Protein–Protein Interactions

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Oral Disinfectants Inhibit Protein–Protein Interactions Mediated by the Anti-Apoptotic Protein Bcl-x_L and Induce Apoptosis in Human Oral Tumor Cells**

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Significant advances during the last decade in the field of small-molecule modulators of protein–protein interactions have raised general awareness of the fact that the modulation of protein–protein interactions has tremendous potential for both basic and applied science.[1] Some of the most advanced orthosteric inhibitors of protein–protein interactions[2–5] are currently in clinical trials. Here, we demonstrate that the bisbiguanide oral disinfectants chlorhexidine[6] (the active ingredient of commercially available Chlorhexamed, Chlorhexal, Periogard, Corsodyl, and Chlorohex) and alexidine[7] (Esemdent), which have been in human use worldwide for many years, target cancer-linked protein–protein interactions between the anti-apoptotic Bcl-2 family protein Bcl-x_L and its pro-apoptotic binding partners. The compounds are shown to induce apoptosis through the mitochondria-dependent apoptotic pathway in oral tumor cell lines.

Our study was based on the hypothesis that some small-molecule drugs already approved for human use against other targets might display hitherto undiscovered activities against protein–protein interactions[8] in their therapeutic concentration range.[9] To test our hypothesis on a protein–protein interaction target with established relevance for human health, we decided to focus on the interaction between the anti-apoptotic Bcl-2 family protein Bcl-x_L and its pro-apoptotic Bcl-2 family binding partner Bad. The interaction between Bcl-x_L and pro-apoptotic Bcl-2 proteins has attracted significant attention from both cancer biologists and medicinal chemists.[2,10–12]

A library of 4088 compounds containing a high proportion of clinically used small molecules was screened against the interaction between Bcl-x_L and a peptide derived from the Bad BH3 domain by using a binding assay based on fluorescence polarization ($Z' = 0.64 \pm 0.11$, see Figure S1 in the Supporting Information).[13] Active compounds in the primary screen (screening concentration: ca. 50 μM) were checked for activities against other protein–protein interactions recorded in a previous screening study.[14] These efforts identified both chlorhexidine (**1**) and alexidine (**2**; Figure 1 A) as potentially selective inhibitors of the Bcl-x_L/Bad interaction (see Figure S2 in the Supporting Information). Robotics-based re-analysis of the screening hits confirmed the dose-dependent inhibition of the interaction between Bcl-x_L and Bad, and also between Bcl-x_L and Bak, another protein of the pro-apoptotic Bcl-2 family, by **1** and **2** with similar activities (see Figure S3 in the Supporting Information). The interaction of Bcl-x_L with Bak, rather than with Bad, was selected for further analysis because of the larger assay window associated with the Bcl-x_L/Bak assay. Manual validation of the activities of **1** and **2** against the Bcl-x_L/Bak interaction confirmed their activities in the low micromolar range (IC_{50}: chlorhexidine: (21.0 ± 1.0) μM; alexidine: (18.2 ± 1.6) μM; Figure 1 B,C, Table 1), and the Supporting Information, Table S1). These activities are well in the concentration range of the oral

