The NADPH Oxidase Inhibitor Imipramine-Blue in the Treatment of Burkitt Lymphoma

Marcel Klingenberg1, Jürgen Becker1, Sonja Eberth2, Dieter Kube2, and Jörg Wilting1

Abstract

Burkitt lymphoma is a rare malignancy arising from B cells. Current chemotherapeutic regimens achieve excellent overall survival rates in children, but less impressive rates in adults. There are cases with poor outcome caused by toxic effects of the therapy, tumor lysis syndrome, or metastatic spread of lymphomas to the central nervous system. Modulators of reactive oxygen species are currently discussed as potential drugs for the treatment of cancer. The NADPH oxidase 4 inhibitor imipramine-blue might satisfy the aforementioned requirements, and was studied here. We used MTT assay, crystal violet assay, and thymidine 3H-incorporation assay to analyze the effects of imipramine-blue on Burkitt lymphoma (BL2, BL2B95, BL30B95, BL41B95), neuroblastoma (KELLY, SH-SY5Y, SMS-KAN), cervix carcinoma (HeLa), breast cancer (MDA-MB231), angiosarcoma (AS-M), human embryonic kidney (HEK293WT), and nonmalignant (FLP1) cell lines. The effects of imipramine-blue on BL2B95 cells in vitro were investigated in xenografts on the chick chorioallantoic membrane (CAM). We report that imipramine-blue is a potent growth inhibitor for several cancer cell lines in vitro with IC50 values comparable to those of doxorubicin (0.16–7.7 μmol/L). Tumor size of BL2B95 cells inoculated in the CAM was reduced significantly (P < 0.05) after treatment with 10 μmol/L imipramine-blue. Lymphogenic dissemination of BL2B95 and the formation of blood and lymphatic vessels in experimental tumors were not affected. We show that imipramine-blue can be used to decrease the viability of cancer cell lines in vitro and in vivo. Imipramine-blue reduces the size of experimental Burkitt lymphoma significantly but does not affect the dissemination of BL2B95 cells, angiogenesis, and lymphangiogenesis. Mol Cancer Ther; 13(4); 1–9. ©2014 AACR.
as part of the cellular defense machinery needed for the killing of microbes. But accumulating evidence suggests a more complex role for ROS in cellular pathways, for example an involvement in proliferation, apoptosis, angiogenesis, and metastasis (13).

It has been shown that tumor cell lines possess high ROS levels, but, depending on the investigated cell line, the experimental alteration of the ROS homeostasis has revealed opposing effects (14–16). A recent study provided evidence for an anti-invasive effect of the new ROS-modulating drug imipramine-blue in a highly invasive RT2 syngeneic astrocytoma rat model. Imipramine-blue is synthesized from the antidepressant drug imipramine and Michler ketone (Fig. 1F). Treatment with imipramine-blue induced the formation of tumors with a more compact morphology. It reduced invasion of tumor cells into healthy tissues and prolonged survival after combination therapy with liposomal doxorubicin (1). The target protein of imipramine-blue, NADPH oxidase 4 (NOX4), is involved in antiapoptotic signaling, including the NF-κB signaling pathway, which is highly active in many non-Hodgkin lymphomas (15, 17–19).

To evaluate the antitumor and antimetastatic effects of imipramine-blue, we treated numerous tumor and normal cell lines in vitro, and lymphoma cells in a xenograft chick chorioallantoic membrane (CAM) tumor model. We chose the BL2B95 lymphoma cell line for the CAM assay, because our previous experiments showed that the EBVþ BL2B95 cells exhibited a high potential for lymphogenic dissemination in the CAM (20), and the infection with EBV has been shown to increase ROS levels (21). Furthermore, the cell line was isolated from a patient with multiple metastases, which involved the central nervous system, the bones, the abdomen and the bone marrow (22). This fact illustrates BL2’s highly aggressive metastasis formation in a living organism and makes this cell line a highly suitable model for in vivo experiments like in our study.

