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# Cytotoxic activities of hydroxyethyl piperazine-based σ receptor ligands on cancer cells alone and in combination with melphalan, PB28 and haloperidol

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**This paper is dedicated to Prof. Dr. Hans-Hartwig Otto on the occasion on his 75th birthday.**

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σ Receptor ligands are attracting interest as possible anti-cancer agents because of their ability to induce cell death by different mechanisms. In this study we investigated the cytotoxic effects of 12 recently developed σ-receptor ligands in a panel of eight different human tumor cell lines by either the crystal violet or MTT assays. The results show that σ ligands have broad cytotoxic activity on a number of human cancer cell lines with $IC_{50}$ values in the low μM range. In addition, apoptosis was observed by the annexin-V/PI double staining method when RPMI 8226 human multiple myeloma cells were treated with a representative σ ligand, (*R*)**-2b**. Combination of (*R*)**-2b** with melphalan led to a higher apoptotic rate than with the drug alone. Likewise, combined treatment of (*R*)**-2b** with the known high affinity $\sigma_2$-agonist PB28 showed an additive effect on the induction of apoptosis in the RPMI 8226 line. In contrast, combinations of (*R*)**-2b** with the known $\sigma_1$-antagonist haloperidol lead to a significant reduction in the cytotoxic activity of (*R*)**-2b**. These results support the idea that (*R*)**-2b** acts as a σ-agonist to cause the death of RPMI 8226 cells.

## 1. Introduction

σ Receptors are a unique group of receptors containing two subtypes ($\sigma_1$ and $\sigma_2$), which show diverse pharmacological profiles (Aydar et al. 2004). The $\sigma_1$ receptor is better characterized due to the fact that it has been cloned from mammalian species. This subtype is a transmembranous 223 amino acid protein with two transmembrane regions, an extracellular loop as well as one C-terminus and one N-terminus, which are intracellularly located (Aydar et al. 2002). The $\sigma_1$ receptor is preferentially localized in the endoplasmic reticulum (ER) and considered to act as an ER chaperone protein because of the ability to translocate to the plasma membrane (Hayashi et al. 2003; Morin-Surun et al. 1999). Moreover, this receptor subtype modulates various ion channels as well as some neurotransmitter systems. It has been intensively studied for the function in the CNS, where it is involved in several disorders like depression and anxiety, psychosis, Parkinson′s disease, Alzheimer′s disease or drug addiction (Maurice et al. 2009).

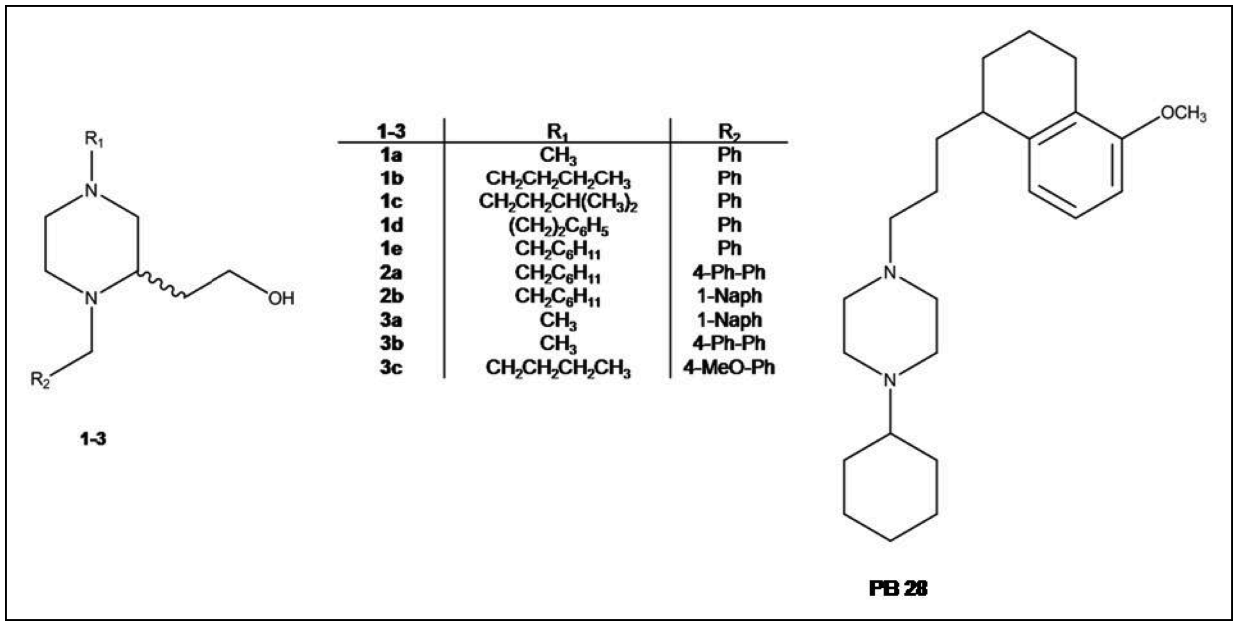
The $\sigma_2$-receptor has not yet been cloned. It is also widely distributed in the CNS but like the $\sigma_1$- subtype high expression levels have also been found in peripheral organs like heart, kidney, liver, intestine, ovaries and testes (Guitart et al. 2004; Hellewell et al. 1994; Jansen et al. 1992). Both subtypes have been shown to be over-expressed in many human tumor cell lines, leading to the development of σ-ligands for diagnostic imaging and cancer treatment emphasizing the important role of these receptors in cancer biology (Colabufo et al. 2004; Wheeler et al. 2000).

