Clofibrate Demonstrates Efficacy in *In Vitro* Treatment of Lymphoma and Multiple Myeloma

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Abstract. *Background/Aim: Multiple myeloma (MM), a hematological malignancy of monoclonal B-lymphocytes, remains largely incurable and novel treatments are urgently required. Aberrant activation of wingless-related integration site (WNT)/β-catenin signaling has been demonstrated in both lymphoma and MM, rendering its signaling molecules attractive for the development of new targeted-therapies. Clofibrate has proven anticarcinogenic effects attributed to peroxisome proliferator-activated receptor alpha (PPARα) agonism, also affecting WNT-associated signaling molecules. Materials and Methods: The antitumor apoptotic effect of clofibrate at doses ranging from 0.1-600 μM was investigated on four human and one murine myeloma cell lines, as well as in two human lymphoma cell lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay. Results: Clofibrate significantly reduced cell viability in all tested myeloma and lymphoma cell lines in a dose-dependent manner, while healthy cells were hardly affected. Conclusion: Given the known safety profile and induction of apoptosis at low effective doses, our data warrant further investigation of clofibrate as a novel therapy agent in MM.*

Multiple myeloma (MM) is a hematological neoplasm characterized by monoclonal malignant secretory plasma cells in the bone marrow and is mostly accompanied by monoclonal protein in peripheral blood or urine (1). The median age at diagnosis is 69 years, and mainly elderly people are affected (2). Innovative therapy strategies including immunomodulatory drugs such as bortezomib, lenalidomide and thalidomide significantly enriched our therapeutic repertoire and markedly improved both treatment outcome and patient survival. Advanced, cell-based therapeutic approaches might also gain increasing importance (3, 4). However, there remains considerable scope for further therapeutic innovations and alternative treatment strategies since the majority of patients might ultimately experience relapse despite state-of-the-art treatment techniques.

Tumor-specific intra- and extracellular signaling pathways involved in tumorigenesis might represent one of the most auspicious approaches in future oncological treatment regimens as they have been proven to be involved in apoptosis induction, differentiation and regulation of cell proliferation. The activation of the wingless-related integration site (WNT) pathway, which is mainly restricted to embryonic development, is one of these tumor-specific signaling pathways that has been shown to induce and maintain oncogenic effects (5-9). In particular, oncogenesis and the tumoral course of lymphoma and MM are often related to immoderate WNT signaling (10-14). Specific inhibition of WNT signaling therefore suppresses progression of lymphoma and MM, and thus renders the WNT signaling cascade a valuable treatment approach (14, 15).

Our recent studies confirmed on the *in vitro* and *in vivo* efficacy of several agents in targeting WNT/β-catenin signaling molecules, particularly in hematopoietic types of cancer (16-29). Clofibrate, the agent under investigation here, has already been proven to possess several anticarcinogenic properties and might, due to its chemical similarity to documented WNT inhibitors, also influence WNT signaling. Here we demonstrate for the first time that clofibrate is effective in the treatment of myeloma and lymphoma cells *in vitro*.

Materials and Methods

Cell lines and culture conditions. Cell lines were obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) or the American Type Culture Collection (ATCC) (LGC Standards, Wesel, Germany) and incubated at 37˚C with 5% CO_2 at 90% humidity.

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The human myeloma cell lines KMS 18, OPM-2, RPMI-8226 and U-266 (all obtained from DMSZ) were cultured in RPMI-1640 medium (PAA, Pasching, Austria), supplemented with 5% heat-inactivated fetal calf serum (FCS; Invitrogen, Darmstadt, Germany) and 1% penicillin-streptomycin (Seromed, Jülich, Germany). Human lymphoma cell lines Raji and SU-DHL-4 were cultured under identical conditions as human myeloma cell lines. MPC-11 (ATCC, LGC Standards GmbH, Wesel, Germany) is a murine plasmacytoma cell line that was also cultured in RPMI-1640 medium supplemented with 5% of heat-inactivated FCS and 1% penicillin-streptomycin.

The human colon fibroblast cell line CCD-18Co was obtained from ATCC (LGC Standards, Wesel, Germany) and cultured in ATCC-formulated Eagle's minimum essential medium (LGC Standards, Wesel, Germany) supplemented with 15% of heat-inactivated FCS and 1% penicillin-streptomycin. Cells were harvested by 0.05% trypsin-EDTA solution (Invitrogen, Darmstadt, Germany), centrifuged at 1,200 × *g* for 7 min and re-suspended in 1 ml media to define the cell count. Media were renewed at least every 3 days.

