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Triarylmethanes, a new class of Cx50 inhibitors

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INTRODUCTION

Connexins (Cx) are a family of proteins with four transmembrane regions, which are encoded by 21 genes in humans and which form hexameric connexons (= hemichannels). These connexons can either function as transmembrane ion channels or assemble into gap junctions (GJ) by the docking of two hemichannels from adjacent cells and directly mediate signaling between cells by passing ions, metabolites and signaling molecules up to 1 kDa in mass. Both hemichannels and GJ channels formed by different connexins play important roles in tissue homeostasis and have therefore been proposed as potential new targets for the treatment of epilepsy, cardiac arrhythmia, cancer, stroke, essential tremor, and corneal wound healing (Nemani and Binder, 2005; Salameh and Dhein, 2009). However, at similar concentrations carbenoxolone also inhibits several other targets such as the enzyme 11β-hydroxysteroid dehydrogenase (IC50 ~5 μM; Monder et al., 1989), voltage-gated Ca2+ currents (IC50 48 μM; Vessey et al., 2004), and the structurally similar pannexin channels (IC50 2–5 μM; Locovei et al., 2007). At even lower concentrations carbenoxolone inhibits P2x7 receptors (IC50 175 nM; Suadicani et al., 2006). Other commonly used connexin blockers like the long-chain alcohols heptanol and octanol, the diphenylborate 2-APB or flufenamic acid are similarly either of low potency or lack selectivity for connexin channels (for a recent review see: Bodendiek and Raman, 2010).

Since the 1980s several endogenous as well as exogenous molecules that modulate GJ channels have been discovered. The peptidic GJ channel activator rotigaptide, which mainly activates Cx43, advanced into clinical trials for the treatment of atrial fibrillation (phase II, terminated in 2007) and endothelial dysfunction (Lang et al., 2008), while GAP-134, an orally available di-peptidic analog of rotigaptide, recently completed phase I clinical trials for the treatment of atrial fibrillation (ClinicalTrials.Gov). However, apart from these exceptions, the development of GJ channel modulators as pharmacological tools and potential therapeutics is still in its infancy. Most existing modulators are either of low potency and exhibit little selectivity either for connexin channels or among individual connexin subtypes. For example, one of the most commonly used GJ channel blockers, carbenoxolone, a more water soluble derivative of the pentacyclic triterpenoid glycyrrhetinic acid, reversibly inhibits GJ currents in human fibroblasts with an IC50 of 3 μM and reduces Cx26 and Cx38 hemichannel currents in Xenopus oocytes with IC50s of 21 and 34 μM, respectively. However, at similar concentrations carbenoxolone also inhibits several other targets such as the enzyme 11β-hydroxysteroid dehydrogenase (IC50 ~5 μM; Monder et al., 1989), voltage-gated Ca2+ currents (IC50 48 μM; Vessey et al., 2004), and the structurally similar pannexin channels (IC50 2–5 μM; Locovei et al., 2007). At even lower concentrations carbenoxolone inhibits P2x7 receptors (IC50 175 nM; Suadicani et al., 2006). Other commonly used connexin blockers like the long-chain alcohols heptanol and octanol, the diphenylborate 2-APB or flufenamic acid are similarly either of low potency or lack selectivity for connexin channels (for a recent review see: Bodendiek and Raman, 2010).

