Rational Design of Polyglutamic Acid Delivering an Optimized Combination of Drugs Targeting Mutated BRAF and MEK in Melanoma


Targeted therapies against cancer can relieve symptoms and induce remission; however, they often present limited duration of disease control, cause side effects, and may induce acquired resistance. Therefore, there is great motivation to develop a unique delivery system, targeted to the tumor, in which several active entities can be combined, the therapeutic index can be increased by reducing systemic exposure, and their synergistic activity can be enhanced. To meet these goals, the biocompatible and biodegradable poly(α-L-glutamic acid) (PGA) is chosen as a nanocarrier that facilitates extravasation-dependent tumor targeting delivery. The RAS/RAF/MEK/ERK pathway when aberrantly activated in melanoma, can lead to uncontrolled cell proliferation, induced invasion, and reduced apoptosis. Here, two drugs targeting this pathway are selected: a MEK1/2 inhibitor (selumetinib, SLM) and a modified BRAF inhibitor (modified dabrafenib, mDBF) that exhibit synergism in vitro. The combination of PGA conjugated to SLM and mDBF (PGA–SLM–mDBF) is synthesized and characterized. PGA–SLM–mDBF inhibits the proliferation of melanoma cells and decreases their migratory and sprouting abilities without inducing a hemolytic effect. Moreover, it exhibits superior antitumor activity in a mouse model of primary melanoma and prolonged survival at a lower dose than the free drugs.

1. Introduction

In the last decade, a greater understanding of key oncogenes and signaling pathways involved in melanoma progression was achieved, leading to new treatment modalities. However, the number of patients with metastatic melanoma continues to rise, ranging from 33% that present brain metastases at diagnosis [1] and up to 75% at the time of death. [2] This may be attributed to the new treatment-induced prolonged lifespan of melanoma patients with primary tumors, enabling the metastatic spread later on. Approximately 50% of all patients with malignant melanoma harbor an activating mutation in the V600 position of the BRAF kinase gene. [3] BRAF kinase is a part of the mitogen-activated protein kinase (MAPK) signaling pathway involved in the regulation of cellular processes such as proliferation and survival. Hence, the BRAF V600 mutation leads to hyper-activation of the MAPK pathway and further uncontrolled tumor growth. [4] To that end, a targeted approach has been developed, relying on inhibition of different key proteins in cancer progression. New targeted therapy agents were developed and some got FDA approval, such as BRAF inhibitors (BRAFi)-vemurafenib, [5] dabrafenib (DBF), [6] encorafenib, [7] and MEK inhibitors (MEKi)-trametinib (TRM), selumetinib (SLM) [8] and binimetinib. [7] Further paramount advances in melanoma therapy include recently approved monoclonal antibodies that target either the programmed cell death-1 (PD-1) receptor on infiltrating T cells, such as pembrolizumab [9] and nivolumab, [10] or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) such as ipilimumab. [11] Initially, monotherapy inhibiting mutated BRAF enzyme in the MAPK pathway showed promising results. Prior to 2011, the 1 year survival rate for patients suffering from advanced or metastatic melanoma was as low as 33%, with a median overall survival (OS) of about 9 months. Treatment with DBF in Phase III clinical trial showed an increase in median OS for up to 20 months [12] and in 1 year survival up to 68%. [13] This new treatment was more efficient.
compared to conventional chemotherapy and became a worldwide standard of care for patients with BRAF-mutant metastatic melanoma. However, most patients treated with BRAFi eventually developed resistance. Consequently, a combination therapy of BRAFi and MEKi was evaluated in numerous clinical trials resulting in statistically significant improvement in response rate, progression-free survival (PFS), and OS compared to monotherapy. In 2015, the FDA approved the combined use of DBF and TRM for the treatment of patients with V600E or V600K BRAF-mutated unresectable or metastatic melanoma. Resistance to BRAFi monotherapy following treatment with DBF or vemurafenib was acquired after 6–8 months in 50% of patients, and the combination therapy delayed the onset time of resistance and the number of grade 3/4 adverse effects increased significantly.

To overcome the serious adverse effects and to improve the efficacy of the combination therapy, we set to deliver both MEKi and BRAFi simultaneously to the tumor site, utilizing a poly(ω-glutamic acid) (PGA) polymer as the drug delivery platform. Polymer–drug conjugates can exploit the leaky angiogenic tumor vasculature and the impaired lymphatic drainage, a phenomenon known as the enhanced permeability and retention (EPR) effect. Thus, the uptake and retention of the stable nanosized carriers by extravasation from the bloodstream to the tumor tissue may be higher in comparison with low molecular weight drugs that distribute evenly in the body.

In addition, polymers as multidrug delivery systems, may improve the drugs’ therapeutic effectiveness while reducing adverse side effects by increasing their solubility, and altering their pharmacokinetic (PK) profiles. In addition, the drugs’ efficacy can be enhanced by delivering and releasing both drugs simultaneously at the tumor site, as opposed to just injecting them simultaneously, where each drug with its own PK may reach the tumor at a different time. Finally, this may lead to a decrease in the treatment dose.

A large number of polymer–drug conjugates were synthesized in order to take advantage of polymers as macromolecular vehicle for low molecular weight ($M_m$) drugs delivery. Jian et al. used hyaluronic acid for the conjugation of nimesulide, resulting in increased solubility of the drug from 10.9 to 600 μg mL$^{-1}$ and high selectivity to CD44-overexpressing HT-29 colorectal tumors. Another example was shown by Luo et al. that synthesized polyethyleneoxy-paclitaxel (PEG-PTX), resulting in fourfold increased solubility of PTX, prolonged half-life and sustained release of the drug in the lung tissue for lung cancer therapy. The importance of macromolecular carriers to enhance the therapeutic efficacy is demonstrated by over 30 polymer therapeutics in advanced stages of development. Among them are Opaxio (CTI BioPharma), a PGA-PTX conjugate, that have reached Phase 3 clinical trials for ovarian cancer, peritoneal cancer and fallopian tube cancer; and Onzeal (Nektar Therapeutics) which exploits PEG for the delivery of irinotecan to treat breast cancer and is in the pre-registration stage.

Previous works in our laboratory focused on the co-delivery of drugs by polymeric backbones such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, PGA, pullulan, poly lactic-co-glycolic acid (PLGA) and PEG among others. These resulted in improved safety profile, PKs, tumor accumulation and efficacy both in vitro and in vivo. Here, we chose PGA as the polymeric backbone bearing a carboxylic group on each monomer, which provides high functionality for the attachment of drugs. In addition, PGA is nonimmunogenic and biodegradable by cysteine cathepsins, a family of enzymes that is highly activated in most tumor tissues. It is not toxic at the concentrations required for therapeutic effect and can be synthesized in different lengths with low polydispersity (D) of 1.1–1.2. The pendant free ω-carboxyl group in each repeating unit of l-glutamic acid is negatively charged at physiological pH, which confers water solubility to the polymer.

We previously reported the successful conjugation of PTX and doxorubicin (DOX) to the same PGA backbone. Accumulation of PGA-PTX-DOX conjugate was much higher in the tumor compared to that in the healthy tissues. Furthermore, a synergistic effect was observed compared to the monodrug conjugates and the cardiotoxicity of DOX was abrogated. Here, we set to deliver both MEKi and BRAFi (SLM and DBF, respectively) simultaneously to the tumor site, by their conjugation to PGA macromolecule, in a ratio that generates a synergistic effect. To our knowledge, this is the first report demonstrating their conjugation to PGA backbone, as a nanomedicine for melanoma. DBF possesses an aromatic primary amine group that does not allow stable covalent conjugation of the drug to PGA. To that end, DBF was modified synthetically to DBF–Lev, DBF–NH$_2$, and DBF–diol (mDBF). We present here the different types of DBF binding to PGA, going from not stable to nonreleasing to stable and cleavable, respectively. SLM, a MEK1/2 inhibitor, exhibited anticancer activity in different tumor types. In 2019, it gained FDA-approval for neurofibromatosis type 1 (NF1). Compared to TRM, which is FDA-approved for melanoma, SLM is less potent, as indicated by a MEK1 kinase IC$_{50}$ of 0.7 × 10$^{-9}$ and 14 × 10$^{-9}$ m, respectively. Despite this fact, we chose SLM due to its functional group that enables facile covalent conjugation to PGA, while TRM has no functional group available for covalent conjugation. We hypothesized that when conjugated to the polymer, SLM will have an equivalent activity with less toxicity versus TRM. Our nanoconjugate, bearing both mDBF (stable and cleavable) and SLM, enhances the drugs’ solubility and stability in physiological conditions and facilitates selective drug release by cathepsins at the tumor site.

