monotherapy (Figure 1B).

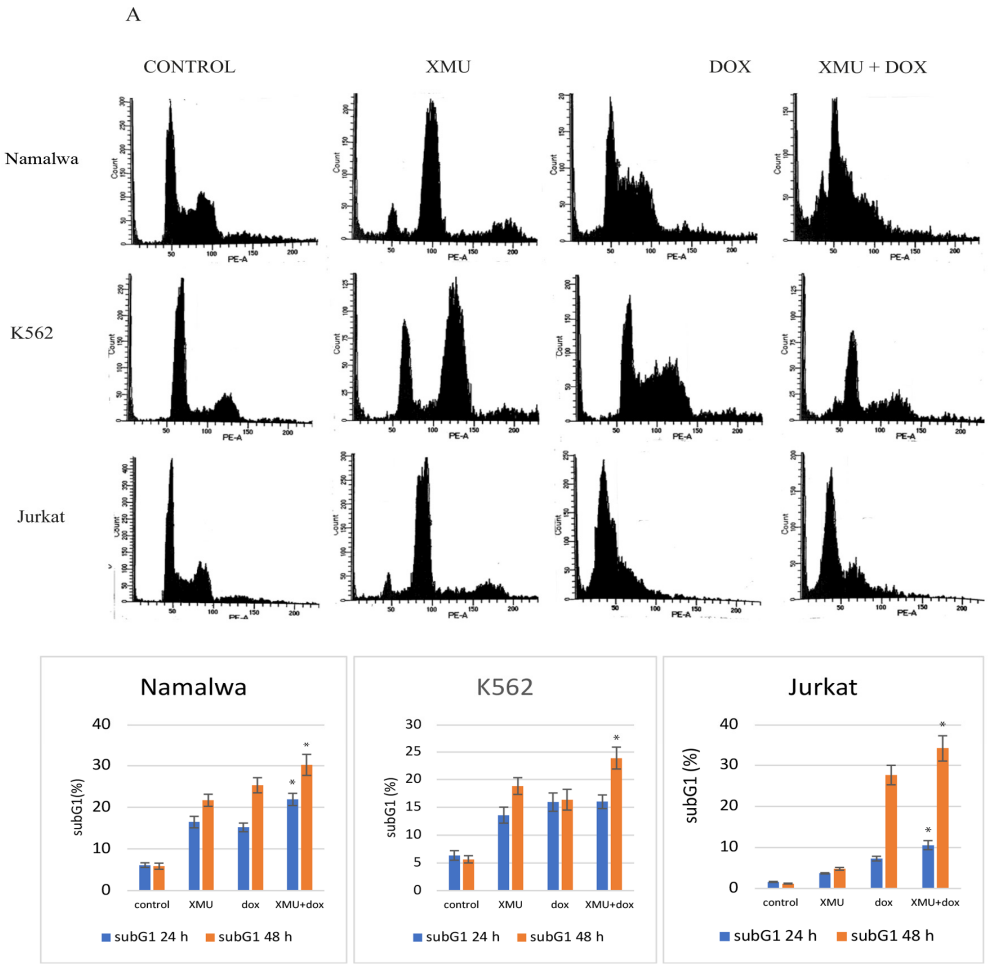

Figure 1. Effect of XMU-MP-1 and doxorubicin on cell cycle and apoptosis. A. Flow cytometry analysis in the Namalwa, K562, and Jurkat cells. **A**. Cell distribution through the cell cycle phases obtained by PI staining of non-treated cells *(only DMSO)* and the treatment with 1.25 μM XMU-MP-1, or with 0.6 μM doxorubicin, or with 1.25 μM XMU-MP-1+0.6 μM doxorubicin for 24 h. **B**. Cell cycle analysis for Namalwa, K562, and Jurkat cells, showing the mean percentage of cells in the sub-G1 phases apoptotic cells. Bar height reflects the mean percentage. Plots show the mean ± SEM for three independent biological experiments. T-tests were performed and asterisks indicate a significant difference compared to the monotherapy cells (*P<0.05).