Drugs and chemical reagents. Clofibrate was purchased from Sigma-Aldrich (Steinheim, Germany) and tested at concentrations ranging from 0.1 to 600 μM for 72 h.

Cell viability assay with 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium-bromide (MTT). The effectiveness of clofibrate was determined by cell viability in MTT assay. Viable cells convert the yellow MTT (Sigma Aldrich, Steinheim, Germany) into purple formazan when taken-up into mitochondria. Cells were plated at 1×10^4 well/100 μl in 96-well plates, and left to adhere overnight in the incubator. Twenty-four hours later, media were removed and renewed containing different concentrations of clofibrate. After 21 h, 1 μl MTT (5 mg/ml) was added to each well and plates were incubated for another 3 h. Then 80 μl of the media were removed and 50 μl of acidified isopropanol was added for cell lysis. After shaking for 10 min, the amount of formazan was measured at 565 nM. The measured amount of formazan in treated cells was compared to that of untreated cells.

Statistical analysis. Values are given as the mean±standard deviation (SD). At least three separate and independent experiments were performed with each cell line. Paired, two-tailed Student's *t*-test was used for statistical analysis. A *p*-value less than 0.05 was considered significant.

Results

Titration of clofibrate. The mean 50% inhibitory concentration (IC_{50}) after 72 h was calculated following titration. Clofibrate concentrations leading to a significant decrease in viability of all tested myeloma and lymphoma cells were, therefore, determined. CCD-18Co colonic fibroblasts serving as healthy controls were also investigated by MTT. IC_{50} values of clofibrate after 72 h of incubation are given in Table I.

Effect of clofibrate on viability of human and murine myeloma cells. The viability of all tested myeloma cells decreased in a concentration-dependent manner following the addition of clofibrate, albeit higher concentrations starting from 300 μM were required for significant reduction of viability in both human and murine myeloma cells. The effects of clofibrate treatment in human myeloma and lymphoma cells were also reproducible in murine myeloma cells. Required clofibrate concentrations for a significant decrease of viability of MPC-11 murine myeloma cells were comparable to those for human myeloma cells. Results were as shown in Figure 1.

Table I. *Inhibitory concentration (IC_{50}) of clofibrate for human lymphoma, human and murine multiple myeloma and control cell lines. CCD18-Co cells served as controls. A total of 1×10^5 cells were cultured with different concentrations of clofibrate for 72 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay. Results represent the mean of data from three independent experiments each.*

	IC_{50} (μM)
Cell line	Clofibrate
KMS 18	>600 μM
OPM-2	278 μM
RPMI-8226	435 μM
U-266	278 μM
MPC-11	412 μM
Raji	272 μM
SU-DHL-4	256 μM
CCD-18Co	>600 μM

Effect of clofibrate on viability of human lymphoma cells. Exposure to clofibrate also significantly reduced lymphoma cell viability in all tested cell lines. The IC_{50} of Raji cells was attained after treatment with 272 μM. SU-DHL-4 lymphoma cells were comparably susceptible to the toxicity of clofibrate. At least 256 μM clofibrate were required to reduce their viability to a level of 50%. Figure 2 presents the respective results.

Effect of clofibrate on viability of healthy controls. We chose CCD-18Co colon fibroblasts in order to analyze the toxicity of clofibrate towards healthy cells. CCD18-Co cells tolerated very high concentrations of clofibrate compared to myeloma and lymphoma cell lines tested, as there was barely a hardly ascertainable reduction of viability after treatment with concentrations as high as 600 μM. Results are shown in Figure 3.

Discussion

MM, a malignant neoplasm of plasma cells is mainly caused by frequent gene mutations or chromosomal translocations (30). State-of-the-art treatment regimens are composed of primary high-dose chemotherapies and facultative hematopoietic stem cell transplantation (30-33). However,