Potent connexin subtype selective modulators are urgently needed to further elucidate the physiological and pathophysiological roles of the different connexins and to perform proof-of-concept studies validating connexins as potential drug targets for various diseases for which they have been proposed as novel inhibitors as pharmacological tools and potential therapeutics is still in its infancy. Most existing modulators are either of low potency and exhibit little selectivity either for connexin channels or among individual connexin subtypes. For example, one of the most commonly used GJ channel blockers, carbenoxolone, a more water soluble derivative of the pentacyclic triterpenoid glycyrrhetinic acid, reversibly inhibits GJ currents in human fibroblasts with an IC50 of 3 μM and reduces Cx26 and Cx38 hemichannel currents in Xenopus oocytes with IC50s of 21 and 34 μM, respectively. However, at similar concentrations carbenoxolone also inhibits several other targets such as the enzyme 11β-hydroxysteroid dehydrogenase (IC50 ~5 μM; Monder et al., 1989), voltage-gated Ca2+ currents (IC50 48 μM; Vessey et al., 2004), and the structurally similar pannexin channels (IC50 2–5 μM; Locovei et al., 2007). At even lower concentrations carbenoxolone inhibits P2x7 receptors (IC50 175 nM; Suadicani et al., 2006). Other commonly used connexin blockers like the long-chain alcohols heptanol and octanol, the diphenylborate 2-APB or flufenamic acid are similarly either of low potency or lack selectivity for connexin channels (for a recent review see: Bodendiek and Raman, 2010).
targets. We therefore screened a small library of compounds containing ion channel modulating pharmacophores for their effects on Cx50 GJ channels. Cx50 was used as an exemplary connexin because it is expressed robustly in expression systems. Cx50 is mainly expressed in the crystalline lens. In lens epithelial cells, it is co-expressed with Cx43 and plays an important role in postnatal lens growth (White et al., 1998; Rong et al., 2002). In fiber cells, where it is co-expressed with Cx46, it has been shown to be an important component of the lens microcirculation, essential for maintenance of lens transparency (Mathias et al., 1997, 2010). Genetic deletion of Cx50 causes mild cataracts and significantly decreases lens growth (White et al., 1998; Rong et al., 2002), while missense and frame shift mutations have been found in families with inherited cataracts (Berthoud and Beyer, 2009; Mathias et al., 2010). To further study the role of Cx50 channels in the lens, a potent and selective blocker would be of great interest. Such an inhibitor is likely to be useful to dissect the contribution of the coupling provided by Cx50 to lens development and transparency. In this study, we describe the design of two Cx50 inhibitors with IC50 of 1.2 and 2.4 μM. Both compounds exhibit excellent selectivity for Cx50 over Cx43, and Cx46, which are also expressed in the lens (<18% inhibition at 10 μM), and strongly reduced junctional currents in primary lens epithelial cells isolated on postnatal day 6, a developmental time-point where Cx50 provides the majority of the coupling in the epithelium. These new pharmacological tool compounds will be useful to further explore the role of Cx50 in lens physiology and pathophysiology and for structure function studies of connexins.

**MATERIALS AND METHODS**

**CHEMICALS AND REAGENTS**

Clotrimazole (CAS No. 23593-75-1), triphenylmethane (CAS No. 519-73-3), triphenylmethyl chloride (CAS No. 76-83-5), triphenylmethanol (CAS No. 76-84-6), 3,3,3-triphenylpropionic acid (T51, CAS No. 900-91-4), (R)-(+)
-\(\alpha\),\(\alpha\)-diphenyl-2-pyridolinemethanol (T52, CAS No. 22348-32-9), (S)-(−)-\(\alpha\),\(\alpha\)-diphenyl-2-pyridolinemethanol (T53, CAS No. 112068-01-6), diphenyl-4-pyridylmethane (T160, CAS No. 3678-72-6), and triphenylmethanamine (T162, CAS No. 5824-40-8) were purchased from Sigma-Aldrich (St. Louis, MO). Tetraphenylethane (T161, CAS No. 630-76-2) was purchased from Alfa Aesar (Ward Hill, MA). 2-Chlorotriethyl chloride (T3-Cl, CAS No. 42074-68-0) and diphenyl-4-pyridylmethanol (T50, CAS No. 1620-30-0) were purchased from TCI America (Portland, OR). T1-T4, T9, T11, T13, T20, T34, T35, T39, T41, T43, T44, T54, T57, T61, T64, T66-T75, T78-T80, T85, and T97 were available in the Wulff laboratory compound library and had been previously synthesized and characterized (Wulff et al., 2000). The remaining compounds were synthesized using Grignard, chlorination and alkylation reactions described as general methods A, B, and C (see also Figure 3). New chemical entities (NCEs) were characterized by melting point (Melting Point B-540, Büchi), \(^1\)H-NMR (Avance 500, Bruker), mass spectrometry (MS: LCQ, Thermo Scientific; HRMS: LTQ-Orbitrap XL Thermo Scientific), and/or combustion analysis (2400 Series II combustion analyzer, Perkin Elmer). All MS and HRMS spectra were recorded with ESI as ionization mode if not stated otherwise. In cases where no sufficient analytical data for previously reported compounds (T109, T117, T129, T141, T143, T144, T154-OH, and T165) were available \(^1\)H-NMR, MS and/or combustion data in addition to melting points are also provided.