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2.1.1. XMU-MP-1 Prevents the Development of Doxorubicin Drug Resistance in the Namalwa and K562 Cells

We have previously shown that XMU-MP-1 has a specific effect on cell death in the hematopoietic tumor cells Namalwa, K562, Jurkat, and others [14].

We set out to investigate whether XMU-MP-1 treatment can prevent the development of doxorubicin resistance. We treated Namalwa and K562 cells with doxorubicin at the concentrations of 0.0375; 0.075; 0.15; 0.3; 0.6 and 0.9 μM, as well as with XMU-MP-1 at the concentrations of 0.625; 1.25 and 2.5 μM, and performed long-term observations of the cells exposed to each compound individually and to the combination of doxorubicin and XMU-MP-1.

We found that the pattern and the rate of cell death strongly depended on the doxorubicin and XMU-MP-1 application regimen, as well as on the cell line.

The most pronounced cell population growth suppression effect was achieved in Namalwa and K562 cells when they were pre-cultivated in the presence of XMU-MP-1 before the addition of doxorubicin.

For the Namalwa cells, the strongest cytotoxic and cytostatic effect was achieved when the cells were pre-incubated with XMU-MP-1 for 2 days, followed by removing XMU-MP-1, and then adding doxorubicin. In this case, the cells died much more efficiently than in the case of cultivation in the presence of XMU-MP-1 and doxorubicin together.

We have demonstrated that pretreatment of the Namalwa cells with XMU-MP-1 suppressed the development of drug resistance to doxorubicin and led to the 100% cell death by around the day 11—16th of cultivation (Figure 2A). This effect was the most pronounced at the XMU-MP-1 concentration of 0.6 μM and doxorubicin concentrations of 0.15 and 0.3 μM, that is, at the XMU-MP-1:Doxorubicin ratio of approximately 4 : 1 - 2 : 1.

Monotherapy with doxorubicin at the concentrations of 0.15 and 0.3 μM initially caused a considerable cell death. However, after about 14--20 days, secondary growth of the cell population began and progressive development of drug resistance could be observed. In the case of the XMU-MP-1 monotherapy, the Namalwa cells died efficiently, and by the day 11--14, no more than 1% of the original cell population remained (from day 0). Nevertheless, after about 25--30 days, the cell population began to increase in size, which means that the formation of drug resistance started. If XMU-MP-1 treatment is performed for only 48 hours, and cells are then washed and cultured in the fresh medium, then after a short period of cell death, the cell population starts to increase and by the day 9 reaches approximately 45--50% of the initial population of the day 0.

The K562 cells die much more efficiently if doxorubicin is added after pre-cultivation in the medium containing XMU-MP-1, and cells are further cultured in the XMU-MP-1 + doxorubicin medium.

Maximum doxorubicin resistance suppression effect is achieved in the K562 cells by pretreating the cells with XMU-MP-1 for 3--5 days and subsequently culturing them in the XMU-MP-1+doxorubicin medium. After 26 days, less than 0.8% of the initial cell population remain alive (control – day 0 of the experiment), and by the day 54, just few live cells are still present. They stop dividing, but die very slowly. For the K562 cells, doxorubicin concentrations of 0.6 and 0.9 μM (0.348 μg/mL and 0.522 μg/mL respectively) and XMU-MP-1 concentration of 2.5 μM are the most effective for the combined use, which means that XMU-MP-1:Doxorubicin ratio is approximately 4 : 1 – 3 : 1. In the case of doxorubicin monotherapy, the cells died rapidly at first, but after about 14--20 days, drug resistance to doxorubicin started to form, and the number of cells began to slowly increase. In the case of XMU-MP-1 monotherapy, cells died rapidly; however, after about 20--25 days, XMU-MP-1-resistant cells started to appear (Figure 2B).

It is well-known that when the K562 cells are cultured in the presence of doxorubicin, they rather quickly develop a multi-drug resistance phenotype [15]. Our results indicate that XMU-MP-1 significantly delays the formation of drug resistance in the K562 myeloblastic leukemia line cells, and also prevents the formation of drug resistance in the Burkitt's B-cell lymphoma Namalwa line.