Materials and Methods

Cell culture

HEK293WT, HeLa, KELLY, MD-MBA 231 cell lines were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig). SH-SY5Y cell line was obtained from American Type Culture Collection. SMS-KAN cell line was kindly provided by Prof. P. Reynolds (School of Medicine, Texas Tech University, Lubbock, TX). AS-M cells were kindly provided by Prof. Dr. P. Ströbel (Department of Pathology, University Medicine Goettingen, Goettingen, Germany). The aforementioned cell lines were not authenticated in the authors’ laboratory. Burkitt lymphoma cell lines (BL2B95, BL2, BL30B95, BL41B95) were obtained from IARC Lyon through G. Bornkamm in Munich, Germany. Burkitt lymphoma cell lines are regularly authenticated via immunoglobulin PCR once a year. Burkitt lymphoma cell lines (BL2B95, BL2, BL30B95, BL41B95) were cultured in RPMI-1640 medium (Lonza) with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (biochrome), 10 mmol/L HEPES (GIBCO), 1 mmol/L sodium-pyruvate (GIBCO), 50 μmol/L α-thioglycerol (Sigma-Aldrich), and 20 mmol/L BCS (bathocuproine disulfonic acid; Sigma-Aldrich). AS-M cells (angiosarcoma) were cultured in EGM-2 (Clonetics). Normal fibroblasts (FLP1), as well as HEK293WT, MD-MBA 231 (breast cancer), KELLY, SH-SY5Y, and SMS-KAN (neuroblastoma), were cultured in RPMI-1640 with 10% FCS and 1% penicillin/streptomycin. All cell lines were incubated at 37°C and 5% CO₂.

CAM assay

Fertilized white Leghorn chick eggs were incubated at 80% relative humidity and 37.8°C. The eggs were windowed at day 3 and the window was sealed with cello-tape. At day 10 of chick development, 1 × 10⁶ BL2B95 cells/egg were applied on the CAM. Cells were resuspended in 50% Burkitt lymphoma medium (with and without imipramine-blue) and 50% Matrigel and incubated for 30 minutes at 37°C, 5% CO₂ before applying them on the CAM. The tumors were surgically removed on day 17 of chick development. Tumors were fixed in 4% paraformaldehyde for 15 minutes, washed 3 times in PBS, and transferred into 10% sucrose for 3 hours at 4°C and 30% sucrose overnight at 4°C. Tumors were then embedded in polyfreeze tissue freezing medium and cut with a cryotome into 4 to 12 μm sections.

Thymidine ³H-incorporation assay

Thymidine ³H-incorporation assays were performed in duplicate in 96-well plates. Cells were seeded at a concentration of 3 × 10⁴ cells/mL in a final volume of 100 μL Burkitt lymphoma medium per well. Imipramine-blue dissolved in Burkitt lymphoma medium was added to a final concentration of 5, 10, and 20 μmol/L. Dimethyl sulfoxide (DMSO) was added in the control groups to a final concentration of 0.2%. Cells were incubated for 24 hours at 37°C and 5% CO₂. After 32 hours, 0.5 μCi thymidine-5-³H solved in 30 μL Burkitt lymphoma medium was added to each well. The cells were then incubated for 16 hours at 37°C and 5% CO₂ and transferred onto a membrane with Inotech cell harvester. The membrane was air dried and 5 mL liquid scintillation cocktail was added. The scintillation was measured with a 1450 MicroBeta Trilux Liquid Scintillation and Luminescence Counter (Wallac).

Methyl violet proliferation assay

The methyl violet proliferation assays were at least performed as sextuplicates in 96-well plates. Cells were seeded at a concentration of 10⁵/mL in a final volume of 50 μL. After 24 hours, imipramine-blue, dissolved in the respective media, was added to a final concentration of 0.1, 1, 5, 10, and 25 μmol/L. DMSO was added in the control groups to a final concentration of 0.25%, which corresponded to the highest amount of solvent in 25
imipramine-blue group. Cells were then incubated at 37°C and 5% CO₂ for 24 or 72 hours. Subsequently, cells were fixed with 50 μL of 5% glutaraldehyde per well and incubated at room temperature (RT) for 30 minutes on a rocking table. Then cells were washed 3 times with purified water and air dried for at least 60 minutes.