In previous work we reported on bridged σ-receptor-ligands with high $\sigma_1$ affinity. (Geiger et al. 2007; Holl et al. 2009a, b; Sunnam et al. 2010). Recently, we described the synthesis, receptor affinity and cytotoxicity of a series of hydroxyethyl substituted σ-receptor-ligands (Fig. 1) (Weber et al. 2014). Two of these compounds, (*S*)**-2a** and (*R*)**-2a**, were able to induce caspase-independent apoptosis as well as an incomplete autophagy in RPMI 8226 cell line (Korpis et al 2014). In the present study the cytotoxic activities of 12 promising σ-receptor ligands were investigated in a panel of eight different human tumor cell lines, a number of which (i.e., A427, 5637, LCLC-103H, MCF-7, RT-4, RPMI 8226) are known to express both σ receptors. (*R*)**-2b** shows high binding affinity for the $\sigma_1$-receptor relative to the $\sigma_2$-receptor, thus it was chosen as a model compound for further investigations (Weber et al. 2014). Here we report the cytotoxic and apoptotic activities of (*R*)**-2b** in the RPMI 8226 cell line in combined treatment with melphalan and two $\sigma_1$-antagonist/$\sigma_2$-agonists, PB28 and haloperidol, that have opposite orders of affinity at the $\sigma_1$/ $\sigma_2$ –receptors ($\sigma_2 > \sigma_1$ and $\sigma_1 > \sigma_2$, respectively). Based on these results, we propose that (*R*)**-2b** acts as an agonist at both subtypes.

## 2. Investigations and results

### *2.1. σ-Receptor-ligands show antiproliferative activity*

The cytotoxic activity of the σ receptor-ligands were evaluated on two human bladder cancer (5637 and RT-4), a human small cell lung cancer (A427), a human large cell lung cancer




| 1-3 | $R_1$ | $R_2$ |
|---|---|---|
| 1a | $CH_3$ | Ph |
| 1b | $CH_2CH_2CH_2CH_3$ | Ph |
| 1c | $CH_2CH_2CH(CH_3)_2$ | Ph |
| 1d | $(CH_2)_2C_6H_5$ | Ph |
| 1e | $CH_2C_6H_{11}$ | Ph |
| 2a | $CH_2C_6H_{11}$ | 4-Ph-Ph |
| 2b | $CH_2C_6H_{11}$ | 1-Naph |
| 3a | $CH_3$ | 1-Naph |
| 3b | $CH_3$ | 4-Ph-Ph |
| 3c | $CH_2CH_2CH_2CH_3$ | 4-MeO-Ph |

Fig. 1: Structures of the tested piperazines with hydroxylethyl side chain (**1–3**) and the $\sigma_1$-antagonist /$\sigma_2$-agonist **PB28**.

(LCLC-103H), a human pancreas cancer (DAN-G), a human breast cancer (MCF-7), a human leukemia cancer (HL60) and on the human multiple myeloma cell line RPMI 8226. For the adherent cell lines (5637, RT-4, A427, LCLC-103H, DAN-G and MCF-7) the crystal violet staining assay was used. For the two suspension cell lines (HL60 and RPMI 8226) the MTT assay was used. Fig. 2Ashows representative dose-response curves for *(S)*-**1e** and (*R*)**-2b** in the A427 line and Fig. 2B shows dose-response curves for (*R*)**-2b** and melphalan in the RPMI 8226 line**.** Interestingly, the shape of the curves for (*R*)**-2b** and melphalan are nearly identical and both compounds gave similar average $IC_{50}$ values.