2. Results

2.1. D4M.3A and 131/4-5B1 Melanoma Cell Lines Harbor a BRAF Mutation

As mentioned, about 50% of melanoma patients harbor activating BRAF mutations, mostly V600E (over 90%). We therefore sequenced our melanoma cell lines to validate whether they harbor a BRAF mutation. We found that the murine D4M.3A cell line bears a V600E mutation, while the human 131/4-5B1 cell line has a V600D mutation in their BRAF gene (Figure S1, Supporting Information). Zebrafish ZMEL1 cell line was previously shown to bear mit-BRAF V600E, P53−/− mutations.
Table 1. Physicochemical characterization of polymer drug conjugates.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Size by DLS [nm]</th>
<th>Drug loading DBF [wt%]</th>
<th>Drug loading SLM [wt%]</th>
<th>Zeta potential [mV]</th>
<th>( M_n ) by MALS [g mol(^{-1})]</th>
<th>Polydispersity index (D)</th>
</tr>
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<tbody>
<tr>
<td>PGA–SLM</td>
<td>6.4 ± 0.4</td>
<td>–</td>
<td>35</td>
<td>–60 ± 6</td>
<td>6.7 ( \times ) 10(^9)</td>
<td>1.39</td>
</tr>
<tr>
<td>PGA–DBF–NH(_2)</td>
<td>10 ± 0.7</td>
<td>31.4</td>
<td>–</td>
<td>NA</td>
<td>1.1 ( \times ) 10(^8)</td>
<td>2.49</td>
</tr>
<tr>
<td>PGA–mDBF</td>
<td>11.7 ± 0.6</td>
<td>32</td>
<td>–</td>
<td>–56 ± 5</td>
<td>2.1 ( \times ) 10(^8)</td>
<td>1.78</td>
</tr>
<tr>
<td>PGA–SLM–DBF–NH(_2)</td>
<td>5.2 ± 0.7</td>
<td>14</td>
<td>27</td>
<td>–7.92</td>
<td>8.2 ( \times ) 10(^7)</td>
<td>1.26</td>
</tr>
<tr>
<td>PGA–SLM–mDBF</td>
<td>11.6 ± 0.6</td>
<td>12</td>
<td>18</td>
<td>–41 ± 2</td>
<td>6.6 ( \times ) 10(^7)</td>
<td>1.26</td>
</tr>
<tr>
<td>PGA–SLM–mDBF–Cy5</td>
<td>11.7 ± 6.7</td>
<td>12</td>
<td>16</td>
<td>–35.82</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Drug loading was optically calculated using a calibration curve or by NMR using internal standard of trifluoroethanol (77 ppm). \(^{30}\)Derivatives of DBF.

2.2. Ethylenamine Addition to DBF Does Not Alter the Cytotoxic Activity of the Drug

In order to facilitate DBF conjugation to PGA, ethylenamine group was added to the drug (Scheme S1 and Figure S2, Supporting Information). To validate that modified DBF retained its cytotoxic activity, human melanoma cells were incubated with dabrafenib–ethylenamine (DBF–NH\(_2\)) and DBF for 72 h at serial concentrations. Both DBF–NH\(_2\) and nonmodified DBF showed a similar potent cytotoxic effect, with an IC\(_{50}\) of 3.5 \( \times \) 10\(^{-9}\) M and 6 \( \times \) 10\(^{-9}\) M, respectively (Figure S3, Supporting Information).

2.3. Synthesis and Physicochemical Characterization of the Conjugates

PGA–DBF–NH\(_2\), PGA–SLM, and PGA–SLM–DBF–NH\(_2\) syntheses were performed as one pot reactions as shown in Scheme S2A–C (Supporting Information) and characterized by \(^1\)H and \(^{19}\)F NMR (Figures S4–S6, Supporting Information). Characterization of the polymer–drug conjugates was performed by dynamic light scattering (DLS) to evaluate the hydrodynamic diameter; the \( M_n \) of each conjugate was measured by multiangle static light scattering (MALS) and the surface charge was evaluated by zeta potential measurements (Table 1).

2.4. PGA–DBF–NH\(_2\) Inhibits the Proliferation of Human 131/4-SB1 Melanoma Cells at a High IC\(_{50}\)

Evaluation of cell proliferation performed on 131/4-SB1 human melanoma cells showed that the PGA–DBF–NH\(_2\) conjugate had a very low inhibitory activity compared to DBF–NH\(_2\). Furthermore, combining the two monodrug conjugates, PGA–SLM and PGA–DBF–NH\(_2\) at the synergistic ratio (10:1) did not show any benefit compared to each monodrug conjugate alone (Figure S7, Supporting Information).

2.5. PGA–SLM–DBF–NH\(_2\) Conjugate Is Barely Degraded by Cathepsin B (CTSB)

We found that no degradation of the conjugate was detected up to 160 h incubation neither in the presence nor in the absence of Cathepsin B (CTSB) (Figure S8, Supporting Information).

2.6. Diol Addition to DBF Does Not Alter the Cytotoxic Activity of the Drug

In order to balance our conjugate’s activity–stability properties (i.e., maintaining its activity while retaining a suitable stability), DBF was modified by the addition of a diol group (Scheme S3 and Figure S9, Supporting Information) and will be referred as modified DBF (mDBF). Next, we evaluated whether this modification retains DBF cytotoxic activity. Murine and human melanoma cells were treated with DBF and mDBF for 72 h, at serial concentrations. Both DBF and mDBF showed similar cytotoxic effect, displaying an IC\(_{50}\) of 4.5 \( \times \) 10\(^{-9}\) M for DBF and 6.5 \( \times \) 10\(^{-9}\) M for mDBF in the murine cell line, and 6 \( \times \) 10\(^{-9}\) M for DBF and 4.5 \( \times \) 10\(^{-9}\) M mDBF in the human cell line (Figure S10, Supporting Information).

2.7. mDBF and SLM Exhibit a Synergistic Inhibitory Effect on Melanoma Cells Proliferation

The ability of mDBF and SLM combination to inhibit the proliferation of murine D4M.3A, human 131/4-SB1 and zebrafish ZMEL1 melanoma cells synergistically was evaluated. Different ratios of the drugs were screened in order to determine the optimal ratio at which the combination treatment decreases the inhibitory concentration of each single drug. The combination treatment showed a synergistic effect on all three cell lines when SLM was at a higher concentration than mDBF (Figure 1A–C). Figure 1D summarizes the combination index (CI) of the treatments at different inhibitory concentrations (IC\(_{20,40,60}\)).

2.8. Synthesis and Physicochemical Characterization of the Polymer-2-Drug Conjugate

PGA–SLM–mDBF synthesis was performed as one pot reaction (Scheme 1A) and characterized by \(^1\)H and \(^{19}\)F NMR (Figure 2A–C). The conjugate was labeled with Cy5 to allow intravital noninvasive imaging to follow its body distribution (Scheme 1B). Characterization of this polymer-2-drug conjugate was performed by DLS for hydrodynamic diameter size evaluation; the \( M_n \) of the conjugate was measured by MALS; and the surface charge was evaluated by zeta potential measurements (Table 1).
Figure 1. Synergistic activity of the combined treatment with mDBF and SLM on different BRAF-mutated melanoma cell lines. Isobolograms of drug combination on A) murine (D4M.3A), B) human (131/4-5B1) and C) zebrafish (ZMEL1) melanoma cells. D) Combination index (CI) for each cell line. In all cell lines the combined therapy resulted in a synergistic effect. All experiments were performed in triplicates and repeated at least three times.

2.9. PGA–SLM–mDBF–Cy5 Conjugate Internalizes into the Cells and Colocalizes with Lysosomes In Vitro

Macromolecular carriers can internalize into cells through endocytosis. Thus, we wanted to evaluate whether the PGA–SLM–mDBF–Cy5 conjugate is colocalized with the lysosomes. D4M.3A and 131/4-5B1 melanoma cells were stained with a lysosome marker, LysoTracker green, and incubated with PGA–SLM–mDBF–Cy5 at 37 °C for 0.5 h. Then, treatment was removed; cells were incubated in their growth medium for additional 0.5, 4, and 24 h. Live cell imaging demonstrated cellular uptake as soon as 0.5 h following treatment with Cy5-labeled PGA–SLM–mDBF conjugate as well as colocalization with lysosomes. Colocalization analysis of cyan (PGA–SLM–mDBF–Cy5) and red (lysosomes stained by LysoTracker green) pixels in D4M.3A and 131/4-5B1 cells showed 90% and 97% colocalization, respectively, 4 h post-treatment removal. Untreated cells served as control in order to determine the threshold for positive Cy5 signal. To further validate the time-course colocalization of our conjugate with lysosomes, live-cell confocal imaging was performed. Cells were treated with PGA–SLM–mDBF–Cy5 at 37 °C for 0.5 h, then washed and incubated in phenol red-free medium. Images were taken at 0.5, 4, and 24 h following treatment removal. Quantification of 5–7 fields demonstrated time-dependent increase in Cy5 colocalization with lysosomes. PGA–SLM–mDBF–Cy5 was reaching 50% colocalization with the lysosome in 131/4-5B1 cells and 80% in D4M.3A cells, 24 h post-treatment.

