

In vitro comparison of cytotoxic effects of bortezomib resistant U266 myeloma cell line (U266/VELR) on combination of ibrutinib with carfilzomib and lenalidomid drugs

İbrutinib'in carfilzomib ve lenalidomid ilaçlarıyla kombinasyonu ile tek başına kullanımının bortezomib dirençli U266 myeloma hücre hattı (U266/VELR) üzerine olan sitotoksik etkilerinin in vitro karşılaştırılması

Hatice Terzi¹, Merve Ergül²

¹Department of Hematology, Medical Faculty, Sivas Cumhuriyet University, Sivas, Turkey

²Department of Pharmacology, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Turkey

Corresponding author: Hatice Terzi, MD, Department of Hematology, Medical Faculty, Sivas Cumhuriyet University, Sivas, Turkey

E-mail: dr.terzi@hotmail.com

Received/Accepted: December 25, 2019 / December 31, 2019

Conflict of interest: There is not a conflict of interest.

SUMMARY


Objective: Multiple myeloma is still one of the incurable hematologic cancers. It is a common problem of all hematologists to look for new combinations in this malignancy, that treatment steps are rapidly depleted.

Method: The cytotoxic activities of carfilzomib, lenalidomide and their combination with ibrutinib were evaluated using the XTT colorimetric assay on U266B1/BR cells. Cells were seeded into 96-well plates at a density of 1×10^4 cells per well, incubated with various concentrations of ibrutinib, carfilzomib, lenalidomide and their combinations for 24h and 48h.

Results: In our study, we found that adding ibrutinib to carfilzomib and lenalidomide combination in Bortezomib refractory myeloma cell line did not increase cytotoxicity.

Conclusions: Further clinical and experimental studies are needed to demonstrate the efficacy of ibrutinib in which many B-cell malignancy has been demonstrated.

Keywords: Multiple myeloma, cytotoxicity, ibrutinib

 Hatice Terzi

 Merve Ergül

ORCID IDs of the authors:

H.T. 0000-0003-3471-1305

M.E. 0000-0003-4661-8087

ÖZET

Amaç: Multipl miyelom hala tedavi edilemeyen hematolojik kanserlerden biridir. Bu maligniteye karşı tedavi adımlarının hızla tükenmesi nedeniyle yeni kombinasyonların ve tedavi seçeneklerinin aranması hematologların ortak bir problemdir.

Yöntem: Karfilzomib, lenalidomid ve bunların ibrutinib ile kombinasyonlarının sitotoksik aktiviteleri U266B1/BR hücrelerinde kolorimetrik XTT testi kullanılarak değerlendirilmiştir. Hücreler, kuyucuk başına 1×10^4 hücre yoğunluğunda 96 kuyucuklu plakalara ekilerek, 24 saat ve 48 saat boyunca farklı konsantrasyonlarda ibrutinib, karfilzomib, lenalidomid ve bunların kombinasyonları ile inkübe edilmiştir.

Bulgular: Çalışmamızda Bortezomib refrakter miyelom hücre hattında karfilzomib ve lenalidomid kombinasyonuna ibrutinib ilavesi sitotoksiste üzerine anlamlı bir etki oluşturmamıştır.

Sonuç: B hücresi malignitesinin fazla olduğu ibrutinibin etkinliğini göstermek için daha ileri klinik ve deneysel çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Multipl myeloma, sitotoksiste, ibrutinib

INTRODUCTION

Multiple myeloma (MM) is a malignancy of differentiated plasma cells and still considered an incurable cancer. The target of treatment is to achieve long-term disease control. Although the treatment of relapsed/refractory multiple myeloma has partially improved, the fact that the disease cannot be cured has not changed. Therefore, additional treatments are needed. Novel combination therapies incorporating monoclonal antibodies have shown significant promise.

Ibrutinib is an oral inhibitor of Bruton's tyrosine kinase that is used in the therapy of refractory chronic lymphocytic leukemia (CLL) and mantle cell lymphoma. BTK is known to be an essential component of B cell receptor (BCR) signalling and is involved in B cell differentiation, proliferation and survival¹. Many studies have reported an essential role of BCR signalling in the pathogenesis of several B cell malignancies. Many preclinical and clinical studies have shown a profound antitumour activity of ibrutinib in different B cell malignancies, including chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), multiple myeloma, diffuse large B cell lymphoma (DLBCL) and Waldenström's macroglobulinemia^{2,3}.

In this study, we aimed to determine the cytotoxic effect of the second generation proteasome inhibitor Carfilzomib and the immunomodulatory agent Lenalidomide, with ibrutinib, on the Bortezomib-resistant Multiple Myeloma cell line.

