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Synthesis and biological effects of new hybrid compounds composed of benzylguanidines and the alkylating group of busulfan on neuroblastoma cells

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ABSTRACT

^{131}Iodine-labelled (*meta*-iodobenzyl)guanidine ([^{131}I]-**mIBG**) and busulfan [butane-1,4-diylbis(methanesulfonate)] are well-established pharmaceuticals in neuroblastoma therapy. We report the design, synthesis, and testing of hybrid molecules—**mBBG** and **pBBG**—which combine key structural features of (*meta*-iodobenzyl)guanidine and busulfan: they contain a benzylguanidine moiety for accumulating in neuroblastoma cells via the noradrenaline transporter and, in the *meta*- or *para*-position, respectively, one of the two identical alkylating motives of busulfan for killing cells. Uptake and toxicity of hybrids **mBBG** and **pBBG** in human neuroblastoma cells compared favorably to their ancestors [^{131}I]-**mIBG** and busulfan.

Neuroblastoma is a malignant tumor of the sympathetic nervous system in childhood with a poor prognosis in stage IV.[1] It usually originates from the adrenal gland. It is the most abundant extracranial solid tumor in childhood making up for 8–10% of all cancers and for 15% of the corresponding mortality.[2] Approximately 1.1 children out of 100,000 under the age of 15 are diagnosed with neuroblastoma. 90% of those who are diagnosed are younger than 10 years, one third is diagnosed in the first year of life.[1,3]

A majority of the cytotoxic drugs currently used in cancer therapy acts non-specifically. Such chemotherapeutics[4] often cause severe side effects and/or long-term damages. The latter are especially severe concerning the treatment of infants. The aim of our project is to develop cytotoxic compounds with a higher specificity than their predecessors. This objective shall be reached by targeting a particular structure in neuroblastoma cells, namely the noradrenaline transporter. A substance with an enhanced specificity for such cells of the sympathetic nervous system is (*meta*-iodobenzyl)guanidine (**mIBG**) [formula: Scheme 1].

An **mIBG** equipped with a radiolabel was first synthesized by Wieland et al. in 1979.[5] In 1984/1985 radio-labelled **mIBG** was introduced independently both in Heidelberg[6] and Tübingen[7] for scintigraphic imaging of neuroblastoma. Following this discovery [^{123}I]-**mIBG** (β^+-emitter, $\tau_{1/2}$ = 13 h) has been used around the world as a routine diagnosis of neuroblastoma and also for an ensuing therapy. The major therapeutical agent, which was developed from the mentioned discovery and which has been used ever since, is [^{131}I]-**mIBG** (β^--emitter, $\tau_{1/2}$ = 8.0 d).[8] Radio-labelled analogs of [^{131}I]-**mIBG** containing iodine-125[9] (β^+-emitter, $\tau_{1/2}$ = 59 d) or astatine-211[10] (mainly α-emitter, $\tau_{1/2}$ = 7.2 h) were prepared as well. They represent therapeutical alternatives for special manifestations of neuroblastoma.[9,10]

The uptake of **mIBG** in neuroblastoma cells is the basis for the aforementioned activities. It was first described in one of our laboratories in cell culture experiments.[11] Later it was shown that this uptake occurs via the noradrenaline transporter.[12] **mIBG** was also shown to be taken up by OCT-expressing cells (OCT: organic cationic transporter).[13] This process is a well known side effect in scintigraphy of neuroblastoma.[8a,14] Very recently, it turned out that the uptake of **mIBG** in OCT-expressing cells can be reduced by glucocorticoids.[15]

Even nonradioactive benzylguanidines—which would not per se be expected to affect cell life [exceptions: a nitro-substituted **mIBG** (Ref. 16) and unsubstituted **mIBG** (Refs. 17–19; see also the next