2.10. PGA–SLM–mDBF Conjugate Is Degraded by CTSB

The degradation by CTSB of the polymer-2-drug conjugate, PGA–SLM–mDBF, with 30 wt% loaded drugs reached up to 40% in 44 h, and 60% in 5 days compared to ≈2% degradation in the absence of CTSB.

2.11. PGA–SLM–mDBF Combined Treatment Inhibits the Proliferation of Murine D4M.3A, Human 131/4-5B1, and Zebrafish ZMEL1 Melanoma Cells

Cytotoxicity of the free drugs, PGA–SLM, PGA–mDBF, and the combined conjugate PGA–SLM–mDBF was evaluated on murine D4M.3A, human 131/4-5B1 and zebrafish ZMEL1 cells. Cells were incubated with the free drugs, their combination, monodrug conjugates and with the conjugate combining both drugs, for 72 h. The drugs ratio used for all treatments was as on the PGA–SLM–mDBF conjugate. PGA–SLM–mDBF conjugate inhibited proliferation of murine D4M.3A cells with IC50 of $100 \times 10^{-9}$ M, which was more potent than each monodrug conjugate alone, however, it was less effective than mDBF alone or the combination of the free drugs. Similar results were obtained in the human 131/4-5B1 cells. The polymer-2-drug conjugate inhibited their proliferation at an IC50 of $100 \times 10^{-9}$ M, which was similar to the PGA–mDBF and PGA–SLM combination, but better than PGA-mDBF alone. Once again, the free drugs and their combination demonstrated better results. Our conjugate inhibited the proliferation of zebrafish ZMEL1 cells, with an IC50 of $3000 \times 10^{-9}$ M which was much more potent than each conjugate alone, and similar to the IC50 of the free drugs. The combination of free SLM and mDBF displayed an IC50 of $80 \times 10^{-9}$ M. The results of all the treatments are summarized (Table S1, Supporting Information).

2.12. PGA–SLM–mDBF Inhibits the Migration of ZMEL1-GFP Cells

Comparison of the inhibitory effect of PGA–SLM, PGA–mDBF, and PGA–SLM–mDBF conjugates on ZMEL1-GFP cell migration was performed. Cells were treated with $1 \times 10^{-6}$ M of free drugs or the equivalent dose of drug in a polymer–drug conjugate. Combined treatment was done with $1 \times 10^{-6}$ M mDBF and $3 \times 10^{-6}$ M SLM to obtain drug ratio of 3:1 SLM/mDBF similarly to the PGA–SLM–mDBF conjugate. Cells were allowed to migrate for 48 h. PGA–SLM–mDBF showed significantly better inhibition of migration compared to the monodrug conjugates or their combination.

2.13. PGA–SLM–mDBF Conjugate Inhibits Melanoma and Endothelial Cells Sprouting in Tumor Spheroids

3D tumor spheroids, formed by a mixture of melanoma cells and brain microenvironment cells (endothelial, astrocytes and microglia), were treated with $3 \times 10^{-6}$ M SLM + $1.75 \times 10^{-6}$ M mDBF and PGA–SLM–mDBF conjugate ($1.75 \times 10^{-6}$ M mDBF, $3 \times 10^{-6}$ M SLM). Melanoma and endothelial cells (EC) sprouting was evaluated every 24 h for 144 h. We found that treatment
Figure 3. PGA–SLM–mDBF–Cy5 in vitro colocalization with lysosomes in murine D4M.3A and human 131/4-SB1 melanoma cell lines. A) Representative bright field and fluorescence imaging of living murine D4M.3A (left) and human 131/4-SB1 (right) melanoma cells stained with LysoTracker green 100 × 10⁻⁹ M (red), a lysosome marker and Hoechst 33342 2.5 μg mL⁻¹ (blue), treated with PGA–SLM–mDBF–Cy5 (cyan) for 30 min and further incubated in medium for 0.5, 4, and 24 h post-treatment removal. B) Colocalization analysis of imaged living cells (dot plot: orange) of the lysosome marker, LysoTracker green, with PGA–SLM–mDBF–Cy5. Yellow indicates cells with red (LysoTracker) and cyan (PGA–SLM–mDBF–Cy5) staining (not in colocalization), orange indicates colocalization and green indicates nongated cells. C) Analyzed cells' percentage of colocalization of cyan (conjugate) and red (LysoTracker green) pixels. D) Representative confocal images of D4M.3A and 131/4-SB1 cells treated with PGA–SLM–mDBF–Cy5 (cyan) and stained with the lysosome marker, LysoTracker red, and with Hoechst 33342 2.5 μg mL⁻¹ (blue). Scale bar = 10 μm. E) Colocalization analysis of living cells following treatment with PGA–SLM–mDBF–Cy5 for 30 min and further incubation in medium for 0.5, 4, and 24 h. The data are presented as mean ± SD. F. PGA–SLM–mDBF conjugate degradation by CTSB. In the presence of CTSB (10 U, pH = 5.5), the conjugate was degraded by 60% in 5 days, while in the absence of CTSB, no degradation was observed.
PGA–SLM–mDBF inhibits melanoma cells proliferation and migration. A) Murine D4M.3A, B) human 131/4-5B1, and C) ZMEL1 zebrafish melanoma cells were treated with the polymer–drug conjugates and free drugs at serial concentrations. In D4M.3A, PGA–SLM–mDBF inhibited proliferation similarly to free DBF (IC$_{50}$ $\approx 100 \times 10^{-9}$ M) but was less effective than the free drugs combination (IC$_{50}$ = $10 \times 10^{-9}$ M). In 131/4-5B1, the IC$_{50}$ of the polymer-2-drug conjugate was $100 \times 10^{-9}$ M, not as effective as the free drugs or their combination, but better than each mono conjugate separately. In ZMEL1, the conjugate inhibited proliferation in a similar manner to the free drugs (IC$_{50}$ = $4 \times 10^{-3}$ M for SLM, $1 \times 10^{-3}$ M for mDBF, and $3 \times 10^{-3}$ M for the conjugate) but was less effective than the free drugs combination ($80 \times 10^{-9}$ M). PGA had no toxic effect on the cells. All assays were repeated at least three times, data represent mean ± SD. D) PGA–SLM–mDBF inhibited significantly ZMEL1-GFP cells migration compared to PGA–SLM, PGA–mDBF and their combination. One-way ANOVA was used for statistical analysis. E) Representative microscope images of ZMEL1-GFP cells migration following treatments. All cells were counterstained with DAPI (blue) for nucleus.

with the polymer-2-drug conjugate inhibited the sprouting of melanoma cells compared to the control treatments, similarly to the combined treatment with the free drugs; however, the inhibitory effect of the free drugs on the endothelial cells was more pronounced than that of the conjugate (Figure 5).

2.14. PGA–SLM–mDBF Conjugate Is Safe for Systemic Administration

Being the largest cellular population represented in the plasma, red blood cells (RBCs) are the first to interact with our treatments. To assess the hematocompatibility of the PGA–SLM–mDBF conjugate, RBC lysis was assessed ex vivo. Our results show negligible hemolysis caused by the conjugate at concentrations up to 8 mg mL$^{-1}$, similar to that of the negative control dextran, while the positive control sodium dodecyl sulfate (SDS) caused extensive hemolysis at concentrations above 0.05 mg mL$^{-1}$. These findings indicate that our conjugate is safe for intravenous (i.v.) administration (Figure 6).

2.15. PGA–SLM–mDBF–Cy5 Accumulates at the Tumor Site

Preferable accumulation of the PGA–SLM–mDBF–Cy5 conjugate at the tumor site following i.v. and intraperitoneal (i.p.) injections in vivo was evaluated by intravital noninvasive imaging using the Maestro system. D4M.3A murine melanoma cells (10$^6$) were subcutaneously (s.c.) inoculated to the mouse upper back. Tumor-bearing mice were injected i.v. or i.p. with PGA–SLM–mDBF–Cy5, SLM-Cy5 or mDBF-Cy5 and the fluorescence was measured at different time intervals for 24 h. The drug–Cy5 molecules were conjugated via an ester linker (Scheme S9, Supporting Information). For each
Figure 5. PGA–SLM–mDBF inhibits cancer cells sprouting in tumor spheroids. Tumor spheroids of D4M.3A murine melanoma (red), murine astrocytes (no color), murine brain endothelial cells (no color), and microglia cells (no color) (1:1:1:1 cell ratio, respectively. A total of 80 000 cells mL\(^{-1}\)) were grown together in reduced growth factor Matrigel sphere. Alternatively, 131/4-5B1 human melanoma cells (red), human astrocytes (no color), and human cerebralmicrovascularendothelial cells (hCMEC/D3, green) (1:1:2 cell ratios, respectively. A total of 80 000 cells mL\(^{-1}\)) were grown together in afull Matrigel sphere. The spheres were either not treated or treated with PGA, SLM+mDBF or PGA–SLM–mDBF and sprouting was evaluated after 72 h for D4M.3A cells and 144 h for 131/4-5B1. Treatment with the conjugate inhibited the melanoma cells sprouting, although to a lesser extent than the free drugs. Scale bar = 400 \(\mu\)m.