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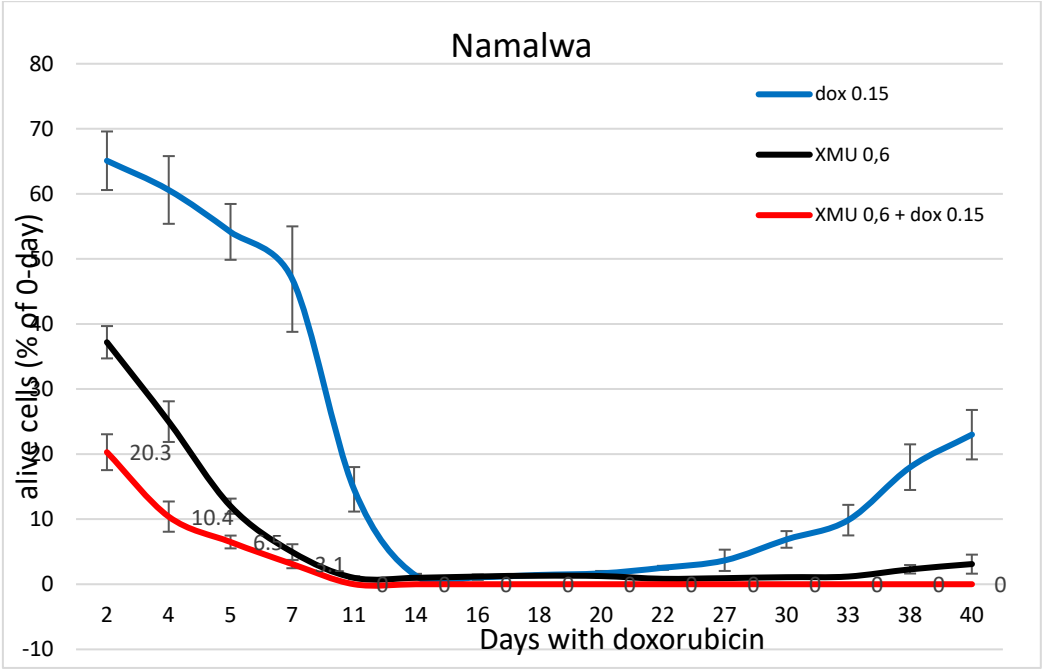
A.



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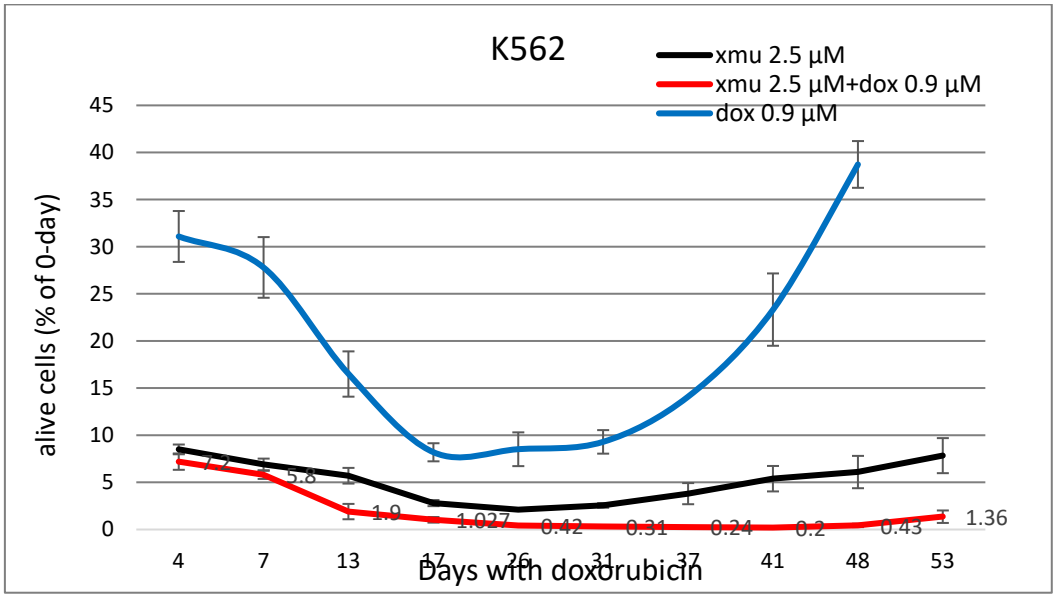