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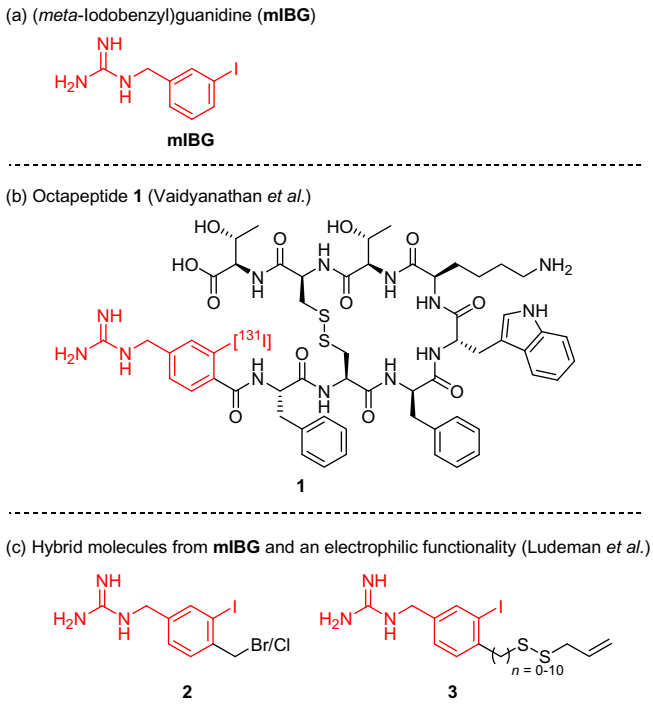
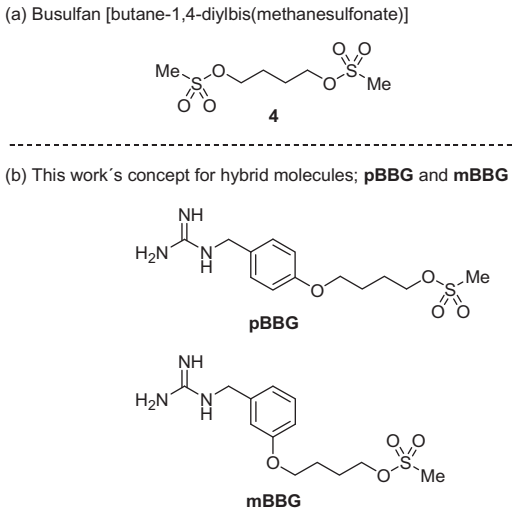


Scheme 1. Benzylguanidine motives in **mIBG** (a), in octapeptide **1**[21f] (b), and in the iodine-containing alkylating agents **2**[22] and **3**[22] (c).

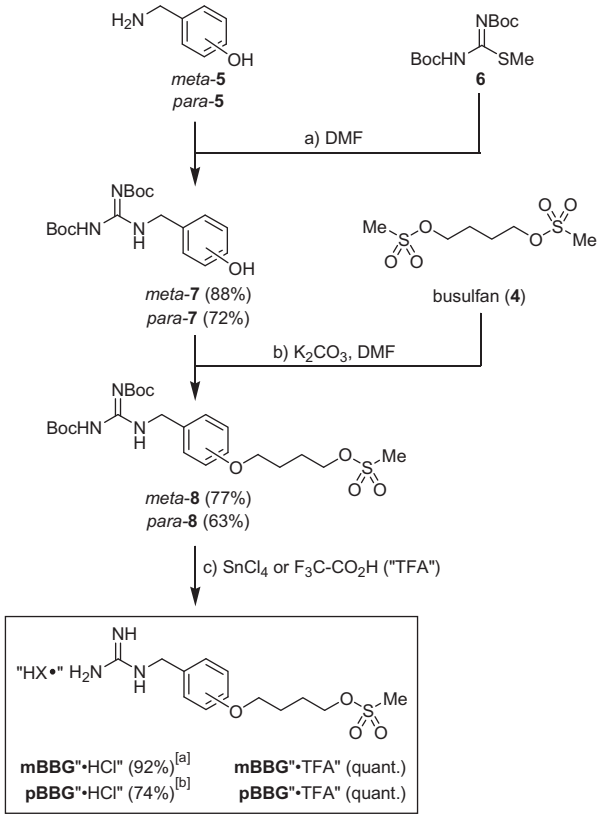
paragraph]—should be modifiable so that they kill neuroblastoma cells. Such a concept has been pursued by at least two groups:[20] Vaidyanathan et al. developed modified—yet always iodine-containing—analogues of **mIBG**,[21] such as the octapeptide **1**[21f] [Scheme 1]. Its cytotoxic effect is most likely due the redox active S—S bond. Ludeman et al. proposed 'A Method for Treating Neuroblastoma' based on a sizable number of **mIBG**s.[22] Each of these molecules contains an electrophile—in substituents of widely varied structures [e.g., Scheme 1 (c)]—for attacking the mitochondrial glutathione of tumor cells. Without an exception the Ludeman compounds display an iodine-substituent in an unvaried **mIBG** substructure.[16,22]



Scheme 2. The bifunctional alkylating agent busulfan (**4**[25]) (a). Our concept for a specific therapy of neuroblastoma (b): hybrid molecules **pBBG** and **mBBG** composed of the alkylating motive of busulfan (**4**) and of the common benzylguanidine motive both of **pIBG** and **mIBG**.