Figure 1. Effect of imipramine-blue on Burkitt lymphoma cell line viability. A, 24-hour MTT assay of Burkitt lymphoma cell lines treated with imipramine-blue. B, effect of 1 μmol/L imipramine-blue for 24 hours on Burkitt lymphoma cell lines (statistical analysis). C, 72-hour MTT assay of Burkitt lymphoma cell lines treated with imipramine-blue. D, effect of 1 μmol/L imipramine-blue for 72 hours on Burkitt lymphoma cell lines (statistical analysis). E, thymidine³H-incorporation assay of Burkitt lymphoma cells incubated with imipramine-blue for 24 hours. F, chemical structure of imipramine-blue. This image is reproduced with permission from The American Association for the Advancement of Science (1). * P < 0.05; ** P < 0.01; *** P < 0.001.
Subsequently, 100 μL 0.1% methyl violet solution was added and incubated for 20 minutes at RT on a rocking table. Cells were washed 3 times with purified water and air dried for at least 30 minutes. After that, 100 μL of 10% acetic acid was added and plates were agitated until the methyl violet was redissolved. Extinction was measured with ThermoMAX microplate reader (Molecular Devices) at 630 nm.

**MTT assay**

MTT cell viability assays were performed in triplicate in 96-well plates. Cells were seeded at a density of 3 × 10⁵ cells/well for 24 hours MTT assay and 10⁴ cells/well for 72 hours MTT assay. Imipramine-blue was diluted in Burkitt lymphoma medium to a final concentration of 0.05, 0.1, 0.3, 1, 2.5, 5, 10, or 20 μmol/L per well. Cells were incubated for 24 or 72 hours at 37°C and 7% CO₂. After 20 or 68 hours, 10 μL of 0.5% MTT in PBS was added to each well and incubated for 4 hours at 37°C and 5% CO₂. Then the plates were centrifuged at 1,000 rpm for 10 minutes, supernatant was discarded and 100 μL of 33% DMSO, 5% formic acid, and 62% isopropanol were added. The plates were shaken until the precipitate was resolved and the extinction was measured with Synergy Mx microplate reader (BioTek) at 540 nm.

**Immunofluorescence analyses**

Immunohistological staining of specimens was performed by incubation for 1 hour with blocking reagent (PBS, 1% BSA, 5% goat serum, 0.2% Triton X-100), 1-hour incubation of primary antibody diluted in antibody solution [TBS (0.05 mol/L, pH 7.2–7.4), 1% BSA, 0.5% Triton X-100] and 1-hour incubation of secondary antibody diluted in antibody solution mixed with DAPI (1:10,000). After each step, specimens were rinsed 3 times with PBS. Samples were mounted with Fluoromount-G (Sigma-Aldrich) and dried overnight at room temperature. Specimens were studied with Zeiss Axio Imager.Z1 (Zeiss) and filter sets 38HE, 43, 49, and 50. Primary antibodies were rabbit-anti-human Prox1 (ReliaTech) at 1:500 dilution, mouse-anti-human HLA A,B,C (BioLegend) at 1:200 dilution, and mouse-anti-Mep21 (chick CD34 homolog; M. Williams, AbLab) at dilution of 1:100.

**Determination of tumor size and vessels**

Pictures of tumor crosssections (H&E stained) were taken with a Leica MZ 16 FA stereo microscope at bright-field mode and tumor area was quantified with ImageJ 1.46r software. Numbers of intratumoral Mep21-positive blood vessels and Prox1-positive lymphatic endothelial cells were counted in immunofluorescence specimens at ×20 magnification.