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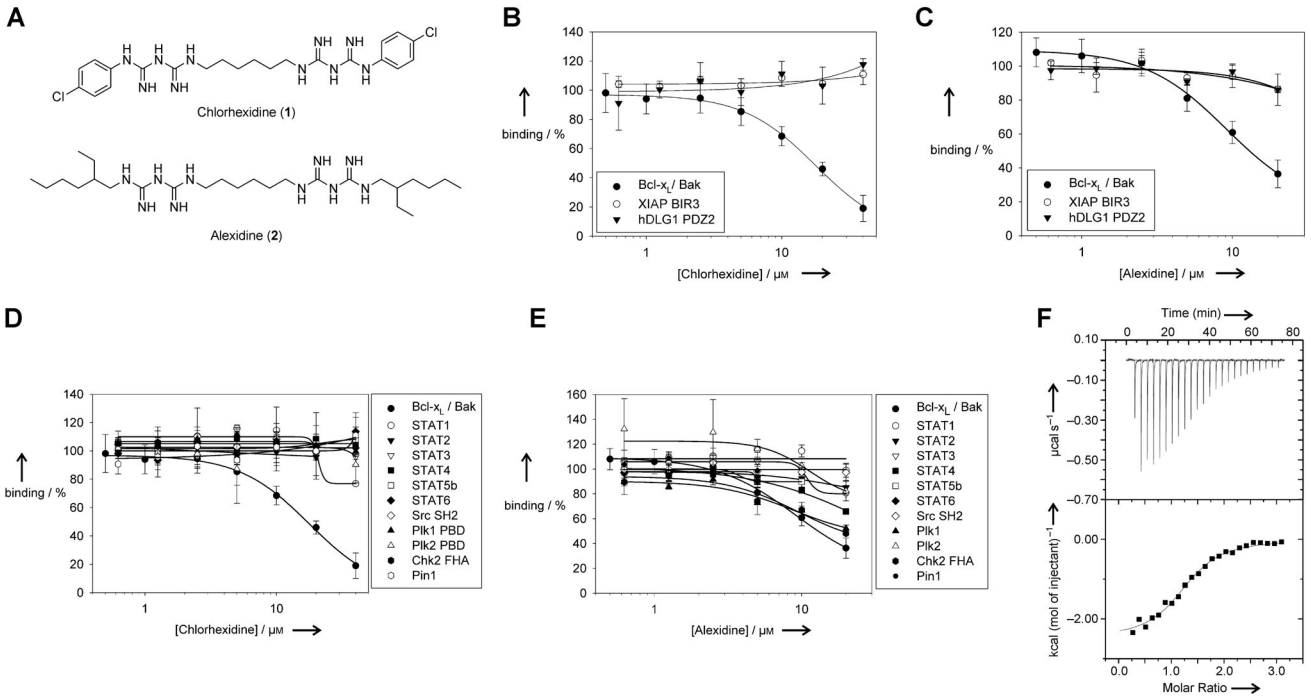
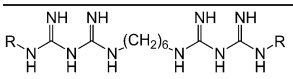
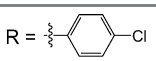
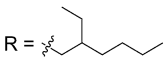
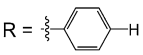


Figure 1. Chlorhexidine and alexidine inhibit protein–protein interactions mediated by Bcl-x_L. The deletion mutant Bcl-x_L Δ45–84, which lacks the flexible loop previously shown to be dispensable for the anti-apoptotic activity of Bcl-x_L, was used in these assays.[18,19] A) Structures of chlorhexidine and alexidine. Activity profile of B) chlorhexidine and C) alexidine against Bcl-x_L, XIAP BIR3, and hDLG1 PDZ2. Activity profile of D) chlorhexidine and E) alexidine against phosphorylation-dependent protein–protein interactions. F) Binding of chlorhexidine to Bcl-x_L, as analyzed by ITC. Parameters deduced from the lower graph: $N = (1.28 \pm 0.0278)$ sites; $K = (9.05 \pm 1.31) \times 10^4$ M^{-1}; $\Delta H = -(2493 \pm 78.14)$ cal mol^{-1}; $\Delta S = 14.2$ cal mol^{-1} deg^{-1}.

disinfectant chlorhexidine (**1**), which is used as a 0.1% and 0.2% (w/v) solution (of the digluconate), corresponding to 1.1 mM and 2.2 mM, respectively. Activity of alexidine (**2**) as an disinfecting agent was often shown at 0.035% (w/v; in the form of the dihydrochloride), which corresponds to 600 μM.[15] Therefore, the concentrations of chlorhexidine and alexidine used in mouthwash solutions are likely to be more than sufficient to inhibit Bcl-x_L in surface-exposed cells in the oral cavity.

Table 1: Activities of bisbiguanides **1**–**3** against the interaction between Bcl-x_L and a Bak-derived BH3-peptide as analyzed in a fluorescence polarization assay.

		Activity
1		$IC_{50} = (21.0 \pm 1.0)$ μM
2		$IC_{50} = (18.2 \pm 1.6)$ μM
3		(28±2)% inhibition at 100 μM

As specificity controls, we tested both compounds against protein–protein interactions mediated by the BIR3 domain of XIAP[16] and the second PDZ domain of hDLG1.[17] Neither of the two protein–protein interactions was inhibited by the two bisbiguanides (Figure 1B,C). Subsequently, extensive specificity analysis was carried out against an 11-member panel of phosphorylation-dependent, structurally diverse protein–protein interactions.[14] These experiments confirmed chlorhexidine as a specific inhibitor of binding mediated by Bcl-x_L, and revealed additional, but weaker, activities of alexidine against other protein–protein interactions (Figure 1D,E). Further specificity analysis demonstrated that some of the remaining anti-apoptotic Bcl-2 family proteins were inhibited by chlorhexidine and alexidine to various extents (see Figure S4 in the Supporting Information), which is advantageous given the functional redundancy amongst proteins of the anti-apoptotic Bcl-2 family.