The simplest nonradioactive **mIBG** is the parent compound itself, namely **mIBG**. Intriguingly, it is active against neuroblastoma cells: associated effects are a sharp decrease of the ATP/ADP ratio[18] and aberrances in glycolysis and oxidative phosphorylation.[17–19] Recently, the HR-NBL1[23] trial of the European SIOP[24] Neuroblastoma Group demonstrated that busulfan [**4**;[25] formula: Scheme 2 (a)] and melphalan (={4-[*N*,*N*-bis(2-chloroethyl)amino]phenyl}alanine) combined make an excellent myeloablative therapy for high-risk neuroblastoma.[26] It is superior to the so-called 'CEM protocol', which is combined treatment with carboplatin, etoposide, and melphalan. The 'CEM protocol' represented the recommended neuroblastoma therapy before it was replaced by the combination busulfan (**4**)/melphalan.[27]

What we hoped would turn out to be anti-neuroblastoma agents were hybrids of the alkylating motive of busulfan [**4**;[25] formula: Scheme 2 (a)] and a benzylguanidine. The alkylating group should be installed in the benzene ring such that a *meta*- (**mBBG**) or *para*-substituted benzylguanidine (**pBBG**) results [formulas: Scheme 2 (b)]. We hoped that the respective hybrid molecules (**mBBG** and **pBBG**) would combine the cytotoxicity of busulfan with the ability of (*meta*-iodobenzyl)- or (*para*-iodobenzyl)guanidines of intruding neuroblastoma cells by means of the noradrenaline transporter. Neither **mBBG** nor **pBBG** contains an iodine atom. This makes our design novel compared to the Ludeman systems[22] and simpler: introducing two substituents into benzene (as



Scheme 3. Synthesis of the hybrid molecules **pBBG** and **mBBG** by deprotection of the *N*,*N*′-Boc-protected precursors *para*-**8** or *meta*-**8** either by HCl or by TFA in the final step. Conditions: (a) **5** (1.0 equiv), **6** (1.1 equiv), DMF, room temperature, 16–24 h; 88% (*meta*-**7**), 72% (*para*-**7**). (b) **7** (1.0 equiv), **4** (3.0–3.1 equiv), K_2CO_3 (5.1–5.2 equiv), DMF, room temperature, 20 h; 63% (*para*-**8**), 77% (*meta*-**8**). (c) $SnCl_4$ (2.0–2.4 equiv), EtOAc, room temperature, 3 h; 92% (**mBBG**"·HCl")[a], 74% (**pBBG**"·HCl")[b]. (c) F_3CCO_2H (TFA)/CH_2Cl_2 1:1 (v:v), room temperature, 1.5 h; quant. [a]Different batches contained 0–20% of an S_N2 product, in which the methanesulfonate group was replaced by an unknown nucleophile.[36] [b]Contained 9% of a S_N2-product where the methane sulfonate group was replaced by an unknown nucleophile.[36]

in this Letter) rather than introducing three (as in Ref. 22) requires fewer synthetic steps.

Our syntheses of **mBBG** and **pBBG** started with commercially available *N*,*N*′-protected thiourea **6**[28] and (*meta*-hydroxybenzyl)- and (*para*-hydroxybenzyl)amine (*meta*-**5** and *para*-**5**), respectively (Scheme 3). Step 1 consisted of stirring the respective (hydroxybenzyl)amines **5** with a slight excess of the guanidinylating agent **6** in DMF at room temperature for 16–24 h. The *N*,*N*′-dicarbamoylated benzylguanidines *meta*-**7** (88% yield) and *para*-**7** (72% yield), respectively, resulted. DMF as a solvent was crucial for attaining acceptable conversions. The likely reason is that the (hydroxybenzyl)amines *meta*-**5** and *para*-**5** were only sparingly soluble in other solvents, for example, in CH_2Cl_2.[29] Step 2 of the synthetic sequence of Scheme 3 consisted of treating the respective (*meta*-hydroxybenzyl)- or (*para*-hydroxybenzyl)guanidine **7** with an excess of busulfan (**4**) and K_2CO_3 in DMF. This delivered the corresponding *N*,*N*′-dicarbamoylated **BBG**-precursors *meta*-**8** (77% yield) and *para*-**8** (63% yield).[30]

The final step of Scheme 3 posed the challenge of removing the Boc groups from the benzylguanidines moiety of *meta*- and *para*-**8** without affecting the sulfonate group. The latter was not only susceptible to rapid hydrolysis but also to (another) nucleophilic substitution (cf. the subsequent paragraph). Whereas we had been able to extract the substrates *meta*-**8** and *para*-**8** of the imminent step from aqueous phases with standard solvents (e.g., *t*BuOMe) and to purify them by flash chromatography on silica gel,[31] isolating the Boc-free guanidines **mBBG** and **pBBG** was incompatible with either technique. It rather required a considerable amount of fine-tuning since **mBBG** and **pBBG** were extremely polar.