The calculated $IC_{50}$ values (half of the maximal inhibitory drug concentration) of the tested ligands are reported in the Table. For comparison, the known chemotherapeutic drug melphalan and the σ-receptor-ligands **PB28** ($\sigma_1$-antagonsit/$\sigma_2$-agonist), haloperidol ($\sigma_1$-antagonsit) and (+)-pentazocine ($\sigma_1$-agonist) were also investigated. In adherent cell lines, compounds with large residues at both N-atoms showed greater antiproliferative activity than compounds with small groups. Whereas ligands with an aliphatic methyl group *(S)*-**1a**, (*S*)-**3a**, (*S*)-**3b**), butyl group (*S*)-**1b,** (*S*)-**3d**) or isobutyl group (*S*)-**1c**) at N4 had no activity in any of the cell lines (except for (*S*)-**3b** in the A427 cells), introduction of the cyclohexylmethyl-moiety (**1e**) led to an $IC_{50}$ value below 20 μM in the A427 cell line (Table). Moreover, replacement of the phenyl-substituent at N1 for the larger naphthylmethyl group (i.e., (*S*)-**2b**, (*R*)-**2b**) or biphenylmethyl group (i.e., (*S*)-**2a**, (*R*) **-2a**) resulted in an increase in potency in almost all adherent cell lines. These findings are in accord with the $\sigma_1$-receptor-binding studies, in which the affinity ($K_i$) increased in the order of methyl < butyl < isopentyl < cyclohexylmethyl (Weber et al. 2014).

Interestingly, the (*R*)-enantiomer is in most cases more effective than the (*S*)-enantiomer. For example, the $\sigma_2$-receptor-rich cell lines MCF-7 and RT-4 were unresponsive to treatment with (*S*)-**2a**, whereas the (*R*)-enantiomer (*S*)-**2a** shows $IC_{50}$ values of about 10 μM. This chiral discrimination is not seen in the RPMI 8226 cell line, here the (*S*)-enantiomer is only slightly more active than the (*R*)-enantiomer (Table). Although (*R*)- **2b** had the greatest binding affinity in this cell line for the $\sigma_1$-receptor, the (*S*)-enantiomer (*S*)-**2b** had the lowest $IC_{50}$ concentration (1.29 μM). The leukemia HL60 cells were resistant to all compounds tested, except for (*R*)-**2b,** which gave an $IC_{50}$ = 17.35 μM.

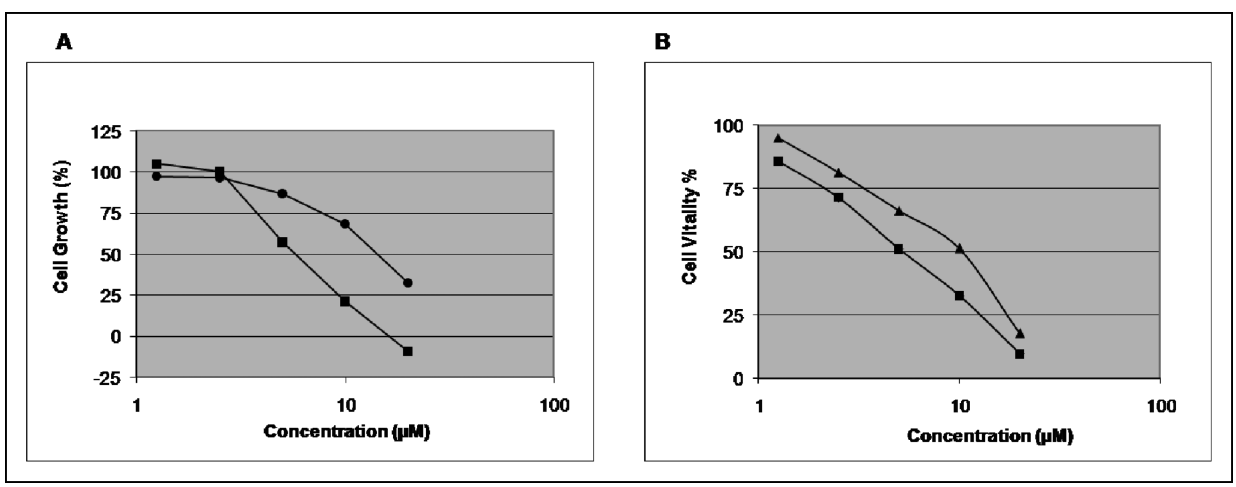



Fig. 2: Representative dose-response curves of (A) A427 cells treated 96 h with (*S*)-**1e** (-●-) or (*R*)-**2b** (- -) after staining with crystal violet and (B) of RPMI 8226 cells treated 72 h with (*R*)-**2b** (- -) or melphalan (-▲) after staining with MTT. Results are in percentage relative to untreated controls (100%).