Figure 2. Pretreatment with XMU-MP-1 prevents the development of doxorubicin drug resistance in the Namalwa and K562 cells. **A.** Namalwa. (XMU 0.6+dox 0.15) – Namalwa cells were incubated for 2 days in the medium containing 0.6 µm XMU-MP-1, then cells were pelleted and transferred to the medium containing 0.15 µM doxorubicin; (XMU 0.6) – cells were incubated in the medium containing 0.6 µM XMU-MP-1, and (dox 0.15) – cells were incubated in the medium containing 0.15 µM doxorubicin. **B.** XMU-MP-1 slows the development of drug resistance to doxorubicin in the K562 cells. (XMU 2.5+dox 0.9) – K562 cells were incubated for 4 days in the medium containing 2.5 µM XMU-MP-1, then cells were incubated in the medium containing 2.5 µM XMU-MP-1+0.9 µm doxorubicin; (XMU 2.5) – cells were incubated in the medium containing 2.5 µM XMU-MP-1, and (dox 0.9) – cells were incubated in the medium containing 0.9 µm doxorubicin. The plots show % of live cells relative to the 0-day control. Shows the mean ± SEM for three independent experiments.

2.1.2. XMU-MP-1 Restores the Sensitivity of the K562 Cells to Doxorubicin

To test whether XMU-MP-1 can restore the sensitivity of drug-resistant cells to the cytostatic, we induced resistance to doxorubicin in the K562 cell line by growing cells in the presence of incremental concentrations of doxorubicin (37--600nM) increasing doxorubicin concentration every two weeks

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during 60 days [15]. Doxorubicin sensitivity of the obtained sublines was measured in the MST assay and by direct counting of live cells under the microscope. Long-term growth in the presence of doxorubicin increased the resistance (IC (50)) of K562 cells in the concentration-dependent way. Two doxorubicin-resistant K562 sublines (DR1 and DR2) (600 nM) and non-resistant original cells (NoDR) were used for further work.

Doxorubicin-resistant K562 cells (DR1 and DR2) were cultivated in the medium containing 0.6 μm doxorubicin for 30 days. The cells were then washed to remove the medium containing doxorubicin and inoculated into the medium containing XMU-MP-1, or 0.9 μm doxorubicin, or XMU-MP-1 + 0.9 μm doxorubicin, or the DMEM medium with only DMSO, cultivated for 8 days, and the number of live cells was then counted. The original cells with no drug resistance (NoDR) were cultured under the same conditions. Upon 8 days of culturing, live cells were counted under the microscope following trypan blue staining. The percentage of live cells in each group was calculated relative to the same cell group cultivated without the addition of XMU-MP-1 or doxorubicin.

As it can be seen from Figure 3, the development of resistance to doxorubicin also increases the resistance of cells to XMU-MP-1. However, their combined action suppresses the resistance to both drugs.