**CHEMICAL SYNTHESIS**

**General method A**

Triaryl methanols were synthesized through a Grignard reaction by stirring 25 mmol of magnesium turnings and 10–15 mmol of the required arylbromide in 50 mL of anhydrous diethyl ether. To initiate the reaction, catalytic amounts of iodine were added. The remaining 15–20 mmol of the required arylbromide were diluted with or dissolved in anhydrous diethyl ether (50 mL) and added slowly allowing gentle reflux. The reaction mixture was refluxed until all magnesium was consumed. Then, a solution of the required benzophenone (25 mmol) in anhydrous diethyl ether (50 mL) was added drop wise and the resulting mixture was heated under reflux for 12–24 h. After completion of the reaction the mixture was cooled to 0°C, poured into 100 mL of cold water and acidified with concentrated HCl. The organic phase was separated, and the aqueous phase was extracted three times with diethyl ether. The organic phases were combined, washed with NaHCO3 (10%) and dried over sodium sulfate. After evaporation of the solvent the crude triaryl methanols were obtained either as solid or as oily residues, which were recrystallized from petroleum ether (40–60°C) several times, if necessary.

**General method B**

The triaryl methyl chlorides were obtained according to McNaughton-Smith et al. (2008) by adding a five-fold excess of acetyl chloride to a stirred solution of the respective triaryl methanol in dichloromethane. After stirring the reaction mixture at room temperature for 12–24 h the solvent was evaporated and toluene (2 × 50 mL) was added and again removed under vacuum to afford the crude triaryl chlorides.

**General method C**

To a solution of the respective triaryl chloride (5 mmol) in anhydrous acetonitrile (100 mL) an excess of the respective amine (10–20 mmol) as hydrogen acceptor was added and the resulting mixture was refluxed for several hours. The progress of the reaction was monitored by TLC. Work up I: The mixture was poured into cold water (400 mL) and kept at 4°C for a few hours. The precipitate was filtered off, thoroughly washed with water to remove any remaining amine, and recrystallized from ethanol. Work up II: the solvent was evaporated and the crude residue was purified by column chromatography and/or recrystallization.

\[-(2-Chlorophenyl)(diphenyl)methyl]-4-methyl-2-phenyl-1H-imidazole (T89) was synthesized from T3-Cl (2.5 g, 7.98 mmol) and 4-methyl-2-phenylimidazole (1.26 g, 7.98 mmol), and triethylamine (1.11 mL, 7.98 mmol) as hydrogen acceptor in anhydrous acetonitrile (100 mL). After 24 h of refluxing the solvent was evaporated to afford a creamy residue, which was dissolved in dichloromethane (200 mL), washed with water (2 × 50 mL), and dried over Na2SO4. Evaporation of the solvent gave the crude product which was recrystallized from petroleum ether (40–60°C)/dichloromethane. T89 was obtained as a white powder (650 mg, 18.7%): Mp 226°C; \(^1\)H NMR (DMSO-\(d_6\)) δ: 1.41 (s, 3H, \(\alpha\),\(\alpha\))
1-Chloro-2-(biphenylmethyl)benzene (T107): To a solution of T3-Cl (0.95 g, 3 mmol) in anhydrous acetone (50 mL) phenol (282 mg, 3 mmol), K₂CO₃ (1.93 g, 14 mmol) and catalytic amounts of KI were added. Afterward the resulting mixture was refluxed for several hours. The progress of the reaction was monitored by TLC. After completion of the reaction K₂CO₃ was filtered off and the solvent was evaporated. The solid residue was dissolved in CH₂Cl₂ and the solution was extracted three times with NaOH (0.5 M). The pooled organic phases were dried over N₅SO₄ and concentrated in vacuo. The residue was recrystallized from ethanol to give an off-white, slightly yellowish powder (80 mg, 7%).

-1-[2-Chlorophenyl]diphenylmethyl]-2-(trifluoromethoxy) aniline (T104) was synthesized from T3-Cl (1.6 g, 5 mmol) and 2-trifluoromethoxyaniline (2.04 mL, 2.7 g, 15 mmol) according to general method C as a slightly yellowish powder (1.4 g, 62%): Mp 123.5°C; ¹H NMR (DMSO-d₆): δ 7.91 (s, 1H, N-H), 6.12 (d, 1H, 3J = 7.8 Hz), 6.60 (dt, 1H, J = 8.2 Hz, 4J = 1.3 Hz), 6.57 (dt, 1H, J = 7.9 Hz, 4J = 1.3 Hz), 7.18–7.38 (m, 14H), 7.58 (d, 1H, 3J = 7.4 Hz); HRMS (ESI) m/z calcd 277.0784 [C₁₉H₁₄Cl]⁺, 178.0480 [C₂H₇NOF₃]⁺; Anal. calcd for C₂₆H₁₉ClF₃NO: C, 68.88; H, 4.22; N, 3.40; found: C, 69.19; H, 4.26; N, 3.10.