**Statistical analyses and IC₅₀-value calculation**

Statistical analyses were performed using the program GraphPad Prism 5. Data of methyl violet and MTT assays were normalized to the internal DMSO control, concentrations (mmol/L) were log-transformed and absorption values were transformed with the term Y = 100 – Y. Data were plotted in log-dose versus response and analyzed with nonlinear regression (log inhibitor vs. normalized response-variable slope). IC₅₀ values and 95% confidence intervals (CI) were calculated by the software automatically. Data of tumor area were normalized to the internal test series DMSO control. Statistical significance was analyzed with an unpaired 2-tailed Student t test at a significance level of 0.05.

**Results**

**Imipramine-blue causes a reduction of Burkitt lymphoma viability and proliferation in vitro**

The treatment with imipramine-blue caused a significant decrease of Burkitt lymphoma cell viability in vitro (Fig. 1A–D). Twenty-four-hour incubation with 1 μmol/L imipramine-blue showed a reduction to less than 25% viability (compared with the DMSO-treated controls) for the Burkitt lymphoma cell lines BL2B95 (P < 0.001), BL2 (P < 0.001), and BL30B95 (P < 0.001). The viability of the BL41B95 (P < 0.01) cell line was only reduced to approximately 75% after 24-hour incubation with 1 μmol/L imipramine-blue (Fig. 1B). Treatment for 72 hours caused a significant decrease of viability to less than 15% in BL2B95 (P < 0.001), BL2 (P < 0.001), BL30B95 (P < 0.001), and BL41B95 (P < 0.05) cell lines (Fig. 1C and D). The thymidine ³H-incorporation assay showed that the proliferative activity of the analyzed Burkitt lymphoma cell lines was completely (<1%) inhibited (Fig. 1E).

**Tumor formation and size in a xenograft CAM model**

BL2B95 cells treated with 10 μmol/L imipramine-blue and DMSO formed lentiform tumors in the CAM, which varied in color between reddish (highly vascularized) and whitish (sparsely vascularized). Hemorrhage in the center of the tumors was found in both imipramine-blue– and DMSO-formed tumors. We determined the blood and lymphatic vessel formation and density with an immunofluorescence approach, and the extinction was measured with Synergy Mx microplate reader (BioTek) at 540 nm.

**Lymphogenic dissemination of BL2B95 cells**

The treatment with imipramine-blue had no obvious effect on the dissemination of the BL2B95 cells via the lymphatic system. Tumor cells were detectable within lymphatic vessels in all 3 groups (DMSO, 1 μmol/L imipramine-blue, 10 μmol/L imipramine-blue). BL2B95 cells were found within lymphatics both in the vicinity of the tumors and at distances of 5- to 10-mm apart (Fig. 2E, a–h).

**Lymphatic and blood vessel formation and density**

Next, we analyzed whether imipramine-blue treatment had an effect on the vascularization of the experimental tumors. We determined the blood and lymphatic vessel density with an immunofluorescence approach, and...
found that imipramine-blue had no effect on the formation of vessels in the tumor. The number of Prox1⁺ lymphatic endothelial cells was unchanged (Supplementary Fig. S1A) as was the number of blood vessels in the treated tumors compared with DMSO control tumors (Supplementary Fig. S1B).

**Effects of imipramine-blue on the viability of other cancer cell lines**

To determine if the effects of imipramine-blue were restricted to Burkitt lymphoma cells, we tested the viability of neuroblastoma (SH-SY5Y, KELLY, SMS-KAN), angiosarcoma (AS-M), cervix carcinoma (HeLa), human embryonic kidney (HEK-293WT), and breast cancer (MDA-MB231) cell lines. The responses of the cancer cell lines to imipramine-blue treatment were compared with the nontumorigenic FLP1 fibroblast cell line (Fig. 3A–C, a–f). This was done to determine if the observed effects were restricted to cancer cell lines or if there were nonspecific cytotoxic effects. The incubation with imipramine-blue caused clearly visible morphologic changes of the cells. FLP-1 cells showed a slight decrease of their diameter, whereas KELLY cells detached from the culture dish most likely by apoptosis (Fig. 3a–f). The cancer cell lines showed distinct responses to imipramine-blue treatment. The highest sensitivity toward imipramine-blue treatment was observed in the neuroblastoma cell line KELLY. These cells responded to the treatment with a viability reduction to less than 10% after 72-hour treatment with 1 μmol/L imipramine-blue (Fig. 3A and B).