This can be attributed to the negative charges of sulfonate groups on the drug–Cy5 conjugates, which are not preferred substrates for hydrolysis by carboxylesterases.\(^{[35]}\) In addition, slower biodistribution presented herein in comparison with reported free Cy5 \(^{[36]}\) or other negatively charged imaging agents such as indocyanine green (ICG) \(^{[37]}\) may indicate that most of the drug-dye conjugates did not hydrolyze during the accumulation assessment.

2.16. PGA–SLM–mDBF Conjugate Inhibits Melanoma Tumor Growth via MAPK Pathway In Vivo

Treatment with PGA–SLM–mDBF conjugate led to a substantial antitumor effect in murine melanoma D4M.3A mouse model, compared with controls. Notably, the PGA–SLM–mDBF conjugate inhibited the tumor growth to a higher extent than the mixture of the free drugs SLM and mDBF at equivalent drug-concentrations. This resulted in a late tumor escape, which kept the tumor growth below 100 mm\(^3\) up to 20 days after treatment withdrawal. In contrast, following treatment with the free drugs, the tumor size kept growing and reached a volume of 300 mm\(^3\) already at the last day of treatment (Figure 8C). The graph of each group was terminated on the day at which the initial number of mice was reduced due to toxicity or large tumor size. Mice treated with PGA–SLM–mDBF conjugate did not suffer from abnormal toxicity during treatments, as indicated from the mice body weight change follow-up (Figure 8D) and survived significantly longer compared to all groups as shown in the Kaplan–Meier survival curve (Figure 8B).

To validate the molecular mechanism of the in vivo tumor growth inhibition shown above, the effect of our polymer-2-drug conjugate on MAPK pathway was evaluated immunohistochemically. The results revealed a marked decrease in phosphorylated ERK1/2 enzyme expression following treatment with both polymer-2-drug conjugate and with free drugs, in comparison to the untreated group (Figure 8E–G). In addition, the cell proliferation and apoptosis, that are directly affected by the MAPK pathway activation, were decreased and increased, respectively, suggesting that our combined nanoconjugate treatment inhibits...
melanoma tumor growth via MAPK pathway inactivation maintaining the original targeted activity of the drugs in vivo following their conjugation to the polymer (Figure 8H,I).

3. Discussion

We synthesized a new polymer-based system combining two targeted therapies for melanoma conjugated to the same polymeric backbone at a synergistic ratio. The conjugates’ hydrodynamic diameter ranged between 6 and 12 nm. This nanometric sized polymer-2-drug conjugate is in the suitable range to exploit the EPR effect and thus benefit from prolonged circulation in the body and preferential accumulation in the tumor site via the leaky blood vessels, in contrast to the free drugs.

The PGA-based conjugate was synthesized via a one-pot reaction, followed by robust purification by washing steps that enable us to avoid column purification. Hence, we were able to scale-up our product up to 1 g of polymer-based conjugate. Via a simple and reproducible synthetic protocol, we were able to conjugate mDBF, a BRAF V600 inhibitor, and SLM, which inhibits MEK1/2 to the same PGA backbone. In the last decade, DBF has become a first-line treatment for patients with unresectable or metastatic BRAF V600E-mutated melanoma. In order to improve the 20 months-OS of melanoma patients, combination therapy of DBF and the MEK 1/2 inhibitor, TRM, was proposed and evaluated in clinical trials. Oral administration of DBF combined with TRM (150 mg, twice a day, BID, and 2 mg, every day, QD, respectively) resulted in an increased median OS to 25 months, a one year survival rate of 74% and up to 51% for two year survival. However, this combination therapy resulted in a significant increase in grade 3/4 adverse events such as vomiting, nausea and elevated blood alkaline phosphatase in 48% and 58% of patients treated with DBF/TRM (150/1 mg) and DBF/TRM (150/2 mg), respectively. While when treated with DBF monotherapy, only 43% of patients presented grade 3/4 adverse events, which required dose adjustments of the drugs. Moreover, Mincu et al. recently published that cardiovascular adverse events were associated with the treatment of BRAF and MEK inhibitors. They revealed increased risk of pulmonary...
Figure 8. Antitumor activity of PGA–SLM–mDBF in D4M.3A tumor bearing mice. A) Timeline (days) of tumor inoculation, treatments, and follow-up. B) Kaplan–Meier survival curve. Mice in the PGA–SLM–mDBF group had the longest overall survival. C) Tumor volume growth curve. PGA–SLM–mDBF conjugate inhibited tumor volume growth more than the combination of free drugs, mDBF + SLM, in equivalent concentrations. D) Body weight change. D4M.3A tumor bearing mice were monitored during the treatment period for weight changes in order to determine the drugs toxicity. All groups presented a stable weight change curve. Data represent mean ± SEM. Statistical significance was determined using two-sided repeated-measures ANOVA (p value < 0.05). N = 5 mice per PGA–SLM–mDBF group, N = 4 mice per DBF + SLM group, N = 3 mice per vehicle group, N = 4 mice per PBS group. E–I) PGA–SLM–mDBF inhibits MAPK signaling pathway resulting in tumor cells death enhancement and proliferation inhibition in parallel to lower expression of pERK1/2 and MEK1/2 compared to an untreated (UT) control. E,H) Representative microscope images of D4M.3A tumor sections of mice treated with PGA–SLM–mDBF, free drugs, or PBS as control. The tissues were stained with FITC-labeled (green) antibodies for cell proliferation and apoptosis markers (Ki67 and caspase 3, respectively) and for phospho-ERK1/2 and phospho-MEK1/2. All tissues were counterstained with Hoechst (blue) for nucleus. Scale bar is 100 μm for all images. F) pERK1/2 expression quantification represented as a relative change from the control in the covered area. G) Distribution from the mean of all pERK1/2 measurement. I) The quantification of proliferation, apoptosis and MEK1/2 expression level represented as a total covered area.
embolism, decrease in left ventricular ejection fraction (up to 26 time higher risk for patients under 55 years old) and arterial hypertension in 2300 patients treated with BRAF and MEK inhibitors compared to treatment with BRAF inhibitors alone. Hence, the aim of our research was to reduce the concentration of the drugs and to alter their biodistribution in order to improve the dose adjustment and tumor selective accumulation while enhancing the efficacy and safety of the treatment. Indeed, we were able to improve the drugs antitumor activity while reducing the dose by up to threefold compared to the free drugs. We managed to lower the dose of DBF from previously used 30 to 10 mg kg\(^{-1}\) and SLM from 25 to 15 mg kg\(^{-1}\).

Polymer conjugates bearing combinations of drugs that increased the efficacy of the treatment were previously reported. For example, Vicent et al. demonstrated a synergistic effect and enhanced cytotoxicity toward MCF-7 breast cancer cell line by conjugating the chemotherapeutic agent DOX and the endocrine therapy (aminoglutethimide, AGM) to the nondegradable HPMA copolymer.\(^{[41]}\) Furthermore, PGA–DOX–PTX combined conjugate, previously evaluated in our laboratory, further emphasized the advantage of using combination polymer therapeutics achieving increased efficacy and safety compared to the combination of the two free drugs.\(^{[26a]}\) This combination achieved simultaneous delivery to the tumor, as opposed to concomitantly administering two free drugs which possess distinct pharmacokinetic profiles, thus, do not necessarily arrive at the same time to the tumor. This preclinical study showed the synergism, enhanced cytotoxic effect and reduced cardiotoxicity of DOX in ovarian and breast cancer mouse models.