-1-(4-[2-Chlorophenyl]diphenylmethyl)[amino]phenylethan-1-one (T105) was synthesized from T3-Cl (1.6 g, 5 mmol) and 4-aminoacetoephene (2.0 g, 15 mmol) according to general method C as a white powder (400 mg, 19.4%): Mp 166.7°C; ¹H NMR (DMSO-d₆): δ 2.30 (s, 3H, -OCH₃), 6.55 (swide, 2H), 7.20–7.25 (m, 6H), 7.29–7.35 (m, 5H), 7.46 (d, 2H, 3J = 8.6 Hz), 7.56 (d, 1H, 3J = 7.4 Hz); HRMS (ESI) m/z calcd 412.1468 [M + H]⁺; found 412.1454 [M + H]⁺; Anal. calcd for C₂₇H₂₂ClNO: C, 78.73; H, 5.38; N, 3.40; found: C, 78.42; H, 5.48; N, 3.38.

-1-[2-Chlorophenyl]diphenylmethyl-4-methoxylanine (T106) was synthesized from T3-Cl (1.6 g, 5 mmol) and p-anisidine (1.85 g, 15 mmol) according to general method C and recrystallized from methanol as a slightly redish-beige powder (1.24 g, 62%): Mp 142.5°C; ¹H NMR (DMSO-d₆): δ 3.52 (s, 3H, -OCH₃), 6.10 (s, 1H), 6.43 (s, 4H), 7.18 (t, 2H, 3J = 6 Hz), 7.26–7.30 (m, 11H), 7.59 (d, 1H, 3J = 7.5 Hz); HRMS (ESI) m/z calcd: 399.1389 [M⁺]; found: 399.1381 [M⁺]; Anal. calcd for C₂₆H₂₁NO: C, 78.09; H, 5.54; N, 3.50; found: C, 77.85; H, 5.62; N, 3.53.

-1-Chloro-2-(phenoxypiphenylmethyl)benzene (T107): To a solution of T3-Cl (0.95 g, 3 mmol) in anhydrous acetone (50 mL) phenol (282 mg, 3 mmol), K₂CO₃ (1.93 g, 14 mmol) and catalytic amounts of KI were added. Afterward the resulting mixture was refluxed for several hours. The progress of the reaction was monitored by TLC. After completion of the reaction K₂CO₃ was filtered off and the solvent was evaporated. The solid residue was dissolved in CH₂Cl₂ and the solution was extracted three times with NaOH (0.5 M). The pooled organic phases were dried over N₅SO₄ and concentrated in vacuo. The residue was recrystallized from ethanol to give an off-white, slightly yellowish powder (80 mg, 7%).

-1-[2-Chlorophenyl]diphenylmethyl]-1,3-benzoizole-2-amine (T103) was synthesized from T3-Cl (1.6 g, 5 mmol) and 2-aminobenzotiazole (2.25 g, 15 mmol) according to general method C and recrystallized from methanol as an off-white powder (1.12 g, 52%): Mp 156.3°C; ¹H NMR (DMSO-d₆): δ 6.95 (s, 1H, 3J = 7.5 Hz), 7.00 (d, 1H, 3J = 8.5 Hz), 7.06 (t, 1H, 3J = 7.3 Hz), 7.20–7.23 (m, 2H), 7.28–7.31 (m, 1H), 7.49 (m, 1H), 7.60 (d, 1H, 3J = 7.5 Hz), 8.96 (s, 1H, N-H); HRMS (ESI) m/z calcd 427.1036 [M + H]⁺; found 427.1060 [M + H]⁺; Anal. calcd for C₂₆H₁₉ClN₂S: C, 73.14; H, 4.49; N, 6.56; S, 7.51; found: C, 73.05; H, 4.39; N, 6.63; S, 8.07.