Incubation with 5 μmol/L imipramine-blue for 72 hours decreased the viability of all analyzed cancer cell lines to less than 25% (Fig. 3C). In contrast, the viability of the nonmalignant cell line FLP-1 remained nearly unaltered (97%; Fig. 3A–C). The statistical analyses of 2 imipramine-blue concentrations (1 and 5 μmol/L) showed significant...
differences in the response between the FLP1 cell line and the cell lines HeLa \((P < 0.001; P < 0.001)\), HEK-293WT \((P < 0.001; P < 0.001)\), SH-SY5Y \((P < 0.01; P < 0.001)\), KELLY \((P < 0.001; P < 0.001)\), SMS-KAN \((P < 0.001; P < 0.001)\), AS-M \((P < 0.001; P < 0.001)\), and MDA-MB-231 \((P < 0.001; P < 0.001)\) after 72-hour incubation. The transformation of the methyl violet and MTT data produced a typical logarithmic dose versus response curve (Fig. 3A). IC\(_{50}\) values and CIs were calculated for each concentration and time-point. Data are summarized in Table 1.

**Discussion**

The most important finding of this study is the reduction of Burkitt lymphoma tumor size after the treatment with imipramine-blue in the chick CAM model. Although we initially assumed to reduce the metastatic behavior of the lymphoma cells, our findings may rather suggest a possible use of imipramine-blue as a cytotoxic drug for Burkitt lymphoma treatment. The dissemination of BL295 cells via the lymphatics was unaltered, as was the ragged appearance of the tumor borders. This seems to be in contrast to the findings of the anti-invasive effects of imipramine-blue in a rodent astrocytoma (RT2) model (1). However, these differences may reside in inherent differences of the chick CAM model and the rat model, different modes of migration of the analyzed tumor cells, or in the different application forms of the drug. The RT2 cell line, which was used in the astrocytoma study, has been shown to migrate mainly amoeboid-like (MMP-independent) \textit{in vivo} (23). This is the same migratory mode as observed in BL295 cells. But against expectations the 2 cell lines show a different response to imipramine-blue treatment. A possible explanation may be a switch of the RT2 cells from amoeboid to mesenchymal migration in the \textit{in vivo} rat model. If this transition took place, RT2 migration would be sensitive to imipramine-blue treatment via inhibition of the NAPDH oxidase complex and the downstream RAC protein pathway. Whether this amoeboid-to-mesenchymal transition occurs in the BL295 cells in the
CAM model is not known. However, the main metastatic route for BL2B95 cells is the dissemination via the lymphatic system, which we found unaltered by imipramine-blue in our experiments. Furthermore, in the CAM study we applied imipramine-blue with a pre-incubation protocol, whereas Munson and colleagues used intravenous imipramine-blue application in the rat model, starting 1 week after the inoculation of the tumor cells (1). Intravenous imipramine-blue application starting 1 week after tumor cell inoculation in the CAM model is impossible because of the limited time window for tumor growth in the egg. We therefore decided to use the preincubation protocol, knowing that this does not reflect the patient situation. ROS inhibitors obviously act in a context-dependent manner. Therefore, the different findings of the in vitro models may also reside in the fact that BL2B95 cells respond to imipramine-blue with apoptosis rather than an inhibition of movement. This is supported by our observation that the viability of Burkitt lymphoma cell line is efficiently inhibited already at very low imipramine-blue concentrations [BL2B95 IC\textsubscript{50} (24 hours imipramine-blue) = 0.16 μmol/L], whereas other cell lines, like HeLa, are less sensitive to imipramine-blue (HeLa IC\textsubscript{50} (24 hours imipramine-blue) = 3.92 μmol/L). This suggests that BL2B95 cells are highly dependent on basal ROS levels to prevent apoptosis. Other cell lines, such as rat RT2 and HeLa, seem to depend to a minor extent on ROS-mediated antiapoptotic mechanisms, and react with an altered cellular movement.