MATERIAL AND METHODS

Cell culture and development of BTZ-resistant MM cell lines

U266B1 (TIB-196, Human Multiple Myeloma cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was routinely grown in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco), and 100 mg/ml streptomycin (Gibco) at 37°C and 5% CO₂. The BTZ-resistant (BR) U266B1 subline (U266B1/BR) was established by stepwise increasing BTZ (LC laboratories, Woburn, MA) concentration over a period of 3 months. These BR cells were adapted to a final concentration of 20 nM BTZ. The stable

genotype of BR cells was confirmed by the BTZ washout experiment for 2 weeks followed by dose-response assays with BTZ.

Preparation of Drug Concentrations

Stock solutions of the drugs (ibrutinib, carfilzomib, and lenalidomide) in DMSO were prepared and each stock solution was sterilized by passing through a 0.2 µm syringe tip filter (DMSO concentration at the highest concentration 0.1%). Then, stock solutions diluted in phenol red-free RPMI-1640 to prepare different concentrations of drugs (0.1 µM - 1 µM - 10 µM - 25 µM - 50 µM - 100 µM) to be applied to the cells. IC₅₀ values were determined by applying the cells at low to high concentrations.

XTT Cell Viability Test

The cytotoxic activities of drugs were evaluated using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) colorimetric assay (Roche Diagnostic) on U266B1/BR cells. Cells were seeded into 96-well plates at a density of 1×10⁴ cells per well in 50 µL of phenol red-free RPMI-1640 culture media, incubated with various concentrations of 50 µL ibrutinib, carfilzomib, lenalidomide and their combinations for 24h and 48h. Following incubation, a mixture of 50 µL XTT labeling solution was added to all the wells and then the plates were maintained at 37°C for 4 h. The absorbance was recorded using an ELISA microplate reader (Thermo) at 450 nm. Evaluation is based on means from at least three independent experiments, each comprising three replicates per concentration level. Cytotoxic effects of drugs and their combinations were determined the dose-response curves were fitted by means of GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

Statistical analysis

The statistical analysis for the assays was performed using GraphPad Prism 7.0 (GraphPad). Data obtained from experiments were expressed as the mean ± standard deviation (SD) and ANOVA test with post hoc Dunnett's test was applied for versus control comparisons. All experiments were repeated three times. P value less than 0.05 is considered statistically significant.

RESULTS

In the present study, our aim was to evaluate the inhibitory effects of ibrutinib, carfilzomib, and lenalidomide on U266B1/BR multiple myeloma cells by applying the drugs in monotherapy and in combination for 24 h and 48 h. Initially, dose-response experiments were performed with each drug alone with an increased concentration (0.1, 1, 10, 25, 50 or 100 μM) and calculated IC_{50} values. As shown in **Figure 1**; ibrutinib, carfilzomib, and lenalidomide showed significant cytotoxicity on U266B1/BR cells at concentrations greater than 10 μM in a dose and time dependent manner ($p <$

0.05). IC_{50} values of ibrutinib, carfilzomib, and lenalidomide were calculated as 67 μM , 81 μM , and 144 μM for 24 h, 121 μM , >100 μM , and >100 μM for 48 h respectively.

Furthermore, a comparison of cytotoxicity between the drug combination showed that ibrutinib+carfilzomib exhibited significant cytotoxicity than ibrutinib+lenalidomide combination. Nevertheless, the combinations did not show significant cytotoxicity to U266B1/BR cells when compared to alone drug application (**Figure 2**).

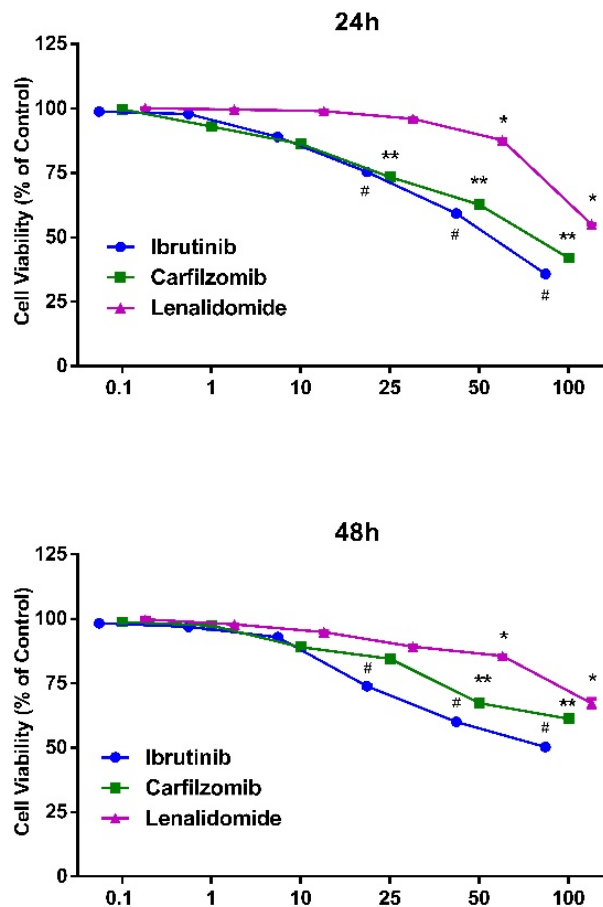


Figure 1: Cytotoxicity as determined by XTT assay. U266B1/BR cells treated with 0.1 to 100 μM of ibrutinib, carfilzomib and lenalidomide for 24 h and 48 h. Data are representative of the mean \pm SEM of three separate experiments done in triplicate.