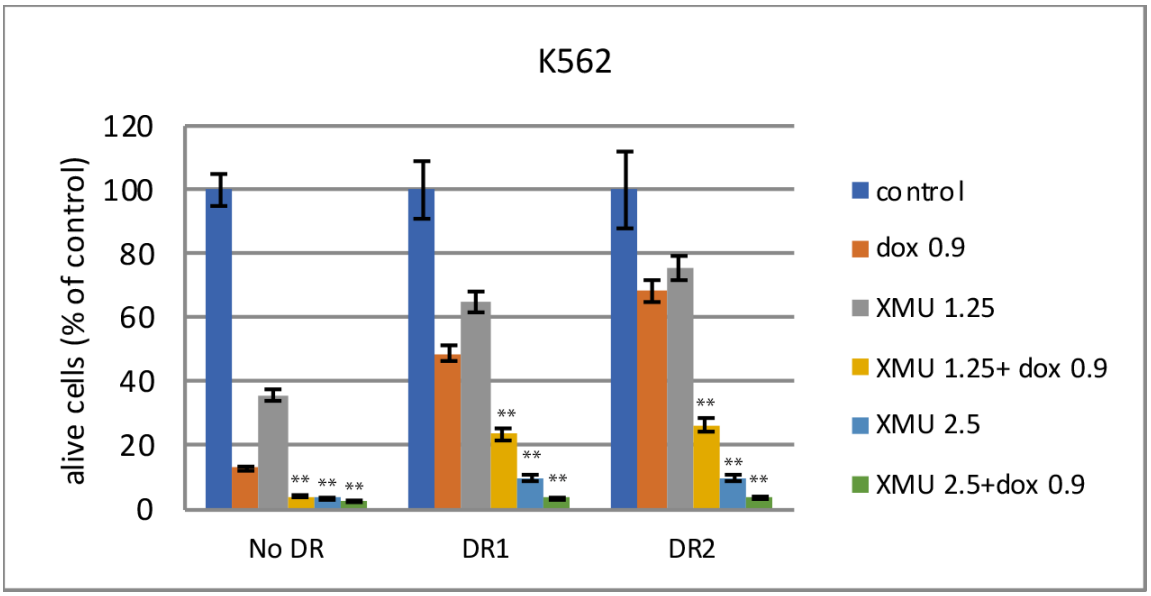

Figure 3. XMU-MP-1 efficiency in reverting the doxorubicin resistance in the K562 cells. Cells with no drug resistance (NoDR) and two cell sublines with drug resistance to doxorubicin (DR1 and DR2) were treated with XMU-MP-1 (1.25 or 2.5 μM) and doxorubicin (0.9 μM) during 8 days. DMSO alone was added to the control group. The plot shows % of live cells relative to the control. The plots show the mean ± SEM for three independent experiments. t-tests were performed, and asterisks indicate P-values relative to the doxorubicin treated cells (**P<0.01).

3. Discussion

Cell signaling pathway inhibitors that control cell proliferation and death can be used in the complex therapy of malignant tumors. A properly chosen signaling pathway inhibitor can perform several functions in a pharmaceutical composition – it can enhance the efficiency of an antitumor component against the tumor cells, including by suppressing drug resistance development, and protect the body from the side effects of the cytostatics. Treatment efficiency depends on many factors, for example, the choice of drug doses, treatment regimens, etc. Suboptimal selection of parameters when using signaling pathway inhibitors may lead to the opposite effect: instead of inhibiting tumor growth, it may promote it. This makes it important to search for the optimal combinations of chemotherapy and drugs with the effects on signal transmission pathways in the

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cells, as well as to expand the repertoire of drugs for antitumor therapy, especially for the tumor cells which are resistant to traditional cytostatics.

In the present work, we have shown that the treatment with XMU-MP-1, the MST1/2 kinase inhibitor in the Hippo signaling pathway, prevents drug resistance development in the lymphoma and myeloma cell lines, and restores the sensitivity of resistant cells to doxorubicin.

A determinant of XMU-MP-1 efficiency in combination with doxorubicin may be its intervention into the mechanisms involved in multidrug resistance development. The mechanisms of multidrug resistance observed in different hematological neoplasms may include overexpression of the ABC transporter protein genes, intensified drug metabolism due to altered molecular targets, defects in the apoptosis mechanism, cell cycle disruption, glutathione-S-transferase overexpression, resistance to the microenvironment, and/or strengthening DNA repair mechanisms [16,17]. The resistance of cancer cells to doxorubicin may be linked to the inhibition of ferroptosis. SH3GL1-activated FTH1 inhibits ferroptosis and confers doxorubicin resistance in diffuse large B-cell lymphoma. [18]. STAT3 mediates the multidrug resistance of Burkitt lymphoma cells by promoting an antioxidant feedback mechanism [19].