As a consequence of its narrower selectivity profile, we focused on chlorhexidine to analyze the thermodynamics of its binding to Bcl-x_L by isothermal titration calorimetry (ITC; Figure 1F). Consistent with the activity of chlorhexidine in the competition assay format, ITC indicated a K_d value of (10.4 ± 0.7) μM. Binding was mediated by both an enthalpic component ($\Delta H = -(2.25 \pm 0.25)$ kcal mol^{-1}) and an entropic component ($\Delta S = (16.0 \pm 1.7)$ cal mol^{-1} deg^{-1}), thus indicating a combination of electrostatic and hydrophobic interactions.

To gain insight into the binding site of chlorhexidine and alexidine on Bcl-x_L, we carried out ^{1}H,^{15}N-HSQC NMR experiments with the protein construct originally used in the NMR-based structural characterization of the Bcl-x_L/Bak interaction.[18] This protein construct lacks the unfolded loop domain (amino acids 45–84) previously shown to be dispensable for the anti-apoptotic function of Bcl-x_L as well as the C-

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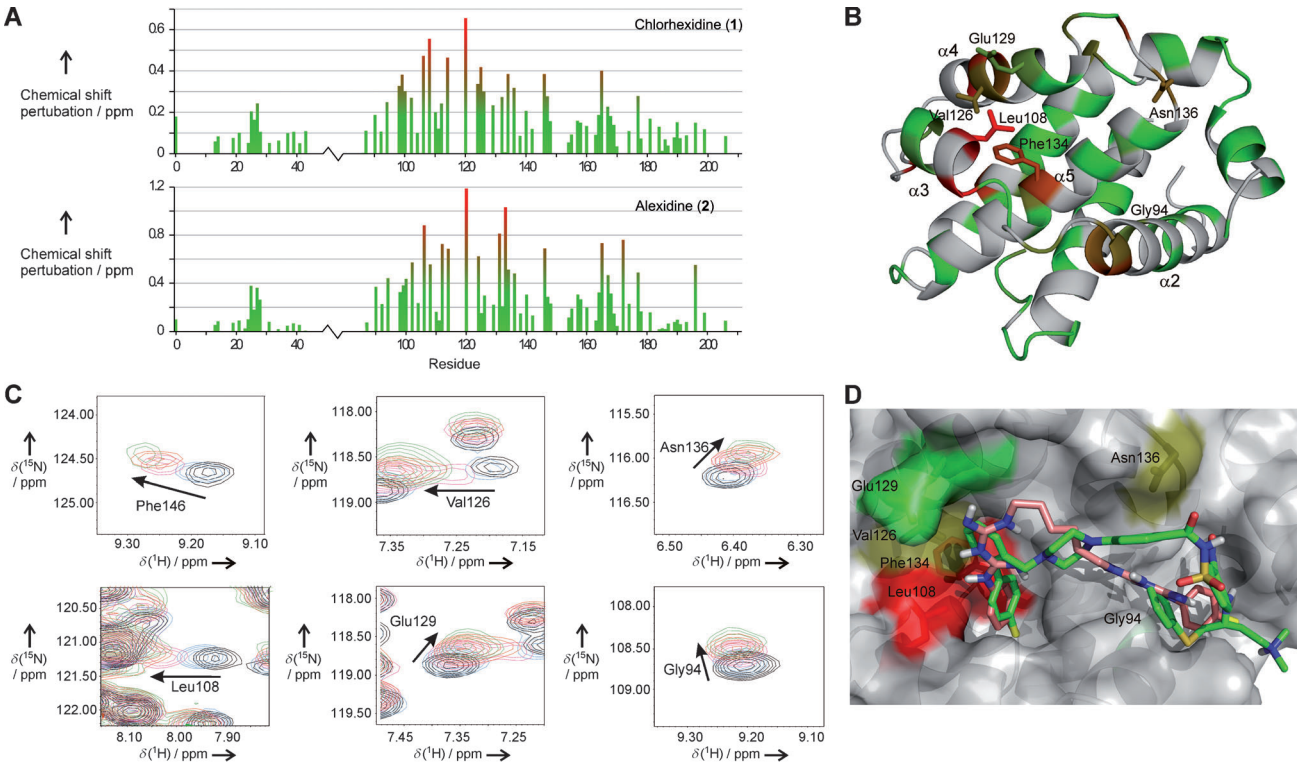