**Table: $IC_{50}$ values (μM) for cell growth inhibition in a panel of human cancer cell lines after treatment with compounds**

| Compounds | A427[a] | 5637[a] | DANG[a] | LCLC-103H[a] | MCF-7[a] | RT-4[a] | RPMI 8226[b] | HL60[b] |
|---|---|---|---|---|---|---|---|---|
| (*S*)-**1a** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**1b** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**1c** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**1d** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**1e** | 13.63 ± 4.89 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**2a** | 9.25 ± 1.00 | 10.54 ± 0.60 | > 20 | > 20 | > 20 | > 20 | 21.32 ± 6.46 | > 20 |
| (*R*)-**2a** | 3.46 ± 1.21 | 6.57 ± 4.76 | 12.21 ± 0.50 | 14.90 ± 1.05 | 8.83 ± 2.08 | 11.31 ± 3.39 | 13.04 ± 4.90 | > 20 |
| (*S*)-**2b** | 9.12 ± 1.98 | 3.65 ± 2.25 | > 20 | 16.18 ± 1.21 | 9.77 ± 3.01 | 5.76 ± 2.89 | 1.29 ± 1.28 | > 20 |
| (*R*)-**2b** | 5.02 ± 1.68 | 4.79 ± 2.94 | 13.30 ± 3.90 | 6.48 ± 0.90 | 9.05 ± 1.07 | 6.68 ± 2.94 | 8.53 ± 3.50 | 17.35 ± 3.35 |
| (*S*)-**3a** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**3b** | 11.29 ± 5.11 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| (*S*)-**3c** | > 20 | > 20 | > 20 | > 20 | n.d. | n.d. | n.d. | n.d. |
| PB28 | 0.81 ± 0.34 | 8.35 ± 6.67 | 4.50 ± 1.07 | 6.99 ± 2.40 | 1.66 ± 0.48 | 8.13 ± 2.53 | 9.29 ± 4.25 | 9.46 ± 3.14 |
| Haloperidol | 9.63 ± 3.67 | 2.25 ± 1.39 | > 20 | 10.87 ± 1.91 | 24.9 ± 9.77[c] | 16 ± 5[d] | 18.39 ± 6.21 | n.d |
| (+)-Pentazocine | > 20 | 3.49 ± 0.90 | 20.08 ± 5.31 | > 20 | > 20[c] | > 20[c] | 17.77 ± 8.30 | n.d |
| Melphalan | 5.13 ± 1.9 [e]) | 1.32 ± 0.14 [e] | 2.65 ± 1.02 [e] | 4.00 ± 0.42 [e] | 3.71 ± 1.19 [e] | 14.25 ± 9.54 [e] | 8.47 ± 0.44 | 1.04 ± 0.48[e] |

[a] Determined by crystal violet method after 96 h treatment [b] determined by MTT method after 48 h (HL60) or 72 h (RPMI) treatment [c] $IC_{50}$ values are from ref (Geiger *et al.*, 2007) [d] $IC_{50}$ value from ref (Sunnam *et al.*, 2010)[e] $IC_{50}$ value from ref (Bracht *et al.*, 2006), n ≥ 3. n.d.: not determined.

The known $\sigma_1$-antagonist/$\sigma_2$-agonist **PB28** gave lower $IC_{50}$ values in the A427, DAN-G and MCF-7 cell lines compared to our ligands, but $IC_{50}$ values were comparable in the 5637, LCLC and RT-4 lines. In the RPMI 8826 cell line (*S*)**-2b** was much more active than **PB28**. As expected for the $\sigma_1$-agonist (+)-pentazocine, only weak cytotoxic activity was found in all cell lines except 5637 (Geiger et al. 2007; Spruce et al. 2004). The $\sigma_1$-antagonist haloperidol gave moderate $IC_{50}$ values in most cell lines, but in $\sigma_1$-receptor rich RPMI 8226 cells the $IC_{50}$ was only 18.39 μM. The $IC_{50}$ values of melphalan, an alkylating agent, were also in the micromolar range and comparable with the σ-ligands.