Here, we had to modify the BRAFi for further conjugation to PGA to create our polymer-2-drug conjugate. First, we altered the DBF to DBF–Lev (Scheme S7, Supporting Information) to have a ketone functional group for hydrazone bond conjugation, which could allow pH-based release. This alteration was performed under the hypothesis that the resulting polymer–drug conjugate should be stable in the bloodstream under physiological pH = 7.4 and that conjugation of the drug through an hydrazine bond can make it stable. Nevertheless, hydrazine bond is sensitive to the acidic environment of the lysosome (pH = 5-5.5) and should release the drug from the conjugate following lysosomal internalization.\(^{[42]}\) However, further characterization revealed that the modified molecule is not stable in water; thus we considered a different modification. To that end, we carried a second attempt to modify the DBF by adding a primary amine group to DBF (DBF–NH\(_2\)). The rationale behind this strategy was that the aminopyrimidine group was not nucleophilic enough for conjugation to PGA. In addition, the conjugation through an amide bond was envisioned to allow greater stability toward hydrolysis of the drug and indeed we showed that DBF–NH\(_2\) retained the anticancer activity of DBF. However, conjugation of DBF–NH\(_2\) to PGA resulted in an amide bond that turned out to be too stable, and the drug was not released at the required conditions. Not surprisingly, combining PGA–DBF–NH\(_2\) with PGA–SLM or combining both drugs on one platform did not reveal an advantage in terms of the anticancer activity of the conjugates. In addition, probably due to its specific conformation, PGA–SLM–DBF–NH\(_2\) was not degraded while incubated in the presence of CTSB; hence neither drug, DBF–NH\(_2\) nor SLM, were released, thus rendering the conjugate inactive. As an alternative, a third attempt was carried out; this time we aimed to balance stability and degradability/drug release by cathepsins. To that end, we conjugated DBF to the polymer through an ester bond to make the conjugate stable in physiological pH and to be predisposed to hydrolysis in acidic pH, in the same manner as SLM. For this, its amine group was modified by an addition of a diol functional group, resulting in a product we named mDBF (Scheme S3, Supporting Information). The mDBF retained its anticancer activity on melanoma cell lines with a similar IC\(_{50}\) as that of DBF. To determine an optimal ratio between SLM and DBF for potent cytotoxic synergistic effect, several combinations were evaluated on different melanoma cell lines. The results showed that when SLM is at a higher concentration than mDBF, these drugs display a synergistic activity inhibiting the proliferation of human 131/4-SB1, murine D4M.3A and ZMEL1 zebrafish melanoma cell lines. We successfully conjugated SLM and mDBF to PGA polymeric backbone in order to achieve this potential synergistic effect. According to the isobolograms, two polymer-2-drug conjugates with SLM and mDBF at different drug ratios of 10:1 and 3:1, respectively, with up to 30 wt\% of loaded drugs were synthesized. Following conjugation, the drugs retained the potent activity in vitro on all tested melanoma cell lines. To our knowledge, no analogs of polymer conjugates bearing BRAFi and MEKi had been reported so far. Our conjugate was designed to be degraded by cysteine cathepsins, which cleave the PGA polymeric backbone enabling the release of the drugs from the conjugate. These enzymes are highly active in malignant compared to healthy tissues,\(^{[26c,43]}\) and mostly located intracellularly within lysosomal compartments.\(^{[46]}\) The PGA–SLM–mDBF–Cy5 conjugate displayed rapid internalization into melanoma cells, shown both via flow and image cytomtery and confocal microscopy. Furthermore, the Cy5-labeled conjugate highly colocalized with the lysosomes. This enabled the lysosomal enzymes to degrade the polymeric backbone in addition to the low pH aqueous environment which promotes the hydrolysis of the drugs from the conjugate. The colocalization with the lysosomal compartments may indicate an alteration in intracellular trafficking resulting in endocytosis pathway rather than diffusion that is more common for small drugs. This is beneficial for reducing acquired resistance by P-glycoprotein (P-gp) efflux pump that had been previously shown to cause resistance to all major classes of anticancer drugs such as camptothecins, taxanes, and kinase inhibitors.\(^{[45]}\)

One of the greatest challenges in nanomedicine design is to increase the drug loading to be as high as possible, while maintaining the proteolytic activity of the overexpressed enzymes (i.e., cathepsins) allowing their access to the polymeric backbone to induce their degradation in the tumor cells and microenvironment. We recently published that cathepsins are highly active in lysates of melanoma and breast cancer \(^{[46]}\) Melancon et al. as well as our group demonstrated that the degradation of the PGA–fluorophore conjugate by cathepsins is dependent on the fluorophore loading;\(^{[47]}\) too high loading may lower the degradation achieved due to steric hindrance to enzyme accessibility (or possibly due to tighter packing, as was observed by the smaller size of PGA–SLM as the loading increased; Table S2, Supporting Information). We found that in the presence of CTSB, there is a time-dependent degradation of our conjugate up to 60% compared to \(\pm 2\%\) degradation in the absence of CTSB. This emphasizes that the conjugate is
degraded in the presence of lysosomal enzymes while it remains intact and stable in acidic conditions and in physiological conditions. This further suggests that the conjugate will remain stable in the bloodstream and in the tumor microenvironment and will release the drugs mainly in the lysosomes of tumor cells. The findings described herein indicate that our conjugate colocalizes with the lysosome compartment. This suggests that our probe is indeed internalized into cells via an endocytotic mechanism, and intracellularly delivered into the lysosomes in a time-dependent manner. The different colocalization percentage between flow and image cytometry and confocal microscopy might result from the lower resolution of the first. However, we wanted to show these two different methods, as they present robust statistics and higher resolution, respectively. Indeed, both methods showed a similar trend of increased colocalization of the conjugate and the lysosomes over time. Release of active drugs from our polymer-2-drug conjugate in the lysosomal compartment resulted in efficient inhibition of proliferation, migration, invasion and sprouting of murine D4M.3A, human 131/4-SB1 and zebrafish ZMEL1 cells compared to monodrug conjugates and their combination. This suggests that indeed we achieved a synergistic activity when both drugs are conjugated to the same polymeric backbone. The fact that the free drugs exhibited lower IC50 than the conjugate in vitro can be attributed to the differences in cell internalization pathways mentioned above. To be activated, the conjugated drugs are required to first internalize by endocytosis and then, to be released from the polymer at an appropriate environment while the free drugs diffuse into the cells. The nanometric size of the conjugate stabilizes the drugs, prolongs their half-lives and increases the circulation time of the conjugate in the bloodstream compared to that of the free drugs. That is translated to a better accumulation in the tumor and thus, an increase of the antitumor activity in vivo compared to free drugs.[48] This advantage could not be observed in vitro where in most evaluations, the free drugs will have a better antitumor activity than nanomedicines due to faster diffusion into the cells which are “bathed” in the treatment for 72 h. To mimic better the tumor microenvironment, we evaluated the sprouting ability of human and murine melanoma cells in the presence of PGA–SLM–mDBF conjugate in a 3D melanoma spheroid model. Here, the inhibitory activity of the free drugs on the melanoma cells combined with stromal cells in 3D was similar to that of the polymer-2-drug conjugate. This may suggest that the non-rigid “spaghetti coiled” structure of the conjugate allowed for enhanced penetration into the sphere, which compensates for its partial loss of activity compared to the free drugs shown in vitro on cells grown in 2D. Furthermore, we showed that our polymer-2-drug conjugate was not hemolytic in concentrations relevant for in vivo studies. This profile allows the long circulation of the conjugate to preferentially accumulate in the tumor and be retained there without causing toxicity to RBC. To summarize, the above results emphasize the advantage of the polymer-2-drug conjugate, PGA–SLM–mDBF, compared to the free drugs. These advantages may enhance the synergistic effect of the conjugated drugs compared to the free drugs that accumulate in the tumor according to the pharmacokinetic profile of each drug at different times as they have different half-lives t1/2 (SLM = 6.5 h;[49] DBF = 2.6 h; TRM = 83 h[50]). Exposure to high concentrations of both drugs in the tumor, while limiting drug release in the bloodstream due to the stable ester covalently bound to PGA results in increased efficacy of the treatment and potentially, decreased side effects. In fact, PGA–SLM–mDBF induced a remarkable tumor growth inhibition in D4M.3A tumor-bearing mice compared to the combination of free drugs. Furthermore, we show that treatment with our PGA–SLM–mDBF at 10 mg kg−1 mDBF-equivalent dose resulted in significantly stronger inhibition of D4M.3A melanoma growth in mice than the FDA-approved combination of free DBF and TRM (Figure S11, Supporting Information) while retaining the ability of the drugs to decrease ERK1/2 and MEK1/2 phosphorylation. We succeeded to reduce the conversion of the DBF compared to previous in vivo studies on A375P melanoma-bearing mice treated with DBF/TRM combination at 30 mg kg−1 DBF and 0.3 mg kg−1 TRM[51] to 10 mg kg−1 equivalent dose of mDBF on the conjugate. SLM was used in combination therapy on HCT-116 colorectal cancer-bearing mice and was potent at 25 mg kg−1 with docetaxel at 15 mg kg−1[52] while in our combination, SLM was reduced to 15 mg kg−1 equivalent dose on the PGA conjugate. The free drugs did not induce any off-target effects at the doses administered; nevertheless, neither the conjugate enhanced any new toxicity as was observed from blood tests that were performed prior and following the treatment with PGA–SLM–mDBF, DBF/SLM and PBS (Figure S16, Supporting Information).