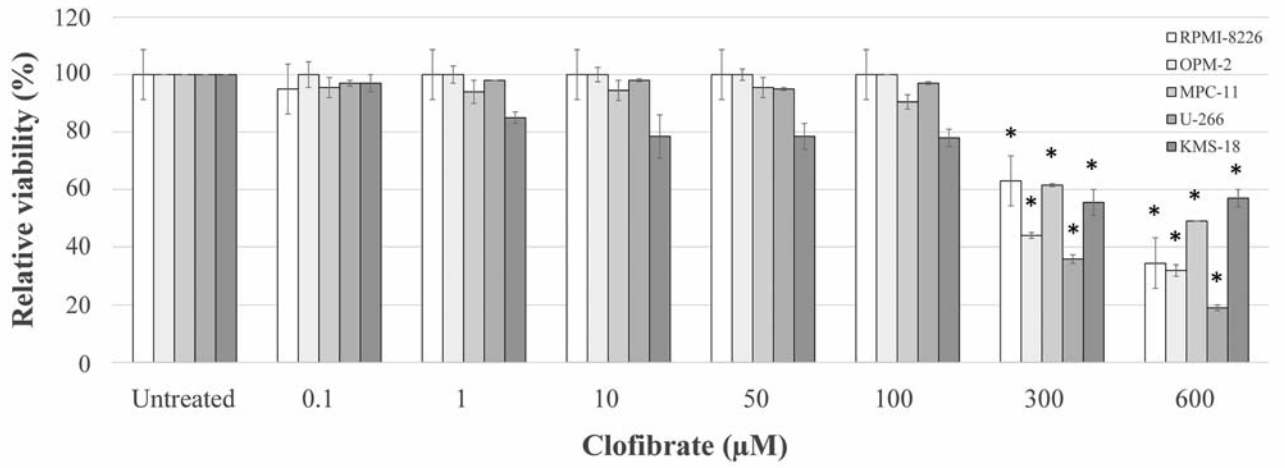

Figure 1. *Effect of clofibrate on viability of KMS-18, OPM-2, RPMI-8226, U-266 and MPC-11 human and murine myeloma cells. Cells were cultured with clofibrate for 72 h. Viability was measured by the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium-bromide (MTT) assay. Results represent data from three independent experiments. Data are shown as the mean±SD. *p<0.05 compared to untreated cells.*

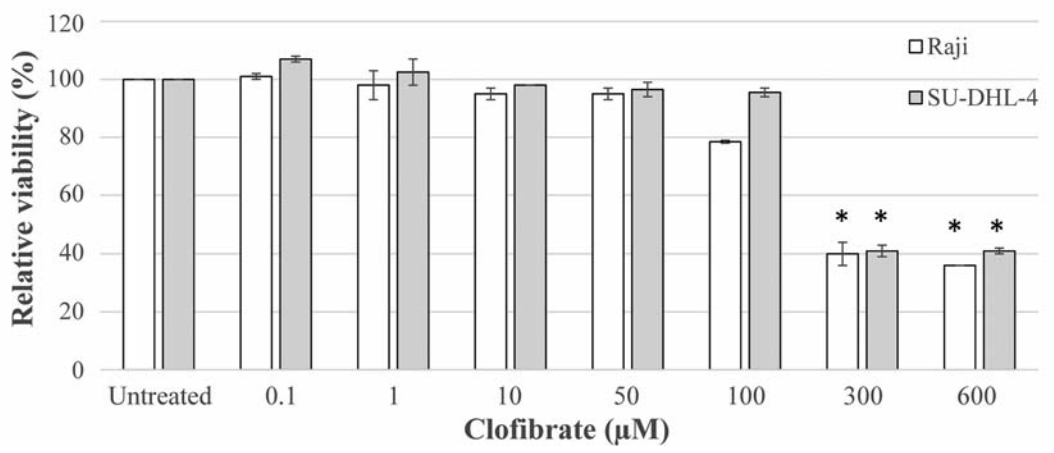

Figure 2. *Effect of clofibrate on viability of Raji and SU DHL 4 human lymphoma cells. Cells were cultured with clofibrate for 72 h. Viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay. Results represent data from three separate experiments each. Data are shown as mean±SD. *p<0.05 compared to untreated cells.*

despite a considerable number of recent treatment innovations, MM currently remains incurable in patients solely treated with chemotherapy (3, 34, 35).

In this regard, targeting tumor-specific signaling pathways involved in tumor differentiation and proliferation represents a promising therapeutic approach in oncology. Canonical WNT signaling is an excellent example of abrogated signaling pathways in MM and might thus represent an ideal candidate to target (10-14). Development and proliferation of MM cells is, among others, dependent on the bone marrow microenvironment, wherein bone marrow stromal cells release WNT ligands, eventually favoring an enhanced proliferation of MM cells (35-37). Hence, the inhibition of WNT/β-catenin signaling suppresses MM growth as evidenced by several *in vitro* and *in vivo* studies (38).

Recently, our laboratory revealed several drugs to be efficient inducers of apoptosis in lymphoma and myeloma cells *in vitro* and partially exhibited *in vivo* efficacy in subsequent animal studies. Four of these drugs were already shown to exert an impact on the WNT pathway through targeting either β-catenin itself or its downstream factors (16-26, 38). Due to their chemical relationship to well-documented WNT inhibitors, such an effect is also conceivable for the remaining agents.