### *2.2. Induction of apoptosis by (R)-2b*

Next, the ability of (*R*)**-2b**, the ligand with the highest affinity for the $\sigma_1$-receptor ($K_i$ value = 0.9 ± 0.2 in the guinea pig brain assay and 7.5 ± 2.7 in the human RPMI 8226 assay) (Weber et al. 2014) to induce apoptosis in RPMI 8226 cells was investigated. The annexin/PI double staining method revealed that apoptotic levels increased from 4.6 ± 0.6 % in untreated control to 18.5 % ± 5.3 after a 48 h treatment with (*R*)**-2b** (Fig. 3A). When the late apoptotic fraction was also considered, the apoptotic level of (*R*)**-2b** rose to 31.8 % of annexin-V and PI positive cells compared to 8.0 % in the control (Fig. 3B). Recently, we also showed that the enantiomers (*S*)**-2a** and (*R*)**-2a** effectively induced apoptotic activity in RPMI 8226 cells (Korpis et al. 2014).

### *2.3. Apoptotic effect of (R)-2b enhanced by the $\sigma_2$-agonist PB28*

To further investigate the nature of the activity of (*R*)**-2b** the known $\sigma_2$-agonist **PB28** was combined in treatment. **PB28** is also a $\sigma_1$-antagonist, but has weaker affinity to σ1- as opposed to the σ2-receptor. The apoptotic fraction of cells treated with **PB28** alone (25% the $IC_{50}$ = 2.3 μM) is in the range of untreated cells but when co-administered with (*R*)**-2b** the apoptotic fraction increases significantly by 48 h (Fig. 4A), indicating an additive effect.

### *2.4. Apoptotic effect of (R)-2b enhanced by the chemotherapeutic melphalan*

To determine if the apoptotic activity of the σ-ligand was also enhanced by a known anticancer agent, RPMI 8226 multiple myeloma cells were treated with (*R*)**-2b** in combination with melphalan. For inducing apoptosis, the compounds were simultaneously added to the cells in concentrations one half their $IC_{50}$ values alone or in combination for 48 h. Monotherapy of melphalan (50% of $IC_{50}$ = 4.25 μM) and (*R*)**-2b** (50%

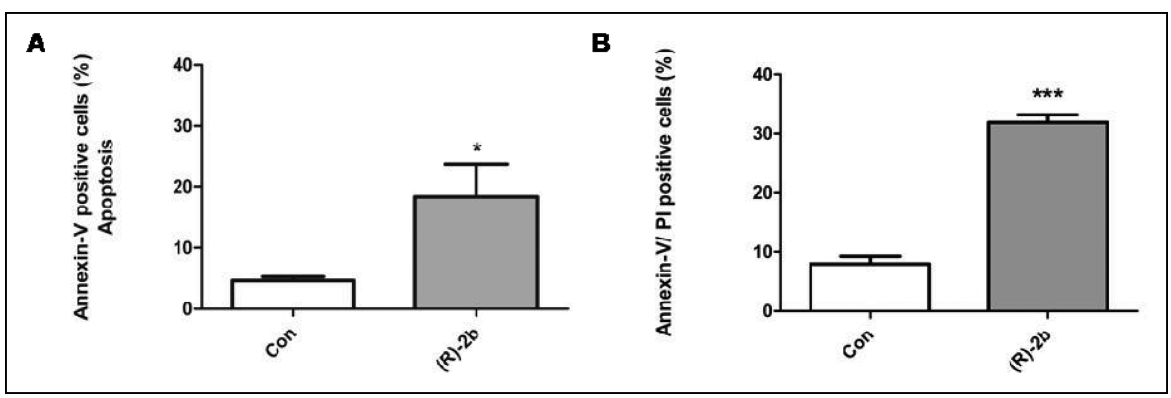



Fig. 3: Apoptotic effects of (*R*)**-2b** in the RPMI 8226 cell line. (A) Early apoptotic cells (annexin-V positive/PI negative cells) after treatment with 15.3 μM (*R*)**-2b** for 48 h. (B) Early and late apoptotic cells (annexin-V and PI positive cells) after treatment for 48 h. Apoptosis was evaluated by determining the percentage of annexin-V positive cells relative to all counted cells. Results are expressed as mean ± SD of at least three independent determinations, Con = untreated cells, $*p < 0.05$, $***p < 0.001$.