Figure 2. Chlorhexidine (**1**) and alexidine (**2**) bind to Bcl-x_L Δ45–84, as shown by ^{1}H,^{15}N-HSQC NMR spectroscopy. A) Chemical shift perturbations (CSPs) per residue for the binding of **1** (upper panel) and **2** (lower panel) to Bcl-x_L. B) Graphical representation of the CSPs for **1** as given in (A) on the structure of unbound Bcl-x_L (PDB entry: 1MAZ).[19] The graphic was generated using PyMOL.[22] C) The amide resonances of Phe146, Val126, Leu108, Glu129, Asn136, and Gly94 in ^{15}N-labeled Bcl-x_L Δ45–84 (100 μM) in the absence of **1** (black) show strong and dose-dependent chemical shifts upon addition of **1** (50 μM, blue; 100 μM, magenta; 200 μM, orange; 400 μM, green). D) Overlay of the docking pose of **1** (carbon atoms shown in pink) with the cocrystal structure of ABT-737 (carbon atoms shown in green) bound to Bcl-x_L (PDB entry: 2YXJ).[20] Amino acids for which CSP are shown in (C) are color coded as in (B).

terminal transmembrane domain.[18,19] Both compounds caused extensive chemical shift perturbations of a similar set of amide resonances (see Figure 2 A–C and Figures S5 and S6 in the Supporting Information). The observed spectral changes are consistent with binding of the small molecules similar to BH3 peptides to the hydrophobic groove of Bcl-x_L. The strongest perturbations were observed in helices α2–α5, which form the binding groove for BH3-domain-derived peptides or small molecules, thus suggesting that the compounds bind to the hydrophobic BH3 binding pocket of Bcl-x_L (see Figure S7 in the Supporting Information).[18] These data are consistent with the displacement of BH3 domain peptides from Bcl-x_L, as observed in the fluorescence polarization assays (see Figure 1 B,C and Figure S3 in the Supporting Information). Amino acids with strong chemical shift perturbations included Phe146, Val126, Glu129, Leu108, Asn136, and Gly94 (see Figure 2 C and Figure S6 in the Supporting Information). The first four of these amino acids are part of a subpocket to which the Bcl-x_L inhibitor ABT-737[11] (see Figure S7C in the Supporting Information) binds, as determined by X-ray crystallography (Figure 2 D).[20] Intriguingly, molecular docking experiments using AutoDock Vina[21] suggested that one of the 4-chlorophenyl rings of chlorhexidine occupies the same hydrophobic subpocket of Bcl-x_L as the 4-chlorobiphenyl moiety of ABT-737.

The binding mode suggested by molecular docking was further interrogated by chemical synthesis and biochemical testing of analogues. Removal of the chlorine atom of chlorhexidine (**1**), as realized in deschlorohexidine (**3**), led to a strong reduction in binding affinity (Table 1). Combined structure–activity data indicate the requirement for appropriate hydrophobic interactions mediated by the outer substituents on the biguanide moieties for binding of the compounds to Bcl-x_L, and show that shorter linker units between the two biguanide moieties are well-tolerated (see Table S1 in the Supporting Information).

Since inhibition of Bcl-x_L is expected to increase the apoptotic rate of tumor cells, which often overexpress Bcl-x_L, we chose to investigate the effects of **1** and **2** on the apoptotic rate of tumor cells derived from oral tissues. Deschlorohexidine (**3**) served as a negative control compound because of its high structural similarity to the more-specific inhibitor **1** but significantly lower activity in vitro. Three tongue squamous cell carcinoma cell lines (SCC-4, SCC-9, SCC-15) and two pharynx carcinoma cell lines (FaDu and Detroit 562) were exposed to the compounds. Apoptosis, as characterized by irreversible degradation of DNA, was visualized by a TUNEL assay (Figure 3 A). Both **1** and **2**, but not the negative control compound **3**, induced DNA strand breaks characteristic of apoptosis in all five cell lines (see Figure 3 B,C and Figure S9 in the Supporting Information).