Screening several Lewis acids, solvents, and solvent mixtures we found trifluoroacetic acid (TFA) or $SnCl_4$ suitable for decarbamoylating *meta*-**8** and *para*-**8** while tolerating their methanesulfonate group. In the former case we stirred the respective precursor *meta*- or *para*-**8** in CH_2Cl_2/TFA (1:1, v:v) at room temperature for 1.5 h.[32] Then we evaporated all volatiles (i.e., the solvent and excess TFA as well as isobutene and CO_2, which are stoichiometric by-products of Boc cleavage) in vacuo. Initially, this seemed to be a reliable procedure for isolating the expected products **mBBG** ·TFA and **pBBG** ·TFA in quantitative yields. Later we realized that TFA could be removed from **mBBG** ·TFA and **pBBG** ·TFA in vacuo to some extent. This phenomenon was a surprise. It was unveiled by preparing several batches of **mBBG**"·TFA" and **pBBG**"·TFA" (for the meaning of the quotation marks: cf. below), respectively, and by finding that the integrals of the respective F_3CCO_2H·**BBG** or F_3CCO_2H·**BBG** ^{13}C NMR resonances (400 or 500 MHz, d_6-DMSO) varied relative to the other ^{13}C NMR integrals. The exact composition of the respective 'trifluoroacetates' remaining unknown, we depict the latter using quotation marks, that is, as **mBBG**"·TFA" and as **pBBG**"·TFA", respectively.[33]

Treatment of the benzylguanidine dicarbamates *meta*- or *para*-**8** with $SnCl_4$ (2.0–2.4 equiv) in ethyl acetate at room temperature for 3 h[34] removed the Boc groups as efficiently as deprotection in TFA/CH_2Cl_2 (vide supra). However, we suspected that rests of Sn(IV) in the resulting hydrochlorides **mBBG**"·HCl" and **pBBG**"·HCl",[35] respectively, might disturb the upcoming cell tests. Therefore, we freed the crude reaction mixtures from excess $SnCl_4$ in vacuo. At 0 °C we quenched the residue with H_2O [$+SnCl_4 \rightarrow SnO_{2(s)}$]. The resulting solution was extracted with a mixture of *i*PrOH and $CHCl_3$ (1:3, v:v). The combined extracts were concentrated to provide **mBBG**"·HCl" and **pBBG**"·HCl" in up to 92% and 74% yield, respectively.[36]

Among the substances synthesized, **mBBG**"·TFA" and **pBBG**"·HCl" were most soluble in aqueous medium and were therefore used for the following experiments with neuroblastoma