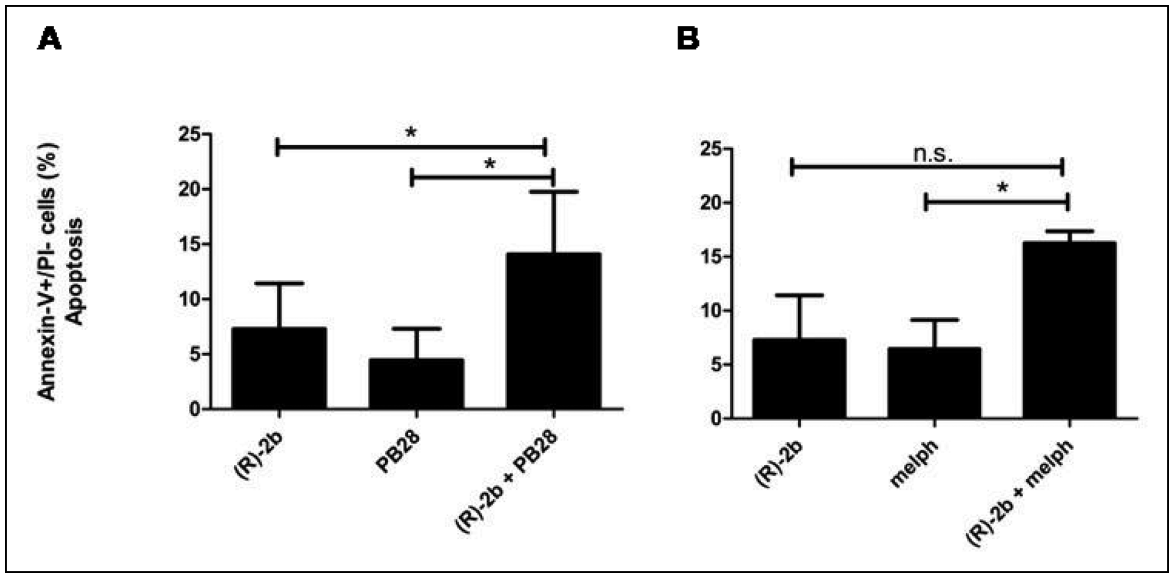



Fig. 4: Apoptotic effects of (*R*)-**2b**, melphalan, and **PB28** in the RPMI 8226 cell line. Early apoptototic fraction of cells (annexin-V positive/PI negative cells) after treatment with (A) 4.26 μM (*R*)-**2b** and 2.3 μM **PB28** or (B) 4.26 μM (*R*)-**2b** and 4.25 μM melphalan (melph), alone or in combination for 48 h simultaneously. Apoptosis was evaluated by determining the percentage of annexin-V positive cells relative to all counted cells. Results are expressed as mean ± SD of at least three determinations, cells, *$p < 0.05$, n.s. not significant. Basel levels of apoptosis in untreated controls (8.3%) have been subtracted from the presented results.

of $IC_{50} = 4.26$ μM) revealed equal apoptotic rates at about 7% (Fig. 4B). Combination treatment with melphalan resulted in significant increases in the fraction annexin-V positive cells, up to ca. 16 %. (Fig. 5B), also an additive effect.

### *2.5. Cytotoxic effects of (R)-2b blocked at lower concentrations by haloperidol*

Combination studies with the σ1-antagonist haloperidol were performed next to further decipher the cause of the cytotoxic effects of (*R*)-**2b**. Treatment of RPMI 8226 cells with haloperidol alone showed a weak cytotoxic effect ($IC_{50}$: 16.39 ± 6.15 μM). Combined treatment of cells with both compounds at low concentrations resulted in a complete loss of cytotoxicity of *(R)*-**2b** (Fig. 5). In fact, 5 μM (*R*)-**2b** together with haloperidol (5 and 10 μM) seemed to stimulate cell growth but at 10 μM of (*R*)-**2b** a weak cytotoxic effect was noted. At higher concentrations of (*R*)-**2b** (20 μM) the antagonistic effect of haloperidol was finally overcome, although cytotoxicity did not reach the same high level seen with (*R*)-**2b** alone. These observations appear to indicate that at low concentrations of (*R*)-**2b** the cytotoxic activity of this compound is antagonized by σ1-antagonism, hence (*R*)-**2b** would appear to be acting as a σ1-agonist. However, haloperidol is a non-selective ligand and also binds to the σ2-subtype but with a lower affinity, so effects at the σ2-subtype cannot be ruled out.