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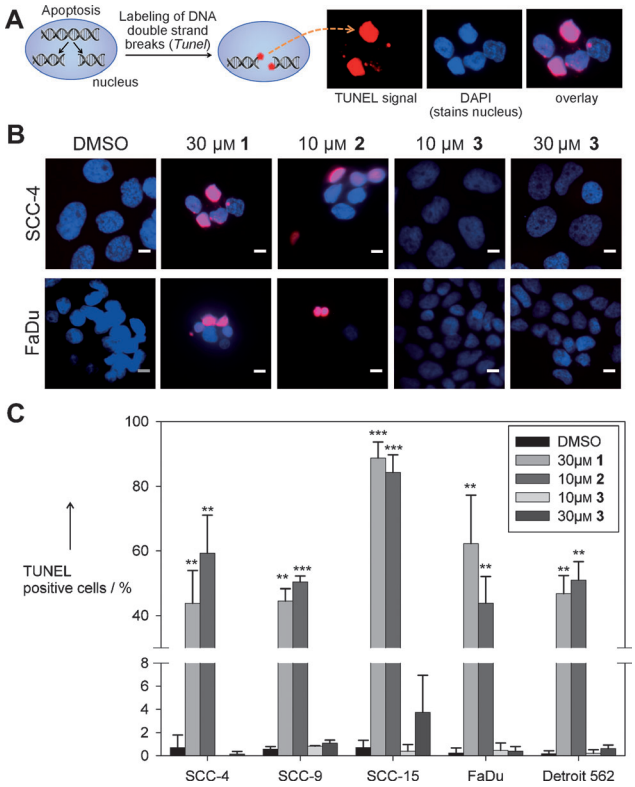

Figure 3. Chlorhexidine (**1**) and alexidine (**2**) induce apoptosis in tumor cell lines derived from the tongue and pharynx, as analyzed by the TUNEL assay. A) Principle of the TUNEL assay. B) DNA strand breaks characteristic of apoptosis induced by treatment of cells with the compounds for 24 h are visualized (red). Cell nuclei are stained with DAPI (blue). Representative fluorescence images are shown. Scale bar: 10 μm. C) Quantification of the experiments as shown in (B) and Figure S9 in the Supporting Information. Data represent mean values and standard deviations from triplicate experiments. At least 100 cells were analyzed in each replicate experiment under each treatment condition. ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test against DMSO control).