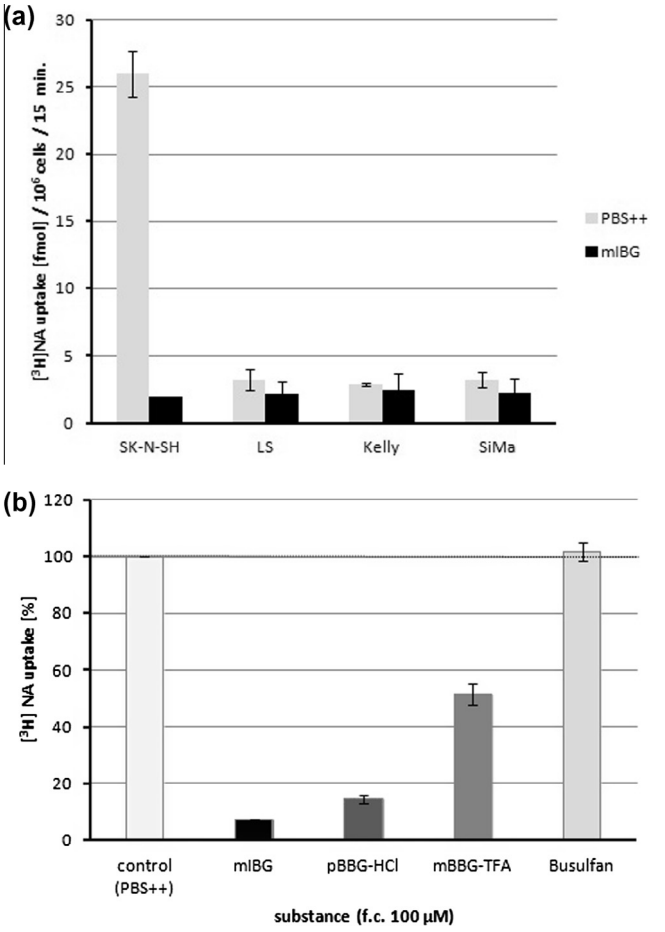


Figure 1. Top (a) uptake of [^{3}H]-NA (0.1 µCi; final concentration 0.1 µM) into different human neuroblastoma cell lines after a 15 min incubation period at 37 °C in pure PBS^{++41} and in the presence of unlabelled **mIBG** [final concentration (f.c.): 100 µM] $n = 3$, mean ± SD. Bottom (b) uptake of [^{3}H]-NA (0.1 µCi; final concentration 0.1 µM) into SK-N-SH cells after a 15 min incubation period at 37 °C in pure PBS^{++41} and in the presence of unlabelled **mIBG**, **pBBG**, **mBBG** and busulfan (**4**), respectively (final concentration: each 100 µM); $n = 3$ independent experiments, mean ± SD).

cells. First, the uptake of **pBBG** and **mBBG** into human neuroblastoma cell lines SK-N-SH, LS, SiMa and Kelly was analyzed by competitive uptake experiments with [^{3}H]-noradrenaline {[^{3}H]-NA}. Cell suspensions were prepared and adjusted to 1×10^6 cells/mL. [^{3}H]-noradrenaline {norepinephrine-(levo[7,^{3}H]); [^{3}H]-NA; specific activity: 12.1 µCi/mmol; Perkin–Elmer; final concentration in the incubation mixture: 1×10^{-7} M} as well as the other compounds used in the experiment (final concentration 1×10^{-4} M) were added in 10 µL portions, respectively. After incubation at 37 °C for 15 min 10 mL ice-cold phosphate buffered saline (PBS[40]) containing Ca^{2+} and Mg^{2+} (PBS^{++41}) was added and the suspensions were centrifuged at 500*g* for 5 min. This procedure was repeated twice. Finally the radioactivity in the cell pellets was measured after lysis with 500 µL distilled water and addition of 10 mL liquid scintillation cocktail (OptiPhase Super Mix, Wallac). It is known that among these four human neuroblastoma cell lines SK-N-SH cells express the noradrenaline transporter. Figure 1a shows that SK-N-SH cells have a much higher uptake of [^{3}H]-noradrenaline than cells of the other neuroblastoma cell lines. The surplus of unlabelled **mIBG** strongly inhibits this uptake process. A significant uptake inhibition in SK-N-SH cells was also obtained by **pBBG**, and—to a lesser extent—by **mBBG**, whereas busulfan [**4**;[25] formula: Scheme 2 (a)] did not show an inhibitory effect at all (Fig. 1b).