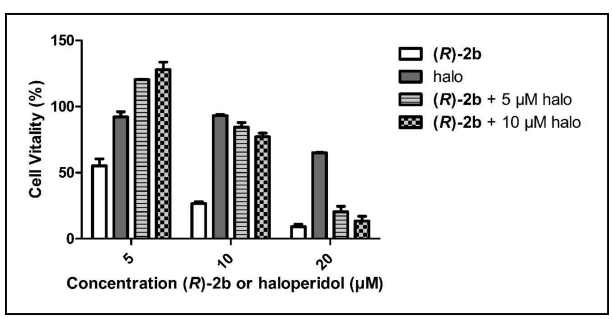



Fig. 5: Combined cytotoxic effects of (*R*)-**2b** and haloperidol in the RPMI 8226 cell line. Cells were treated with 5, 10 and 20 μM (*R*)-**2b** (white bar) or haloperidol (gray bar); 5, 10 and 20 μM (*R*)-**2b** and additionally 5 μM haloperidol (light gray bar with vertical line) or 5, 10 and 20 μM (*R*)-**2b** and additionally 10 μM haloperidol (light gray squared bar) for 72 h and stained with MTT. Results are in percentage relative to untreated controls (100%). Figure shows mean ± SD of at least three independent determinations.

## 3. Discussion

Due to the observation that σ-receptors are over-expressed in certain tumor cell lines, there has been increasing interest in developing σ-receptor-ligands as potential anticancer drugs. σ-Ligands are known to induce cell death by caspase-dependent and independent mechanisms depending on the structure of the ligand and the tumor cell line. For example, evidence indicates that $\sigma_1$-antagonists induce a caspase-dependent apoptosis whereas $\sigma_2$-agonists initiate a form of programmed cell death that does not require caspase activation (Crawford et al. 2002; Ostenfeld et al. 2005; Riganas et al. 2012; Spruce et al. 2004). Based on this knowledge, we investigated several new σ-receptor-ligands with varying substitutents at both aromatic N-atoms for their antiproliferative activity in a panel of eight human tumor cell lines. Compounds with large substituents at both N-atoms inhibited the growth of almost all cell lines in the low μM range, except for the leukemia cell line HL60, which was inherently resistant to all compounds tested.

The RPMI 8226 multiple myeloma cell line is known to expresses high levels of the $\sigma_1$ subtype (Brune et al. 2012). Thus, we used this cell line to study the cytotoxic effects of one of the more active of the new compounds, (*R*)-**2b**, in more detail. Previous work showed that (*R*)-**2b** has high affinity to sigma receptors in the RPMI 8226 line (Weber et al. 2014). As already described for (*S*)-**2a** and (*R*)-**2a** (Korpis et al. 2014), compound (*R*)-**2b** was also able to induce apoptosis in the RPMI 8226 line. Cell cycle analysis of RPMI 8226 cells treated with (*R*)-**2b** or **PB28** at the $IC_{90}$ value showed no evidence for an arrest in any particular phase of the cell cycle (data not shown).

Despite initial responsiveness of many cancers to chemotherapy, relapses occur very often accompanied by the development of drug resistance. Combination treatments with therapeutic drugs could provide a therapeutic benefit and improve therapy of many cancers. Thus, the combination of (*R*)-**2b** with melphalan, an alkylating agent with a long history of use against MM, and the $\sigma_2$-agonist/$\sigma_1$-antagonist **PB28** in subtherapeutic concentrations were investigated. These combinations led to significant

increases in the proportion of apoptotic cells as compared to drug alone. Combination with the non-selective $\sigma_1$-antagonist haloperidol first led to a decrease in antiproliferative activity of (*R*)**-2b**, suggesting its role as a $\sigma_1$-agonist. The combination of (*R*)**-2b** with **PB28** demonstrated that this ligand could also act as an agonist at the $\sigma_2$-subtype due to the increased apoptotic rate after the combination treatment. Agonists of $\sigma_2$-receptors as well as $\sigma_1$-receptor-antagonists are known to have antiproliferative activity, whereas $\sigma_2$-receptor-antagonists and $\sigma_1$-receptor-agonists have been reported to be neuroprotective. Cytotoxic activity of (*R*)**-2b** appears associated with activation of both receptor subclasses. However, further experiments will be needed to unravel the intrinsic activity of this interesting σ-receptor-ligand.