Here, we were able to show that imipramine-blue inhibits growth of various Burkitt lymphoma cell lines in vitro and in vivo. Imipramine-blue possesses a high therapeutic potency in vitro, comparable to that of doxorubicin, for the cell lines SH-SY5Y, MDA-MB231, HeLa, and HEK-293WT (24–27). The most sensitive cell lines toward imipramine-blue treatment were BL30B95, BL2, BL2B95, and the neuroblastoma cell line KELLY with IC\textsubscript{50}(24 hours) values lower than 0.8 μmol/L (0.79, 0.49, 0.16, and 0.78 μmol/L). The aforementioned most sensitive cell lines (BL30B95, BL2, BL2B95, KELLY) are considered to belong to the “reactive oxygen driven tumor” phenotype. This tumor phenotype is characterized by high ROS and AKT levels, and wild-type p53 (28–30). Therefore it is not surprising that the tumor cell lines of this phenotype are very sensitive to imipramine-blue treatment. Our in vitro studies were extended to several cancer types, which are characterized by metastasis formation and partially known to spread via the lymphatics (31–33). This included angiosarcoma, neuroblastoma, and breast cancer cell lines. There was no correlation between imipramine-blue sensitivity and the growth rate of the treated cancer cell lines. Nevertheless, all analyzed cell lines responded to the imipramine-blue treatment effectively. Whether the observed effects rely mainly on NOX4 inhibition or if off-target effects are involved is still unclear. It was shown by Munson and colleagues (1) that imipramine-blue application in vivo influences a number of genes in RT2 tumors (1). Among these, MFHAS1 may be of special interest. MFHAS1 is a potential oncogene that may cause B-cell lymphoma and is downregulated at the transcriptional level by imipramine-blue treatment (1,34). The inhibition of MFHAS1 may explain the high sensitivity of the Burkitt lymphoma cell lines toward imipramine-blue treatment.

Of note, the nonmalignant FLP1 fibroblasts were the least sensitive [IC\textsubscript{50} (24 hours) = 7.77 μmol/L] of all studied cell lines. To determine whether this is an incidental finding or if it hints toward a general lower sensitivity of nonmalignant cells toward NOX4-inhibition, additional studies, for example on endothelial cells, are needed to clarify the therapeutic window for imipramine-blue.

Table 1. IC\textsubscript{50} values of cell lines treated with imipramine-blue

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer/cell type (viral infection)</th>
<th>IC\textsubscript{50} 24-hour imipramine-blue/(95% CI), μmol/L</th>
<th>IC\textsubscript{50} 72-hour imipramine-blue/(95% CI), μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLP1</td>
<td>Fibroblast cell line</td>
<td>7.70/(6.57–9.04)</td>
<td>7.16/(6.13–8.37)</td>
</tr>
<tr>
<td>HEK-293WT</td>
<td>Embryonic kidney (HAdV-5\textsuperscript{+})</td>
<td>4.11/(3.49–4.84)</td>
<td>2.36/(2.24–2.50)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix carcinoma (HPV18\textsuperscript{+})</td>
<td>3.92/(3.41–4.49)</td>
<td>2.02/(1.83–2.23)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast carcinoma</td>
<td>1.91/(1.64–2.22)</td>
<td>1.11/(0.88–1.40)</td>
</tr>
<tr>
<td>AS-M</td>
<td>Angiosarcoma</td>
<td>1.81/(1.56–2.10)</td>
<td>0.98/(0.91–1.06)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblastoma</td>
<td>1.74/(1.17–2.58)</td>
<td>1.79/(1.13–2.84)</td>
</tr>
<tr>
<td>SMS-KAN</td>
<td>Neuroblastoma</td>
<td>1.60/(0.97–2.64)</td>
<td>1.17/(0.61–2.24)</td>
</tr>
<tr>
<td>KELLY</td>
<td>Neuroblastoma</td>
<td>0.78/(0.52–1.17)</td>
<td>0.14/(0.11–0.18)</td>
</tr>
<tr>
<td>BL418B95\textsuperscript{a}</td>
<td>Burkitt lymphoma (EBV\textsuperscript{+})</td>
<td>1.11/aD</td>
<td>0.78/aD</td>
</tr>
<tr>
<td>BL30B95\textsuperscript{a}</td>
<td>Burkitt lymphoma (EBV\textsuperscript{+})</td>
<td>0.79/aD</td>
<td>0.47/(0.31–0.70)</td>
</tr>
<tr>
<td>BL2\textsuperscript{a}</td>
<td>Burkitt lymphoma</td>
<td>0.49/(0.42–0.57)</td>
<td>0.75/aD</td>
</tr>
<tr>
<td>BL2B95\textsuperscript{a}</td>
<td>Burkitt lymphoma (EBV\textsuperscript{+})</td>
<td>0.16/(0.13–0.21)</td>
<td>0.45/(0.35–0.59)</td>
</tr>
</tbody>
</table>