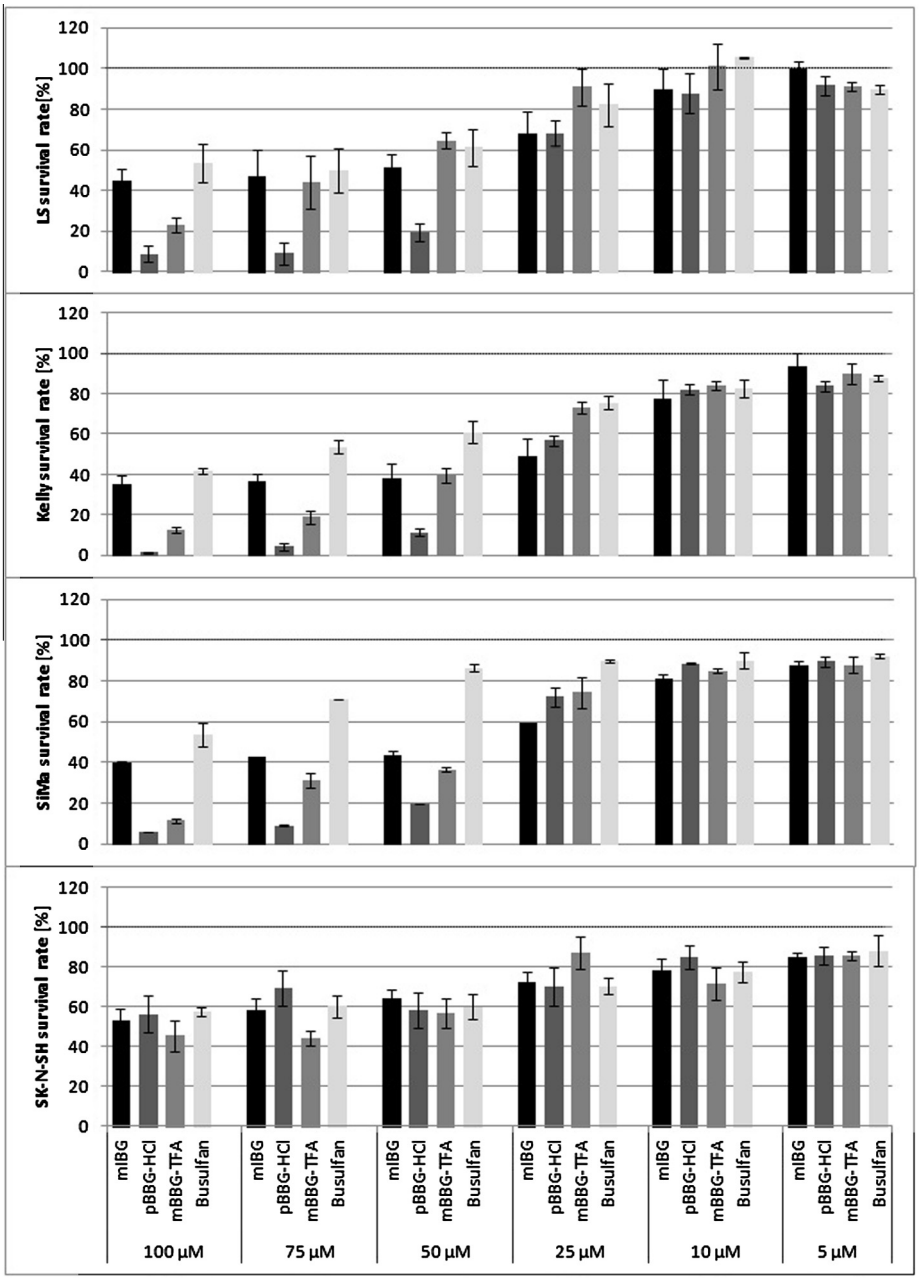


Figure 2. Concentration-dependent influence of **mIBG**, **pBBG**, **mBBG**, and busulfan (**4**) on cell proliferation/vitality on four different human neuroblastoma cells compared to untreated controls, set as 100%. MTT-test; mean ± SD, $n = 3–5$ independent experiments.

Next, the cytotoxic, that is, antiproliferative effects of **pBBG** and **mBBG** on neuroblastoma cells compared to **mIBG** and busulfan [**4**;[25] formula: Scheme 2] were investigated using the MTT-test according to Mosmann.[37] After an incubation period of 72 h, **pBBG** and—to a lesser extent—**mBBG** were more effective in a dose-dependent manner (f.c. 10–100 µM) than **mIBG** or even busulfan (**4**), indicating their powerful potential for neuroblastoma treatment (Fig. 2). Surprisingly, SK-N-SH cells which incooperate [^{3}H]-noradrenaline much better than LS, Kelly and SiMa cells, were affected in the MTT-assay to a lesser extent by **mIBG**, **pBBG**, **mBBG**, and busulfan (**4**). Compared to the other cells, SK-N-SH proliferate much slower (doubling time approx. 30 h compared to 20–24 h in the case of LS, Kelly, and SiMa). This may explain the latter discrepancy. Further experiments with systematic time-dependent uptake proliferation, and cytotoxic studies should clarify this unexpected effect.

By analyzing the influence of **mIBG** on cell metabolism, Loesberg et al. observed that **mIBG** enhanced the rate of glycolysis (enhanced lactate formation) in neuroblastoma and lymphosarcoma cells.[18] This observation is of special interest since many tumor cells, among them neuroblastoma cells, preferentially metabolize glucose to lactate instead of CO_2 and H_2O even in the presence of oxygen ('aerobic' glycolysis, Warburg effects).[38,39] Based on the assumption that one mol glucose can theoretically be metabolized at the most to two mol lactate (100%), all three neuroblastoma cell lines used in the experiments shown in Figure 3 followed significantly the Warburg characteristics: LS: 81 ± 19%; SiMa: 98 ± 2%; Kelly: 74 ± 26% glucose converted to lactate (mean ± SD; $n = 3$). We were interested whether **pBBG** and **mBBG** behave similar to **mIBG** concerning their influence on the glucose metabolism. For this purpose, glucose consumption and lactate production were measured with commercial assays (Roche, Mannheim,