Whole-transcriptome analysis of the Namalwa cells treated with XMU-MP-1, which we performed earlier [14], did not reveal any significant decrease in the ABC transporter mRNAs expression, including ABCC1(MRP1), ABCC2(MRP2), MVP, and ABCG2(BCRP). It seems that the combination of XMU-MP-1+doxorubicin may inhibit resistance development in hematological tumor cells through the mechanisms that are different from acting on the ABC transporter genes. Although it cannot be excluded that the effects they exert on the Hippo signaling pathway may extend to the post-transcriptional modifications of ABC transporters, reducing their activity by these means [20].

The suppression of drug resistance may be associated with the development of mitotic catastrophe when the two drugs act together. Mitotic catastrophe is one of the defense mechanisms that senses mitotic damage and prevents the survival and/or reproduction of cells with mitotic errors. Induction of mitotic catastrophe seems to be a promising strategy for increasing the sensitivity of tumor cells to chemotherapy. Usually, mitotic catastrophe is caused by chemotherapeutic agents in the low doses, which are not enough to cause rapid cell death [21]. The characteristic biochemical sign of mitotic catastrophe is mitotic arrest and the presence of multinucleated polyploid cells. This state is considered to be the preliminary stage of cell suicide via apoptosis, necroptosis, or autophagy [21]. Mitotic catastrophe can result in the elimination of cells with defective mitosis and genomic instability, thereby preventing carcinogenesis. However, some cells may avoid mitotic catastrophe, which results in their premature exit from mitosis and transition to interphase.

In hematological tumor cells, XMU-MP-1 like doxorubicin causes mitotic catastrophe. We have previously shown that XMU-MP-1 significantly changes the expression of the cell cycle regulatory genes, which leads to mitosis arrest in the G2/M phase and apoptosis activation [14]. At the same time, doxorubicin blocks the cell cycle at the G1/S stage.

We suggest that the combination of XMU-MP-1 and doxorubicin increases the probability of the cell's "non-return" and cell death by inhibiting the key regulators of cell cycle and, thereby enhancing the individual cytotoxic effects of doxorubicin and XMU-MP-1. This may lead to the suppression of drug resistance development. However, this hypothesis needs further investigation.

Another mechanism which may account for the enhanced effect of doxorubicin when combined with XMU-MP-1 can be connected to the fact that in hematological tumors, DNA damage leads to the activation of a proapoptotic pathway based on the nuclear relocation of the ABL1 kinase. Previous studies have shown that low cellular levels of YAP1 can block the ABL1-induced apoptosis in hematological malignancies. At the same time, genetic inactivation of MST1 restores YAP1 levels causing cell death both in vitro and in vivo [22]. Hence, the simultaneous use of the two tested compounds can lead to an increase in the death rate in the Namalwa cells.

If XMU-MP-1 has the effect of preventing or delaying the development of drug resistance, as our results indicate, it should become an indispensable part of the cytotoxic arsenal.

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Another advantage of using the cytostatic+XMU-MP-1 composition is the dual effect of XMU-MP-1. On the one hand, XMU-MP-1 potentiates the effects of the cytostatic in hematological tumors and thereby makes it possible to reduce the effective dose of doxorubicin. On the other hand, XMU-MP-1 mitigates the toxic effects of chemotherapy and radiation therapy. Pretreatment with XMU-MP-1 significantly reduced the damage caused to the small intestine by total body irradiation with a dose of 9 Gy, increased the average survival time in mice exposed to a lethal radiation dose, and restored the impaired function of hematopoietic stem cells after total body irradiation [12]. XMU-MP-1 stimulates platelet recovery [13], alleviates the cytotoxic effects of paclitaxel on hair follicle cells [23], activates liver regeneration in humans [6], and improves cardiomyocyte survival and preserves cardiac function, which results in a lower fibrosis level [10].

It is possible that the combined use of doxorubicin and XMU-MP-1 may reduce the effective dose of doxorubicin used in the treatment of hematological cancers, and thereby significantly reduce the life-threatening toxic effects of doxorubicin such as free radicals formation, which underlies the drug's cardiotoxicity, thrombocytopenia, leukopenia, and necrotizing colitis. Our previous studies have also shown that XMU-MP-1 enhances the antitumor activity of other chemotherapeutic drugs such as etoposide and cisplatin towards Burkitt's lymphoma cells [24]. Thus, XMU-MP-1 can potentially become an effective component in the combination therapy of hematological tumors.