4. Conclusions

Here we show several synthetic approaches by fine-tuning the functional groups on the free drug DBF in order to allow a linker in the polymer-2-drug conjugate which balances stability and drug release. This is highly important for an ideal activity at the target site. Indeed, our conjugate presented a prolonged inhibition of tumor growth compared to previous reports of other polymer-2-drug conjugates[24a] and combination of BRAF/MEK targeted therapies.[53] The Kaplan–Meier survival curve clearly demonstrates the effect of the treatment on the OS of the mice. Lowering the dose of the free drugs used in our conjugate has the potential to decrease the adverse effects of the drugs and abrogate their acquired resistance. The fact that the two targeted therapies that were loaded on the PGA backbone are already FDA-approved together with the strong anticancer efficacy, the ease of preparation in one-pot, purification and scale-up of the nanosized polymeric-2-drugs conjugate have a strong impact on the clinical relevancy of the proposed nanomedicine, making this conjugate highly translatable to clinical trials.

5. Experimental Section

**Materials:** SLM was purchased from Petrus Chemicals (Herzliya, Israel) in minimum 99% purity. DBF and all other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich (Rehovot, Israel) or Tzamal-Dchem (Petch-Tikva, Israel). All reactions requiring anhydrous conditions were performed under an Argon atmosphere. Chemicals and solvents were either AR grade or purified by standard techniques. Spectra were recorded on Bruker Avance I and Avance III (Bruker MA, USA) 400 MHz (1H), 376 MHz (19F), and 100 MHz (13C) spectrometers. Mw and polydispersity index (D) analysis of the PGA polymers were performed on Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, US) equipped with a multivariate static light scattering (MALS)
Synthesis and Characterization of the Conjugates

PGA Synthesis: PGA was synthesized from O-benzyl protected glutamic acid (O-BzGLu)-OH by NCA polymerization from a suspension of H-Clu(OBz)-OH, 9.5 g, 40 mmol) in dry THF (60 mL) was heated to 50 °C. Triphosgene solution (6 g, 20 mmol) in dry THF (10 mL) was added. The reaction mixture was stirred under reflux for 3 h at 50 °C under argon atmosphere. Then, the solvent was evaporated up to 50% of monomer, Scheme S5, Supporting Information. The reaction solution was precipitated in cold hexane, filtered and recrystallized from a mixture of 5:3 toluene:THF in 40 °C, up to full conversion, which was monitored using thin layer chromatography (TLC) at 60% EtOAc/hexane. DCM was evaporated under pressure, and silica column was used with a gradient eluent that started from 60% to 80% EtOAc/hexane. We obtained 17 mg DBF–Lev (55% yield) (Scheme S7, Supporting Information).

1H NMR (400 MHz, CDCl3) δ 8.14 (d, 1H), 7.79 (t, 1H), 7.63 (t, 1H), 7.57 (m, 1H), 7.46 (t, 1H), 7.04 (t, 2H), 6.68 (d, 1H), 2.77 (m, 1H), 2.52 (d, 2H), 0.24 (m, 1H), 2.07 (s, 5H), 1.5 (s, 9H) (Figure S13, Supporting Information).

DBF–ethyleneamine (DBF–NH2) was synthesized from N-[3-[5-(2-Chloro-4-pyrimidinyl)-2-(1,1-dimethylthyl)-4-thiazolyl]-2-fluorophenyl]-2,6-difluorobenzenesulfonamide (358 mg, 1 mmol) that was dissolved in dry toluene (5 mL) with N-Boc-ethylenediamine (1.6 mL, 10 mmol) under argon atmosphere, and stirred overnight at 60 °C, up to full conversion, which was monitored using TLC (5% (19 MeOH/ EtOAc)/DCM). Toluene was evaporated under pressure, and silica column was used with a gradient eluent that started from 5% (19 MeOH/EtOAc)/DCM up to 20% (19 MeOH/EtOAc)/DCM. The obtained amine-protected product (255 mg) was then deprotected using 4 M HCl/1,4 dioxane (1.5 mL) for 20 min. The reaction was dried by HV with liquid nitrogen trap to obtain 215 mg of pure product (38% yield) (Scheme S1, Supporting Information).

DBF–diox (mDBF) was synthesized from N-[3-[5-(2-Chloro-4-pyrimidinyl)-2-(1,1-dimethylthyl)-4-thiazolyl]-2-fluorophenyl]-2,6-difluorobenzenesulfonamide (358 mg, 1 mmol) that was dissolved in dry toluene (5 mL) with (±)-3-amino-1,2-propanediol (0.39 mL, 5 mmol) under argon atmosphere. The mixture was stirred overnight at 60 °C up to full conversion, using TLC (5% MeOH/DCM) to monitor the reaction progress. Toluene was evaporated under pressure and silica column was used with a gradient eluent that started from 5% MeOH/DCM up to 10% MeOH/DCM. The final product obtained in 500 mg after drying in HV (85% yield) (Scheme S3, Supporting Information).

1H NMR (400 MHz, MeOD) δ 8.16 (d, 1H), 7.61 (m, 1H), 7.47 (m, 2H), 7.28 (t, 1H), 7.10 (t, 2H), 6.64 (s, 1H), 3.66 (t, 2H), 3.14 (t, 2H), 1.47 (s, 9H) (Figure S3, Supporting Information).

Synthesis of PGA–Drug Conjugates: PGA was synthesized as described above. The PGA polymer obtained had Mn of 10 kDa and a D of 1.16, as characterized by MALS. PGA–SLM (Scheme S2A and Figure S4A, Supporting Information) was synthesized using PGA (90 mg, 0.69 mmol monomer) with SLM (17 mg, 0.04 mmol) in dry DCM (145 mL). After 24 h, the reaction mixture was collected and washed three times with DDW and lyophilized. The powder was washed with acetonitrile three times. For dialysis, the conjugate was dissolved in 0.5 m NaHCO3, placed in a 20 mL dialysis vial, stirred at RT for 4 h, and dialyzed against DDW and lyophilized. The powder was washed with acetonitrile three times. For dialysis, the conjugate was dissolved in 0.5 m NaHCO3, placed in a 20 mL dialysis tube with MWCO = 3.5 kDa and stirred for 2 days in DDW that were replaced three times. HPLC was performed following dialysis. The product was lyophilized and 67 mg of dry conjugate with a yield of 50% by weight was obtained. The conjugate was dried by lyophilization and characterized by HPLC. Drug loading was determined using spectrophotometer and calibration curve. The absorbance of the conjugated drug was measured and compared to that of the free drug. The synthesis of PGA–DBF–NH2 (Scheme S2B and Figure S5A, Supporting Information) and PGA–mDBF (Scheme S8 and Figure S14A, Supporting Information) was done using the same general procedure that was used for the PGA–SLM conjugate synthesis.
Synthesis of PCA–SLM–DBF–NH₂ Conjugate: PCA–SLM–DBF–NH₂ conjugate was synthesized using PCA (50 mg, 0.4 mmol monomer, M₆ = 10 kDa, PDI = 1.16), SLM (27.4 mg, 0.06 mmol) and DBF–NH₂ (13.5 mg, 0.024 mmol) dissolved in dry DMF (2 mL). The reaction was cooled in an ice bath for 15 min, then BOP-Cl (40.6 mg, 0.16 mmol), DMAP (39 mg, 0.32 mmol) and DIPEA (0.047 mL, 0.26 mmol) were added. The mixture was stirred for 4 h in an ice bath, and then heated to RT overnight. The reaction was followed by TLC (10% MeOH/DCM). Next, the hydrolysis was done by cooling the reaction in an ice bath and addition of 10% NaCl for 10 min followed by addition of 0.5 M HCl (0.2 mL) and stirring at RT for 1 h. The precipitant was collected and washed three times with DDW. Then, the sample was lyophilized to obtain a white powder that was further triturated with ACN three times up to a complete wash of the free drugs. For dialysis, the conjugate was dissolved in 0.5 M NaHCO₃, placed in a 20 mL dialysis tube with MWCO = 3.5 kDa and stirred for 2 days in DDW that were replaced three times a day. HPLC was done following the dialysis completion. The product was lyophilized, and 40 mg of dry conjugate with a yield of 44% by weight was obtained. The drug loading was measured by addition of 0.001 mmol of the internal standard CF₃CH₂OH to 4 mg of the conjugate and peaks area calculation. The drug ratios were: 6 mol%, 14% w/w of DBF–NH₂ and 15 mol%, 27% w/w of SLM (Scheme S2C and Figure S6A,B, Supporting Information).