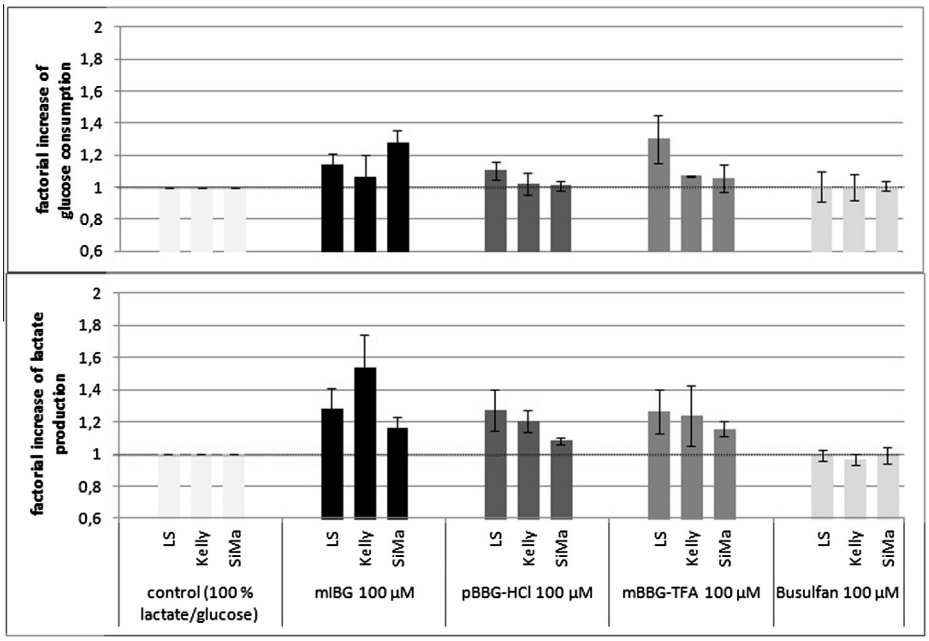


Figure 3. Influence of **mIBG**, **pBBG**, **mBBG**, and busulfan (**4**) on glycolysis (glucose consumption and lactate production) in fast proliferating neuroblastoma after 12 hours incubation period at 37 °C in PBS^{++}[41] containing 10 mM glucose. Fractional changes in glucose consumption (top) and lactate production (bottom) in the presence of **mIBG**, **pBBG**, **mBBG** and busulfan were related to the untreated control cells, set as 1.0; mean ± SD, $n = 3$ independent experiments. Further information: see text.

Germany) in the supernatant of approx. 500.000 seeded cells in 24 well plates (2 mL PBS^{++},[41] containing approx. 10 mM glucose) during a 12 h incubation period at 37 °C. Fractional changes in glucose consumption and lactate production in the presence of **mIBG**, **pBBG**, **mBBG**, and busulfan [**4**;[25] formula: Scheme 2 (a)] were related to the untreated control cells (Fig. 3). In these experiments, it could be confirmed that **mIBG** enhanced glucose consumption and lactate production also in the three investigated neuroblastoma cell lines. Most characteristically, **mBBG** and **pBBG** caused also enhanced lactate production (glucose consumption was only significantly enhanced in LS cells), indicating that the new synthesized compounds still retained features of **mIBG** after iodine replacement by the alkylating group. In contrast, these effects on glycolysis were not observed after treatment with busulfan (**4**). Further experiments should clarify in which way glycolysis is influenced by **mIBG**, **mBBG**, and **pBBG** on a molecular level and whether these features contribute to their cytotoxic effects and could be used for therapy.

In summary the present study discloses the syntheses of salts of **mBBG** and **pBBG** [formulas: Scheme 2 (b)], that is, of hybrid molecules combining the benzylguanidine moiety of **mIBG** [formula: Scheme 1 (a)] with the methanesulfonate moiety of busulfan [**4**; formula: Scheme 2 (a)]. The *N*,*N*′-dicarbamoylated precursors *meta*-**8** and *para*-**8** of **mBBG** and **pBBG**, respectively, were obtained in two steps from the (hydroxybenzyl)amines *meta*-**5** or *para*-**5**, thiourea **6**, and busulfan (**4**; Scheme 3); their overall yields were 68% and 45%, respectively. Boc removal from the dicarbamates *meta*-**8** and *para*-**8** was straightforward in trifluoroacetic acid (TFA)/CH_2Cl_2 (1:1, v:v) but at best tedious if not outright troublesome using $SnCl_4$. In the former case we obtained the trifluoroacetates of **mBBG** and **pBBG**, in the latter case contaminated[36] hydrochlorides. We showed that **mBBG** and **pBBG** retain features of **mIBG**, that is, uptake via the NAT and an enhancing effect on glycolysis in human neuroblastoma cells. Gratifyingly, **mBBG** and **pBBG** were even more cytotoxic towards these cells than their ancestors **mIBG** and busulfan (**4**). Further studies shall clarify the mechanism of action of **mBBG** and **pBBG**. They will also address their effect on glycolysis, the time-dependent uptake into NAT-positive and NAT-negative neuroblastoma cells, and potential therapeutic applications.