Cytotoxic activity against hematological tumors and the ability to overcome drug resistance allow us to consider XMU-MP-1 as a promising candidate drug in combination with anthracyclines for the treatment of hematological tumors, especially the drug-resistant ones.

This is the first report on the ability of XMU-MP-1 to prevent drug resistance development. The clinical implications of these findings are of huge importance. They suggest that anthracycline-sensitive tumors should probably be treated with XMU-MP-1 from the very outset to get maximum benefit from combining these drugs.

4. Materials and Methods

4.1. Cell Lines

Human cell lines: B-cell lymphoblastoid Burkitt lymphoma Namalwa, T-cell lymphoblastoma Jurkat, and human chronic myeloid leukemia K-562 were obtained from the Russian Cell Culture Collection, Institute of Cytology, St. Petersburg, Russia. Cells were maintained in DMEM (GIBCO, Thermo FisherScietific, United States) with 10% FCS (FBS; HyClone, United States), 100 U/mL penicillin, and 100 μg/mL streptomycin, 5% CO_2.

4.2. Cell Cycle Assay by Flow Cytometry (FCM)

Cells were inoculated into 60 mm^2 dishes and treated with 1.25 μM XMU-MP-1, or with 0.6 μM doxorubicin, or with 1.25 μM XMU-MP-1+0.6 μM doxorubicin. Only DMSO was added to the control group. Measurements were taken in three sessions. 24 h and 48 h after the treatment, treated cells were fixed in 70% ethanol and stored at -20^{0}C. For the *FCM* assay, frozen cells were washed with PBS, stained with 50 μg/mL propidium iodide (PI), and treated with 100 μg/mL RNase A (Sigma, cat. no. R-4875) at 37^0 C for 60 min. Cells were analyzed in the BD LSRFortessa Cell Analyzer using the provided BD Bioscience software (Becton Dickinson, San Jose, CA, United States). Cells were distributed into the G1, S, G2/M, and sub-G1 subpopulations based on their fluorescence.

4.3. In Vitro Cell Viability Assay

To test the cytotoxicity of mono- and combined treatments, cells were inoculated into 96-well plates at the density of 30,000 (Namalwa) or 10,000 (K562) cells per well in 100 μL of the DMEM medium (PanEco, Russia) supplemented with 10% fetal calf serum (BioSera, France). Drug combinations were added to the wells. Cells were cultured at +37 °C in 5% CO_2 in the cultural plates (TRP, Switzerland) in DMEM (PanEco, Russia). XMU-MP-1 cytotoxicity was assessed using the

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CellTiter 96 AQueous One Solution kit (Promega, United States). Antiproliferative activity of XMU-MP-1, doxorubicin, and XMU-MP-1+doxorubicin was studied by treating cells with XMU-MP-1 at the concentrations of 0.6; 1.25, and 2.5 μM, doxorubicin, at 0.0375; 0.075; 0.15; 0.3; 0.6, and 0.9 μM and their combinations (at least three replicates for each compound concentration). Cells with only DMSO and no tested compounds added served as the control. After 48 and 72 h of incubation, antiproliferative and cytotoxic effects of XMU-MP-1 were analyzed according to the manufacturer's protocol. The optical density of solutions was measured at 490 nm using the Chameleon V plate reader (Hydex Oy, Finland). The amount of the formazan product calculated based on the absorbance rate at 490 nm is in direct proportion to the number of live cells in the culture.

4.4. In Vitro Model System to Study Drug Resistance Development

For each drug and their combinations were determined optimal concentrations for long-term cultivation were chosen. Cells were inoculated into the 25 cm flasks in the amount of 4 million cells per flask.