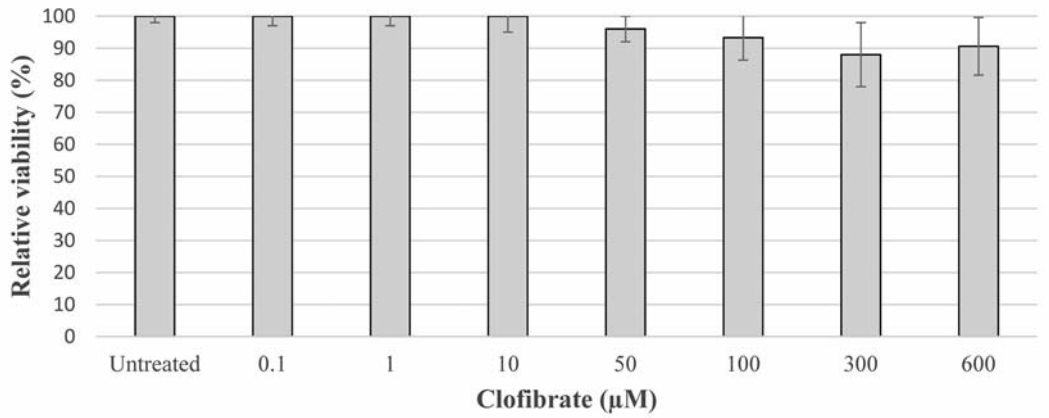

Figure 3. *Effect of clofibrate on viability of CCD18-Co cells which served as controls. Cells were cultured with clofibrate for 72 h. Viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay. Results represent data from three separate experiments each. Data are shown as mean±SD. *p<0.05 compared to untreated cells.*

Clofibrate is an aryloxyisobutyric acid derivative with antihyperlipidemic activity. Although its exact mechanism of action has not been fully characterized, clofibrate may increase the activity of extrahepatic lipoprotein lipase, thereby enhancing the conversion of very-low-density lipoprotein to low-density lipoprotein and eventually to high-density-lipoprotein. This is accompanied by a slight increase in secretion of lipids into the bile and ultimately into the intestine. Besides its current therapeutic use in hyperlipidemia, it is of major importance that clofibrate possesses the capacity to act as an agonist of the peroxisome proliferator-activated receptors (PPARs), ligand-activated transcription factors of the nuclear receptor superfamily. Three types of PPARs have been identified: PPARα, PPARβ/δ, and PPARγ. The PPARα subtype in particular is involved in fibrate-activated signal transduction and is expressed in numerous tissues such as liver, heart, kidney, skeletal muscle and others (39-41).

Several studies suggest a potential utilization in anticancer therapy, particularly due to PPARα ligands, beyond their prescription as lipid-lowering drugs. They suppressed tumor growth in mice through direct and indirect angiogenesis inhibition (42). In addition, clofibrate inhibited proliferation, induced differentiation, and increased the proportion of G_0/G_1 cells in HL-60 leukemic cells by the down-regulation of c-Myc and cyclin D2 expression human leukemia cells (43). Another study demonstrated that clofibrate induces extensive apoptosis of human T-leukemia Jurkat cells by the induction of endoplasmic reticulum stress and activation of caspases 2 and 3. Quite recently, *in vitro* treatment with clofibrate was found to lead to a significant reduction of breast cancer cells by inhibiting nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and extracellular-signal-regulated kinase 1/2 (ERK1/2) activation, reducing cyclin D1, cyclin A, cyclin E, and inducing pro-apoptotic P21 levels in breast cancer cells (44) – this is of major importance since the majority of these factors also play an essential role in the WNT pathway.

Interestingly, PPARα ligands other than clofibrate have frequently been described as potential inducers of apoptosis in leukemia cells (45-47); however, the actual impact of clofibrate on MM and lymphoma has not yet been addressed.

Our data indicate that clofibrate also affects the proliferative capacity of multiple myeloma and lymphoma cells. Clofibrate significantly reduced the viability of all tested myeloma and lymphoma cell lines. Thereby both human and murine cells were equally affected in a dose-dependent manner. Doses of approximately 300 μM significantly reduced cell viability in all myeloma and lymphoma cell lines and, interestingly, CCD-18Co colonic fibroblasts serving as healthy controls tolerated much higher concentrations, thus emphasizing favorable tolerability.

Due to its influence on WNT-associated signaling molecules in other malignancies, clofibrate might also interfere with signaling molecules of the WNT and its associated signaling pathways in lymphoma and multiple myeloma. Clofibrate revealed significant cytotoxic potential towards both MM and lymphoma cells and hardly affected the viability of healthy controls. Given the known safety profile of clofibrate, our data warrant further investigation of its potential as novel therapy for patients with MM and lymphoma.

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