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29. The ^{1}H NMR spectra (300–500 MHz) of the (hydroxybenzyl)guanidine *meta*-**7** in $CDCl_3$ changed both with the concentration of *meta*-**7** and with the temperature during signal acquisition. Sample concentrations could be (unintentionally) (1) such that the chemically distinct $NCO_2C(CH_3)_3$ groups displayed a single singlet rather than two singlets and/or (2) such that peaks of the benzyl group were shifted by about as much as the 'spread' of an *ortho* coupling. The interference of these variations was attributed to differential intermolecular hydrogen bonding. As a consequence, the chemical shifts in all benzylguanidines of this study (**7**, **8**, **mBBG**, and **pBBG**) might depend likewise on the concentration and/or the temperature (we did not probe such effects, though).
30. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of a given batch of compound *para*-**8** in $CDCl_3$ was registered two times. To our considerable surprise one spectrum showed two $NCO_2C(CH_3)_3$ resonances (δ = 153.2 and 163.7 ppm) for the two $NCO_2C(CH_3)_3$ groups (which is what we expected) while the other spectrum showed a single $NCO_2C(CH_3)_3$ resonance (δ = 153.2 ppm) whereas the signal at δ = 163.7 ppm had disappeared. Under the latter conditions the spectrum showed two dramatically weakened $NCO_2C(CH_3)_3$ resonances. We attributed these phenomena tentatively to the quadrupole moments of the three nitrogen atoms of the guanidine moiety.
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33. Sometimes impurities formed during the deprotection of compound *meta*-**8** with TFA. These impurities displayed a ^{1}H NMR triplet at δ = 4.46 ppm (500 MHz, d_6-DMSO) and a ^{13}C NMR resonance at δ = 68.3 ppm.
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35. How much HCl the desired guanidines **mBBG**·HCl and **pBBG**·HCl contained was not ascertained experimentally. However, we had discovered circumstantial evidence that the analogous guanidines **mBBG**·TFA and **pBBG**·TFA lost TFA to some extent, when these compounds were dried in vacuo (cf. main body of the text). By analogy, HCl losses from **mBBG**·HCl and from **pBBG**·HCl must be considered as possibilities until the contrary is proved. Therefore, we denote these guanidine specimens as **mBBG**"·HCl" and **pBBG**"·HCl", respectively, attempting to indicate that they might contain less than a full equiv of HCl.
36. **mBBG**"·HCl" and **pBBG**"·HCl" were obtained together with up to 20% and 9%, respectively, of an impurity. These impurities showed characteristic triplets at δ_H = 3.71 ppm (400 MHz, d_6-DMSO). The distinctness of this value from the ^{1}H NMR chemical shifts of *n*PrCH_2Cl (δ = 3.62 ppm) and *n*PrCH_2OH (δ = 3.38 ppm) in d_6-DMSO (400 MHz;Abraham, R. J.; Byrne, J. J.; Griffiths, L.; Perez, M. *Magn. Reson. Chem.* **2006**, *44*, 491) seems to rule out that the methanesulfonate group in compounds **mBBG** and **pBBG** had been exchanged by S_N2 attacks of a chloride ion or H_2O. Moreover, the ^{1}H NMR shifts (500 MHz, d_6-DMSO) of $C_6H_5CH_2NH_2$ (δ = 3.72 ppm) or 2-MeO–C_6H_4–CH_2NH_2 (δ = 3.71 ppm) published by Swain, C. J.; Seward, E. M.; Cascieri, M. A.; Fang, T. M.; Herbert, R.; MacIntyre, D. E.; Merchant, K. J.; Owen, S. N.; Owens, A. P.; Sabin, V.; Teall, M.; VanNiel, M. B.; Williams, B. J.; Sadowski, S.; Strader, C.; Ball, R. G.; Baker, R. *J. Med. Chem.* **1995**, *38*, 4793 are virtually identical with that of the mentioned impurities. At this point we held the latter for the respective benzylamines, which would be released from compounds **mBBG** and **pBBG** hydrolytically. However, we observed one decomposition of compound **mBBG**, which falsified this interpretation: The signal of $CH_2OSO_2CH_3$ (δ = 4.27 ppm) decreased while the triplet of the impurity (δ = 3.71 ppm) increased (400 MHz ^{1}H NMR analysis, d_6-DMSO); concomitantly the signals of both $CH_2OSO_2CH_3$ (δ = 70.1 ppm) and $CH_2OSO_2CH_3$ (δ = 36.6 ppm) decreased and the signal of the impurity (δ = 45.2 ppm) increased (125 MHz ^{13}C NMR analysis, d_6-DMSO)—but the intensity of the signal of the guanidine–carbon remained unchanged.
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40. 'PBS' is for 'phosphate-buffered saline' and usually refers to a salt solution without Ca^{2+} or Mg^{2+}.
41. 'PBS^{++}' designates PBS,[40] to which both Ca^{2+} and Mg^{2+} were added.