The following culturing options were used for the Namalwa cells:

1. Cultivation for 48 hours in the complete DMEM medium in the presence of XMU-MP-1 (Sigma) (0.625, 1.25, or 2.5 μM), then cell pelleting and resuspending in the complete DMEM medium, adding doxorubicin (0.15 μM or 0.3 μM), and then long-term culturing
2. Cultivation for 48 hours in the complete DMEM medium containing XMU-MP-1 (0.625, 1.25, or 2.5 μM) followed by cell pelleting, resuspending in the fresh medium, and adding XMU-MP-1 (0.625, 1.25, or 2.5 μM)
3. Cultivation for 48 hours in the complete DMEM medium containing DMSO alone followed by cell pelleting, resuspending in the fresh medium, and adding doxorubicin (0.15 μM or 0.3 μM)
4. The control cells were cultured in the complete DMEM medium containing only DMSO and no tested compounds, only DMSO. The control cells were passaged 1/10 every 5 days.

The following culturing options were used for the K562 cells:

1. Cultivation for 96 hours in the complete DMEM medium in the presence of XMU-MP-1 (Sigma) (1.25 or 2.5 μM), then cell pelleting and resuspending in the fresh medium containing XMU-MP-1 (1.25 or 2.5 μM) + doxorubicin (0.6μM or 0.9 μM), and then long-term cultivation.
2. Cultivation for 96 hours in the complete DMEM medium containing XMU-MP-1 (1.25 or 2.5 μM) followed by cell pelleting and resuspending in the fresh medium containing XMU-MP-1 (1.25 or 2.5 μM)
3. Cultivation for 96 hours in the complete DMEM medium with DMSO alone, followed by cell pelleting and resuspending in the fresh medium containing doxorubicin (0.6 μM or 0.9μM)
4. Cultivation in the complete DMEM medium containing only DMSO and no tested compounds. The control cells were passaged 1/10 every 5 days.

The medium was changed every 5 days. Cells chemosensitivity was analyzed by direct cell counting after trypan blue staining using microscope and recording when the survived cells reach the initial density of $2x10^{6}$/8ml (so-called "repopulation"). Toxicity of different concentrations and combinations of the tested compounds was determined based on the cells' viability relative to the control.

4.5. Assessment of Doxorubicin Drug Resistance Overcoming to

To test whether XMU-MP-1 can acts as a drug resistance modulator in the short term, two K562 sublines (DR1 and DR2) resistant to doxorubicin at the concentration of 0.6 μM were obtained.

The original K562 cells with no drug resistance (NoDR) and the derived sublines (DR1 and DR2) were inoculated into the 25 cm flasks in the amount of 4 million cells per flask and treated with 0.9 μM doxorubicin, as indicated above, in combination with XMU-MP-1 (1.25 or 2.5 μM) or without XMU-MP-1 for 8 days. Cells from each subline that were not treated with doxorubicin and XMU-MP-1 were used as the controls.

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The chemosensitivity of the cells was then analyzed by direct cell counting after trypan blue staining using a microscope. Drug resistance of cells using different concentrations and combinations of the tested compounds was estimated based on the cells' viability relative to the control.

4.6. Statistics

All experiments were repeated at least three times. Statistical analysis of the results from the cell proliferation assay, and the flow cytometry assay was conducted using the GraphPad software v10 (GraphPad Software, San Diego, CA, USA). Student's t-tests were used to generate p-values. Error bars represent the standard error of the mean (S.E.M.). (* $P < 0.05$ and ** $P < 0.01$).

5. Conclusions

This work provides the first evidence that XMU-MP-1, the Hippo signaling pathway MST1/2 kinase inhibitor, prevents or significantly inhibits the development of drug resistance in lymphoma and myeloma cell lines. This effect of XMU-MP-1suggests that it has the potential to further enhance the efficiency of doxorubicin in the treatment of hematological tumors. The cytotoxic activity and the ability to overcome drug resistance allow considering XMU-MP-1 as a promising candidate drug for antileukemic therapy.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, E.P. and A.S.; methodology, E.P.; software, A.S.; validation, E.P. and A.S.; formal analysis, E.P.; investigation, E.P. and A.S; resources, E.P. and A.S.; data curation, E.P.; writing—original draft preparation, E.P.; writing—review and editing, A.S.; visualization, E.P.; project administration, E.P. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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