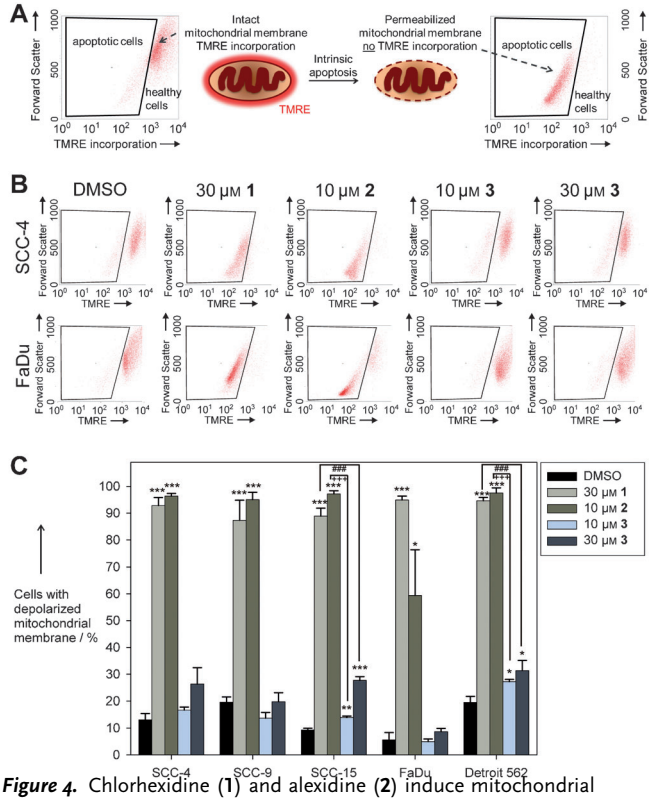

Figure 4. Chlorhexidine (**1**) and alexidine (**2**) induce mitochondrial membrane depolarization in tumor cell lines derived from the tongue and pharynx. A) Principle of the assay. B) Mitochondrial accumulation of the fluorescent dye tetramethylrhodamine ethyl ester (TMRE) is strongly reduced in cells exposed to **1** and **2** for 24 h, but only to a significantly lesser extent in cells exposed to **3**. Single cells are sorted according to TMRE content (*x* axis) and cell size (forward scatter, *y* axis) by flow cytometry. Experiments were carried out in triplicate; representative flow cytometry images are shown. C) Quantification of the experiments as shown in (B) and Figure S10 in the Supporting Information. Data represent mean values and standard deviations from triplicate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test of compounds against DMSO control). ### $p < 0.001$ (Student's *t*-test of 30 μM **1** against 30 μM **3**). +++ $p < 0.001$ (Student's *t*-test of 10 μM **2** against 10 μM **3**).

Anti-apoptotic Bcl-2 family proteins such as Bcl-x_L inhibit the intrinsic apoptosis pathway by binding to and sequestering pro-apoptotic proteins of the Bcl-2 family. Small-molecule-mediated release of the inhibition by anti-apoptotic proteins of the Bcl-2 family leads to oligomerization of the pro-apoptotic Bcl-2 proteins Bak and Bax located in the mitochondrial membrane, thereby causing depolarization of the mitochondrial membrane and induction of the intrinsic apoptosis pathway. To investigate whether apoptosis induced by chlorhexidine and alexidine is triggered by the intrinsic pathway we carried out mitochondrial membrane depolarization assays using the same five tumor cell lines as used in the TUNEL assay. This assay type exploits the fact that the cationic dye tetramethylrhodamine ethyl ester (TMRE) rapidly accumulates in cells with intact, active mitochondria, but fails to do so in apoptotic cells with depolarized membranes (Figure 4A). Both **1** and **2** led to a strong reduction of TMRE uptake, consistent with depolarization of the mitochondrial membrane leading to the subsequent induction of apoptosis by these agents (see Figure 4B,C and Figure S10 in the Supporting Information). In contrast, deschlorohexidine (**3**), which lacks strong activity against Bcl-x_L in vitro, had a significantly weaker effect on TMRE uptake.

To provide further evidence that the induction of apoptosis in tumor cells is mediated, at least in part, by inhibition of the Bcl-x_L/Bak interaction, we performed co-immunoprecipitation assays in FaDu cells (see Figure S11 in the Supporting Information). These experiments confirmed that both **1** and **2** interfere with binding between Bcl-x_L and Bak, while the negative control compound **3** only had a minor effect. Combined cell-based assay data demonstrate the potent effects of **1** and **2** in inducing apoptosis in tumor cell lines derived from human oral tissue through the intrinsic apoptosis pathway, and suggest that this effect is mediated, at least in part, by the inhibition of Bcl-x_L. Our data are in line with previous reports on the induction of cell death by **1** in L929 fibroblasts,[23] and the apoptosis-inducing activity of

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alexidine in head and neck cancer cell lines, including FaDu cells.[24]

To assess whether treatment of oral tumor cells with **1** and **2** would preferentially target tumor cells over nontransformed cells, we carried out cell viability assays using SCC-4 and FaDu cells, as well as normal human gingival progenitor cells (HGEP). Cell viability of both tumor cell lines was significantly more affected by **1** and **2** than the nontransformed cells (see Figure S12 in the Supporting Information), thus suggesting the possibility of a therapeutically useful concentration range for the compounds. These data are consistent with the reported lack of cytotoxicity of **2** on primary pancreatic islet cells[25] and the preferential inhibition of cancer cells versus nontransformed cells by **2**,[24] and provides a molecular explanation for the latter observation.

In summary, we have identified the anti-apoptotic protein Bcl-x_L as a target of two oral disinfectants. To our knowledge, this represents the first report providing cell-based mechanistic evidence that compounds in current widespread, everyday household use in the industrialized world can exert an inhibitory effect on disease-relevant protein–protein interactions at physiologically relevant concentrations. In addition, our data add cancer as a potential new therapeutic indication for established oral disinfectants. Thus, while the scientific community awaits the clinical approval of the first orthosteric small-molecule inhibitor of an intracellular protein–protein interaction, our discovery indicates that the inhibition of protein–protein interactions may already be a potential mode of action of some of the existing therapeutic compounds. We believe our data will significantly influence the scientific community's perception of the druggability of protein–protein interactions and the nature of druggable targets.

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