In conclusion, we found that various hydroxyethyl piperazines have potent cytotoxic activity in a number of human tumor cell lines. Furthermore, (*R*)**-2b**, a compound with high affinity for $\sigma_1$-binding sites in RPMI 8226 cells, augmented the apoptotic effects of the anticancer drug melphalan as well as another well-known σ-ligand **PB28** in cell culture. On the other hand, the $\sigma_1$-antagonist haloperidol attenuated the cytotoxicity of (*R*)**-2b**. Future studies will aim to understand the signal transduction pathways influenced by these new σ-ligands.

## 4. Experimental

### *4.1. Materials*

The tested σ-receptor-ligands were prepared as described (Weber et al. 2014). RPMI 1640 medium was obtained from PAN-Biotech (Aldenbach, FRG), Accutase from PAA Laboratories GmbH (Cölbe, FRG), fetal bovine serum, penicillin/streptomycin, Trypsin/EDTA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT), crystal violet, melphalan and **PB28** (sold as a racemate) were purchased from Sigma (Taufkirchen, FRG), glutaraldehyde was obtained from Roth (Karlsruhe, FRG), the annexin/PI apoptosis kit was obtained from Miltenyi Biotec (Teterow, FRG). The water for all experiments was supplied by a Milli-Q water purification system from Millipore.

### *4.2. Cell culture*

All cell lines were obtained from the DSMZ (Braunschweig, FRG) and grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, penicilin (10 000 U/ml) and streptomycin (10 mg/ml) at 37 °C in a humidified incubator with 5 % $CO_2$ atmosphere.

### *4.3. In vitro cytotoxicity assay*

Cell viability of the suspension cells (HL60 and RPMI 8226) was analyzed using the MTT assay. Cells were seeded out in 96-well plates at a density of 10 000 cells/well and incubated for 48 h (HL60 cells) or 72 h (RPMI 8226) with increasing concentrations of the substances. Controls included cells without drug and medium plus solvent but no cells. 20 µl MTT (2.5 mg/ml) was added to each well followed by a 4 h incubation.100 µl/well of 0.04 N HCl in isopropanol was added to dissolve the MTT-formazan product and the absorbance of each well was measured at λ= 570 nm with a microplate reader (Anthos, Salzburg, Austria). The relative cell viability was expressed as the percentage of the control that was treated with vehicle only.

Growth inhibition of adherent cells was measured with crystal violet staining. Cells were plated out into 96 well microtiter Plates 24 h prior to testing at a density of 500 cells/well (LCLC-103H), 1000 cells/well (DAN-G; MCF-7; RT-4) or 2000 cells/well (A427) in 100 µl medium. One plate for each cell line served as a control plate ($t_0$) and was fixed with glutaraldehyde after 24 h.

In the primary screening the tumor cells were incubated with a 20 µM solution of the test compound at 37 °C for 96 h. Compounds that reduced cell growth by greater than 50 % compared to untreated controls were further tested to determine $IC_{50}$ values, where five serial dilutions of the substances in DMSO were prepared. After 96 h the medium was discarded and cells were fixed with 1% glutaraldehyde solution for 20 min. Staining of cells was done with 100 µl/well of a crystal violet solution (0.02% in water) for 30 min followed by a 15 min washing step. To redissolve the dye, stained cells were treated with 100 µl in 70% ethanol/water for 2 h under gentle shaking. The optical density in the wells was measured at 570 nm with an Anthos plate reader. Concentrations giving T/C values between 10 and 90 % were used to estimate the $IC_{50}$ values, which were calculated by least-squares analysis of the dose-response curves as described previously (Bracht et al. 2006).

### *4.4. Apoptosis staining*

RPMI 8226 cells ($7.5 \times 10^5$) were treated with the substances and incubated for desired times. Then cells were harvested and double-stained with annexin-V and propidium iodide (PI) by using the protocol of the manufacture of the annexin V-FITC apoptosis detection kit. After 15 min incubation in the dark, cells were washed and subsequently analyzed by flow cytometry using a Macs Quant flow cytometer and software (Miltenyi Biotec, Bergisch Gladbach, Germany). A total of $10^4$ cells were scored in all determinations. Experiments were repeated at least three independent times.

### *4.5. Statistical analysis*

The results of independent experiments are expressed as the means ± S.D. The significance of differences between experimental conditions was calculated by using GraphPadPrism software. Significant differences between two groups were tested for with the two-side paired *t*-test. When comparisons between three or more groups were done, analysis of variance (ANOVA) was done followed by a multiple comparison test with a Dunnetts or Bonferroni test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ was considered statistically significant.

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