Synthesis of PCA–SLM–mDBF Conjugate: PCA–SLM–mDBF was synthesized using PCA (800 mg, 6.2 mmol monomer, M₆ = 10 kDa, PDI = 1.16), SLM (425 mg, 0.93 mmol) and mDBF (183 mg, 0.31 mmol) dissolved in dry DMF (40 mL). The reaction was cooled in an ice bath for 15 min, then BOP-Cl (635 mg, 2.5 mmol), DMAP (610 mg, 5 mmol), and DIPEA (0.252 mL, 1.56 mmol) were added. The mixture was stirred for 4 h in an ice bath, and then heated to RT overnight. The reaction was followed by TLC (10% MeOH/DCM). Next, the hydrolysis was done by cooling the reaction in an ice bath and addition of 10% NaCl for 10 min followed by addition of 0.5 M HCl (2 mL) and stirring at RT for 1 h. The precipitant was collected and washed three times with DDW. Then, the sample was lyophilized to obtain a white powder that was further triturated with ACN three times up to a complete wash of the free drugs. For dialysis, the conjugate was dissolved in 0.5 M NaHCO₃, placed in a 20 mL dialysis tube with MWCO = 3.5 kDa and stirred for 2 days in DDW that were replaced three times a day. HPLC was done following the dialysis completion. The product was lyophilized, and 990 mg of dry conjugate with a yield of 70% by weight was obtained. The drug loading was measured by addition of 0.001 mmol of the internal standard CF₃CH₂OH to 4 mg of the conjugate and peaks area calculation. The drug ratios were: 3.8 mol%, 12% w/w of mDBF and SLM, 2 mol%, 35% w/w of SLM and peaks area calculation. The drug ratios were: 3.8 mol%, 12% w/w of mDBF and 7.3 mol%, 18% w/w of SLM.

Synthesis of mDBF–Cy5 and SLM–Cy5: mDBF–Cy5 and SLM–Cy5 were synthesized using mDBF or SLM (1 equiv.), Cy5–COOH (2 equiv.), EDC (2 equiv.), and DMAP (3 equiv.) in dry DCM under Argon atmosphere. The reaction took place for 48 h with a second addition of 2 equiv. of EDC after 24 h. At the end of the reaction, the solvent was evaporated, and the product was separated by silica column with gradient from 2% MeOH/DCM up to 20% MeOH/DCM. The reactions yields were 87% for mDBF–Cy5 and 98% for SLM–Cy5 (Scheme S9 and Figure S15, Supporting Information).

Cathespin B (CTSB) Activity Assay: In order to evaluate CTSB-mediated conjugate degradation, the conjugates were dissolved at a concentration of 1 mg mL⁻¹ in freshly prepared activity phosphate buffer (0.1 M, pH = 5.5) containing: 50 × 10⁻³ M NaCl, 1 × 10⁻³ M ethylenediaminetetraacetic acid (EDTA) and 5 × 10⁻³ M glutathione (GSH). Bovine spleen CTSB (0.5 U mL⁻¹) or CTSB from human liver enzyme (10 U mL⁻¹) were then added and the mixture was incubated at 37 °C. The amount of the released drugs or degradation was assessed by HPLC at sequential time points compared to a calibration curve of each of the free drugs or conjugates area under the curve measurements at t = 0. For nonenzymatic hydrolytic cleavage assessment, polymers were incubated in an enzyme-free activity buffer.

Cell Culture

Cell Lines: 131/4-SB1 human melanoma cells were a kind gift from Robert Kerbel’s laboratory (University of Toronto, Canada), and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin, 12.5 IU mL⁻¹ nystatin, 2 × 10⁻⁵ M l-glutamine. D4M.3A murine melanoma cells were a kind gift from David Mullins’ Laboratory (Dartmouth College, Hanover), and were cultured in advanced DMEM/F12 medium supplemented with 5% FBS, 100 IU mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin, 12.5 IU mL⁻¹ nystatin, and 1% glutaMAX. Human astrocytes were purchased from ScienCell (Carlsbad CA, USA). Cells were cultured in basal astrocyte medium (AM) supplemented with 2% FBS, 1% astrocytes growth supplement (AGS), and 1% penicillin/streptomycin solution.

Human cerebral microvascular endothelial cells, hMEC/D3 were purchased from Merck (Germany) and cultured in EndoGRO-MV complete medium. Murine endothelial cells (EC) and astrocytes were isolated from brains of adult C57BL/6 mice. Briefly, the extracted brains were minced and incubated with Collagenase/dispose solution (Worthington, Lakewood N, USA) for 50 min at 37 °C. Red blood cell (RBC) lysis was performed using RBC lysis buffer followed by Percoll gradient for myelin separation. Cell suspension was incubated with CD31 microbeads for EC separation and final isolation was done on MACS MS magnetic columns. The remaining cell suspension contained the murine astrocytes. Cell type and purity were determined by FACS. Murine astrocytes were cultured in AM, as described above. Murine EC were cultured in endothelial cell medium (ECM) consists of basal medium, supplemented with 5% FBS, 1% of endothelial cell growth supplement (ECGS) and 1% penicillin/streptomycin solution. All cell lines were grown at 37 °C in 5% CO₂. Cells were routinely tested for mycoplasma contamination with a mycoplasma detection kit. ZMEL1 zebrafish melanoma cells were derived from a transgenic zebrafish with the mitf-BRAFV600E and p33−/− background, as previously described. Cells were cultured in DMEM supplemented with 10% FBS, 100 IU mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin and 1% GluMAX. Cells were grown at 28 °C in 5% CO₂.

BRAF Sequencing: V600 mutations were evaluated in all melanoma cell lines. Genomic DNA was extracted from the cells using QiAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. DNA concentration was determined by nanodrop and an RT-PCR reaction was done to detect the BRAF gene. BRAF primers for human cell lines were: forward—TGCTTGGCTCTGATAGAAATG; reverse—TCAGGGCAAAAAATTATCCA, and for murine cell lines: forward—TCCAGGCAGCTAATGTCATC; reverse—GTGAGTATCGGAACTGTGAAA. The PCR product was run on a 2% agarose gel, and the BRAF amplicon (222 bp for human cells and 565 bp for murine cells) was purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison WI, USA). The gene was then sequenced using the forward primer in a 3′ End capillary electrophoresis DNA sequenc.
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The Effect of PGA–SLM–mDBF on Cell Proliferation: Cells were plated on 24 well plates (7.5 × 10^3—D4M.3A, 15 × 10^3—131/4-S5B1) and allowed to attach for 24 h. Cells were incubated with the conjugates and free drugs, dissolved in the appropriate cell culture medium at serial concentrations, for 72 h. mDBF-containing treatments concentrations ranged from 0.001 × 10^−9 to 10 000 × 10^−9 M, according to mDBF-equivalent concentra-
tion, while the concentration-range for the SLM-containing treatments was (0.0003–30 000) × 10^−9 M, according to SLM-equivalent concentration. These concentrations were selected in order to have the same molar ratio of mDBF and SLM in all the controls as in the PGA–SLM–mDBF conjugate (3:1 SLM:mDBF, respectively). PGA was used at concentrations equiva-

tion with phenol red-free trypsin (0.5% trypsin–EDTA X 10 solution diluted 1:40 in 2.5× washes, detached by trypsin–EDTA and counted by Coulter Counter (Beck-

cell viability assay.

Tumor Spheroids: Multicellular tumor spheroids were prepared using the hanging-drop method as reported before.217 3D tumor spheroids were formed using a mixture of murine or human melanoma cells and brain microenvironment cells (murine astrocytes, microglia and endothelial cells, or human astrocytes and endothelial cells) to simulate the in vivo charac-
teristics of melanoma brain metastasis. Briefly, cells suspension of human astrocytes, GFP-labeled hCMC/D3 cells and mCherry-labeled 131/4-S5B1 cells (80 000 cells mL−1; 1:2:1 ratio) was prepared in full astrocyte me-
dia (AM) supplemented with 0.25% w/v methyl cellulose. Alternatively, murine astrocytes, endothelial cells, and microglia cells isolated from C57BL/6 mice and mCherry-labeled D4M.3A cells suspension (80 000 cells mL−1; 1:2:1:1 ratio) were prepared in full AM supplemented with 0.25% w/v methyl cellulose. Cells were deposited in 25 μL droplets on the inner side of a 20 mm dish and incubated for 48 h at 37 °C when the plate is fac-
ing upside down thus allowing spheroid formation. Spheroids were then embedded either in full (human spheroids) or reduced (murine) Matrigel (BD, Franklin Lakes NJ, USA), seeded onto a 96-well plate and treated with the different conjugates or free drugs, dissolved in the appropriate cell culture medium at 3 × 10^−6 M SLM-equivalent concentration and 1.75 × 10^−6 M DBF-equivalent concentration for 144 h. 3D spheroid invasion was visualized using EVOS imaging system.