NOTE: IC\textsubscript{50} values were calculated on the basis of methyl violet assay data; IC\textsubscript{50} is the concentration that inhibits 50% of proliferation; aD is the ambiguous data, which means no reliable calculation of 95% CIs was possible.

\textsuperscript{a}IC\textsubscript{50} values calculated on the basis of MTT cell viability assay data instead of the methyl violet assay.
Furthermore, we plan to test imipramine-blue in combination with classical chemotherapeutic drugs (CHOP/CODOX-M/IVAC) to analyze the possibility of synergistic effects that may improve chemotherapeutic regimens for the treatment of patients with Burkitt lymphoma. Because imipramine-blue encapsulated in PEGylated liposomes accumulates in brain tumor sites and diffuses into the brain (1), its application may be of high value for the treatment of brain metastases of lymphomas.

Our observations are in line with several publications that described the beneficial effects of ROS inhibition in vitro as well as in clinical trials (15, 35–37). On the other hand, numerous publications and clinical trials with ROS-inducing drugs have shown that an experimental upregulation of ROS may kill cancer cells as well (14, 38–40). As noted above, ROS levels act in a highly context-dependent manner, and the effects must be determined for each tumor entity. Therefore, it is still under debate whether ROS decrease or increase may be more beneficial for the treatment of cancer. Cancer cells may either be affected by the accumulation of ROS above a cytotoxic threshold, or by the inhibition of ROS below a concentration, which is essential for signaling processes (10). Thereby, we assume that the upregulation of ROS may not be the preferred approach, as it may severely affect normal cells. A systemic treatment with drugs that increase intracellular ROS levels above a threshold may induce apoptosis and severe side-effects in healthy tissues. This is supported by observations of unwanted side-effects in a phase II clinical trial of the pro-oxidant drug triapine, where patients developed neutropenia, hypoxia, methemoglobinemia, and dyspnea, without any beneficial effects (41).

In summary, our study shows that the NOX4 inhibiting small molecule imipramine-blue is able to inhibit the proliferation of BL2B95 cells in vivo and in vitro. Several other cancer cell lines are significantly influenced by imipramine-blue, too. Thereby, imipramine-blue shows a potency that is comparable to that of doxorubicin. The most sensitive cell lines are the lymphoma cell lines BL2, BL2B95, and BL30B95, as well as the neuroblastoma cell line KELLY. In an animal model with xenographed BL2B95 cells, imipramine-blue caused a reduction of the tumor size, but the dissemination of lymphoma cells via the lymphatics and tumor-induced angiogenesis remained unaltered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Klingenberg, D. Kube, J. Wilting
Development of methodology: M. Klingenberg, J. Becker, J. Wilting
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Klingenberg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Klingenberg, J. Becker, S. Eberth, D. Kube, J. Wilting
Writing, review, and/or revision of the manuscript: M. Klingenberg, J. Becker, D. Kube, J. Wilting

Study supervision: J. Wilting

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