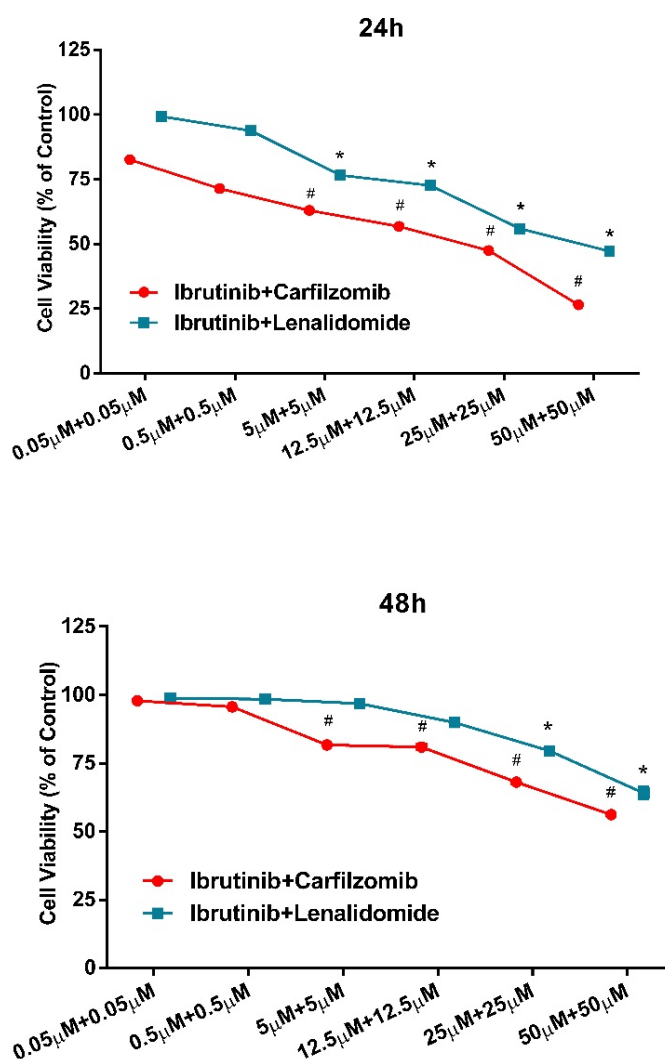


Figure 2: Cytotoxicity as determined by XTT assay. U266B1/BR cells treated with 0.05+0.05 to 50+50 µM of ibrutinib+carfilzomib and ibrutinib+lenalidomide for 24 h and 48 h. Data are representative of the mean ± SEM of three separate experiments done in triplicate.

DISCUSSION

Multiple myeloma (MM) is a malignancy characterized by accumulation of malignant plasma cells within the bone marrow (BM). MM is considered mostly without definitive treatment because of the inability of standard of care therapies to overcome drug-resistant relapse⁴. The increasing number of therapeutic options for patients with multiple myeloma (MM), both in the newly diagnosed and relapsed/refractory settings, has led to improved outcomes including prolonged survival⁵.

Both of these classes of drugs, the IMiDs (including thalidomide, lenalidomide and pomalidomide) and the PIs (including bortezomib, carfilzomib and ixazomib), act through multiple mechanisms to cause myeloma cell death and

prevent cell proliferation^{6,7}. Although these drugs were initially used in relapsed myeloma, the use of IMiDs and PIs in induction therapies has resulted in better patient outcomes, including higher response rates and lower toxicity, when compared with traditional cytotoxic chemotherapy^{8,9}.

Ibrutinib (Imbruvica™) is an irreversible, potent inhibitor of Bruton's tyrosine kinase (BTK). BTK is known to be an essential component of B cell receptor (BCR) signalling and is involved in B cell differentiation, proliferation and survival^{10,11}. Many studies have reported an essential role of BCR signaling in the pathogenesis of several B cell malignancies¹². BTK became a significant goal for the treatment of B cell-derived cancers. Inhibition of BTK disrupts the survival and

proliferation of malignant B cells by inducing apoptosis¹³.

In a clinical study performed by Treon et al., 63 patients with Waldenström's macroglobulinemia were treated with ibrutinib and concluded that it could be used as an alternative^{2,10}.

We planned this study considering that standard treatment can be combined with ibrutinib in patients with relapse/refractory multiple myeloma. However, we found that the combination of carfilzomib, lenalidomide and ibrutinib in the bortezomib refractory cell line did not provide an advantage to standard treatment.

CONCLUSION

In conclusion, we think that the combination of ibrutinib to standard therapy should be studied in more detail in different myeloma cell lines and clinical trials.

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