Migration Assay: Migration assays were performed using 3 μm tran-
swell inserts (Corning Inc. Corning NY, USA) and 24-well companion plate (Corning Inc. Corning NY, USA). ZMEL1-GFP cells (1 × 10^5 cells) were seeded onto the upper chamber of the transwell and incubated for 1 h. Cells were then treated with 1 × 10^−6 M free or PGA-conjugated mDBF and SLM (2.9 μM free equivalent dose) and allowed to migrate for 48 h to the lower chamber containing DMEM supplemented with 10% FBS. Cells were then fixed using 4% PFA for 15 min and stained with Hoechst (Thermo Fisher Scientific, Waltham MA, USA) (1:2000) for nuclear staining. The fluores-
cence of the migrated cells was imaged using Zeiss inverted microscope integrated with AxioCam 503S mono camera by X20 objective. Total area of migrated cells from the captured images per membrane was analyzed using NIH image software.

Cellular Uptake and Colocalization with Lysosomes of

PGA–SLM–mDBF–Cy5 Image Stream Flow Cytometry: D4M.3A or 131/4-S5B1 cells were seeded onto 10 cm petri dishes 48 or 24 h before treatment, 1 × 10^5 cells per well or 2 × 10^5 cells per well respectively. Cells were treated with 250 × 10^−6 M (Cy5-equivalent concentration) Cy5-labeled PGA–SLM–mDBF for 0.5 h. Then, treatment was removed, the cells were washed three times with PBS and incubated with growth medium for 0.5, 4, and 24 h. In the last 0.5 h, 100 × 10^−9 M LysoTracker Green DND-26 (Life Technologies, Carlsbad, CA, USA) and 2.5 μg mL−1 Hoechst 33242 trichloroethylene tri-
hydrazide (mDBF-1,000× concentration) (Invitrogen, Carlsbad, CA, USA) were added to the 10 cm Petri dishes. Then, cells were detached from the dish by incuba-
tion with phenol red-free trypsin (0.5% trypsin EDTA X 10 solution diluted in PBS, Gibco by Life Technologies, Carlsbad CA, USA) for 5 min. Further, 6 mL of 2% FBS in PBS solution was added and the cells were centrifuged for 7 min at 1700 rpm in 4 °C. The cells were resuspended in 50 μL 2% FBS in PBS solution and analyzed using an ImageStreamX Mark II Imaging Flow Cytometer for colocalization or cellular uptake by fluorescence signal evaluation. Similarity (colocalization) threshold was set as above 1.5 (AU) fluorescence signal intensity of red and green overlapping pixels.

Confocal Microscopy Imaging: D4M.3A or 131/4-S5B1 cells (262000 cells) were seeded onto 35 mm culture dishes with a glass bottom (Ibidi GmbH, Martinsried, Germany) and incubated for 24 h. Cells were treated with 2.9 μg mL−1 PGA–SLM–mDBF–Cy5 conjugate or with 69.6 ng mL−1 Cy5 (100 × 10^−9 M Cy5-equivalent concentration) for 0.5 h. LysoTracker (Life Technologies, Carlsbad, CA, USA) and Hoechst 33042 (Thermo Fisher Scientific, Waltham MA, USA) were added to the wells at 100 × 10^−6 M and 2.5 μg mL−1, respectively, for 0.5 h. Cells were then washed three times and fresh phenol-red free medium was added. Cells were im-
aged using Leica SP8 confocal imaging systems (X60 magnification) (Le-
ica Microsystems, Wetzlar, Germany), at 0.5, 4, and 24 h post-treatment. Fresh lysotracker and Hoechst were added prior to the 24 h imaging. Colo-
calization with lysosomes was assessed using the Imaris software (Bit-
plane, Zurich, Switzerland). Averages and SD were calculated based on 5–7 fields.

Red Blood Cell Lysis Assay: Fresh blood was obtained from male Wistar rats by cardiac puncture and collected in heparinized tubes. Erythrocytes were washed three times with PBS, and then 2 g reuspended with PBS to a 2% w/v solution. The RBC solution was incubated with serial dilu-
tions of the PGA–SLM–mDBF conjugate, starting from 5 mg mL−1 poly-
mer concentration for 1 h at 37 °C. Dextran (Mw 70 kDa, Sigma-Aldrich, Rehovot, Israel) and PBS were used as negative controls, and sodium do-
decyl sulfate (SDS) as positive control. After centrifugation, the super-

tellate:EtOH:saline (vehicle). The control groups were treated with PBS

In Vivo Evaluation of PGA–SLM–mDBF

PGA–SLM–mDBF–Cy5 Conjugate Biodistribution: Male C57BL/6 mice were inoculated subcutaneously (s.c.) with 1 × 10^5 D4M.3A cells at a dorsal site (upper back). Mice bearing≈260 mm^3 tumors were treated i.v. (n = 5 mice per group) or i.p. (n = 3 mice per group) with 200 μL PGA–SLM–mDBF–Cy5 (3:1), mDBF–Cy5 and SLMy-Cy5 at 100 × 10^−6 M mDBF-equivalent concentration. Tumor accumulation was monitored by flow cytometry (MACSQuant, Miltenyi Biotec, Germany). Mice were anesthetized using ketamine (100 mg kg−1), xylazine (12 mg kg−1), treated with a depilatory cream (Veet) and placed inside the imaging system at different time points post-treatment (1, 30, 60, 180, 360, 1080, and 1440 min). Multispectral image-cubes were acquired through 590–750 nm spectral range in 10 nm steps using excitation (635 nm) and emission (675 nm) filter set. Mice auto-fluorescence and undesired back-

Evaluation of Antitumor Activity and Toxicity of the Conjugate in Com-
parison with Free Drugs: Male C57BL/6 mice were inoculated s.c. with 1 × 10^5 D4M.3A cells. Mice bearing≈25 mm^3 tumors were treated with DBF, SLm, or with the conjugate bearing the two drugs, PGA–SLM–mDBF. Treatments were administered i.p. in doses equivalent to 10 and 15 mg kg−1 for mDBF and SLM, respectively (n = 5 for all groups). The conju-
gate was dissolved in PBS, while free drugs were dissolved in 1:1:8 chro-
mophor:EIOH:saline (vehicle). The control groups were treated with PBS

called vs. width^2 x length x 0.52. Body weight was also moni-
tored every other day. Mice were euthanized when tumor volume reached 1000 mm^3 or when they lost more than 15% of their body weight.
**Immunohistochemistry:** Frozen OCT-embedded D4M.3A primary tumors were cryosectioned into 5 μm thick sections. Immunostaining of the frozen sections was performed using the BOND RX automated IHC stainer (Leica Biosystems, Wetzlar, Germany). Briefly, slides were incubated with goat serum (10% goat serum in PBS X1 + 0.02% Tween-20) for 30 min to block nonspecific binding sites. Slides were then incubated with rabbit antimonouse Ki67 (1:50 dilution, Novus biologicals, Centennial, CO, USA), rabbit antimonouse cleaved caspase 3 (1:30 dilution, Cell Signaling, Danvers, MA, USA), rabbit antimonouse phospho-MEK (1:50 dilution, Cell Signaling, Danvers, MA, USA) and with rabbit antimonouse phospho-ERK (1:28 dilution, Novus biologicals, Centennial, CO, USA). After 1 h incubation, slides were incubated for an additional 1 h with the secondary antbody goat antirabbit Alexa-488 (1:350 dilution, Abcam, Cambridge, UK) and with rabbit antimouse phospho-ERK (1:28 dilution, Novus biologicals, Centennial, CO, USA), rabbit antimouse cleaved caspase 3 (1:30 dilution, Cell Signaling, Danvers, MA, USA) and with rabbit antimonouse phospho-ERK (1:28 dilution, Novus biologicals, Centennial, CO, USA). After 1 h incubation, slides were incubated for an additional 1 h with the secondary antibody goat antirabbit Alexa-488 (1:350 dilution, Abcam, Cambridge, UK) followed by Hoechst fluorescent dye (1:5000) for additional 10 min for nuclei counterstained. The tissues stained were then fixed and mounted on a glass microscope slide with a glass coverslip using ProLong Gold antifade reagent (Invitrogen). Fluorescence images were captured using a fluorescence microscope (Evos FL Auto, LifeTechnologies) at 40x magnification.

**Statistical Analysis:** Normalization, if performed, was noted at the relevant figure. All in vitro studies data are presented as mean ± SD, while the data from the in vivo studies are presented as mean ± SEM. Sample size (n) for each statistical analysis was added for each experiment. Statistical significance was determined using two-sided Student’s t-test, one-way ANOVA or two-sided repeated-measures ANOVA with P value testing level. The Kaplan–Meier curve was created to assess survival of mice in vivo. The software used for statistical analysis was GraphPad Prism 8.

**Ethics Statement:** All animal procedures were performed in compliance with Tel Aviv University and approved by the Institutional Animal Care and Use Committee (Protocol No. 01-18-027).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

BRAF inhibitors, MEK inhibitors, melanoma, polyglutamic acid, polymeric nanomedicines, targeted therapies

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