-1-[2-Chlorophenyl]diphenylmethyl]-2-(trifluoromethoxy) aniline (T104) was synthesized from T3-Cl (1.6 g, 5 mmol) and 2-trifluoromethoxyaniline (2.04 mL, 2.7 g, 15 mmol) according to general method C as a slightly yellowish powder (1.4 g, 62%): Mp 123.5°C; ¹H NMR (DMSO-d₆): δ 5.91 (s, 1H, N-H), 6.12 (d, 1H, 3J = 7.8 Hz), 6.60 (dt, 1H, J = 8.2 Hz, 4J = 1.3 Hz), 6.57 (dt, 1H, J = 7.9 Hz, 4J = 1.3 Hz), 7.18–7.38 (m, 14H), 7.58 (d, 1H, 3J = 7.4 Hz); HRMS (ESI) m/z calcd 277.0784 [C₁₉H₁₄Cl]⁺, 178.0480 [C₂H₇NOF₃]⁺; found 277.0773 [C₁₉H₁₄Cl]⁺, 178.0465 [C₂H₇NOF₃]⁺; Anal. calcd for C₂₆H₁₉ClF₃NO: C, 68.88; H, 4.22; N, 3.40; found: C, 69.19; H, 4.26; N, 3.10.
2-[[2-Chlorophenyl]diphenylmethyl][sulfanyl]pyrimidine (T112) was synthesized from T3-Cl (1.6 g, 5 mmol) and 2-mercaptopyrimidine (1.68 g, 15 mmol) according to general method C and recrystallized from methanol as an off-white powder (1.16 g, 60%); Mp 151.6˚C; 1H NMR (DMSO-d6) δ: 7.00 (t, 1H, J = 4.8 Hz), 7.20 (t, 2H, J = 7.2 Hz), 7.26 (t, 4H, J = 7.6 Hz), 7.33–7.37 (m, 7H), 7.81–7.82 (m, 1H), 8.28 (d, 2H, J = 4.9 Hz); HRMS (EI) m/z 389.0879 [M + H]+; found 389.0869 [M + H]+; Anal. calc'd for C23H18O2S: C, 76.19; H, 5.10; S, 17.71; found: C, 76.26; H, 5.03; S, 17.65.

(2-Methoxyphenyl)diphenylmethanol (T117) was synthesized from 2-bromoanisole (3.09 mL, 4.68 g, 25 mmol) according to general method A as an off-white powder (4.9 g, 17 mmol, 67.6%); Mp 128.7˚C; Lit. 128–129˚C (Baeyer, 1907); 1H NMR (DMSO-d6) δ: 3.67 (s, 3H, -OCH3), 5.28 (s, 1H, -OH), 6.54 (d, 1H, J = 7.7 Hz), 6.84 (t, 1H, J = 7.6 Hz), 6.97 (d, 1H, J = 8.2 Hz), 7.25–7.32 (m, 11H); HRMS (ESI) m/z calculated 273.1279 [M-OH]+; found 273.1270 [M-OH]+; Anal. calc'd for C20H18O2: C, 82.73; H, 6.25; found: C, 82.45; H, 6.20.

-N-[2-Methoxyphenyl][diphenylmethyl]-1,3-thiazol-2-amine (T122): In a first-step compound T117 was chlorinated with acetyl chloride according to method B to afford 1-(chlorodiphenylmethyl)-2-methoxybenzene (T117-Cl). Without further purification and characterization T117-Cl (3.7 g, 9.9 mmol) was immediately reacted with 2-aminopyrimidine (5.02 g, 52.75 mmol) according to general method C. T122 was obtained as an off-white powder after recrystallization from ethanol (1.46 g, 42%); Mp 183.1˚C; 1H NMR (CDCl3) δ: 8.07 (s, 1H, phenyl-CH3), 6.26 (d, 1H, J = 4.8 Hz, thiazole-H4), 6.86 (s, 1H, N-H), 7.04 (d, 1H, J = 4 Hz, thiazole-H3), 7.12 (d, 2H, J = 8 Hz), 7.20–7.27 (m, 12H); HRMS (ESI) m/z calculated 357.1426 [M + H]+; found 373.1375 [M + H2O]+; found 357.1420 [M + 1]+, 373.1537 [M + H2O]+; Anal. calc'd for C23H20N2S: C, 77.49; H, 5.65; N, 7.86; S, 9.89; found: C, 76.87; H, 5.67; N, 7.7; S, 9.48.

-N-[2-Methylphenyl][diphenylmethyl]-pyrimidin-2-amine (T125): (2-Methylphenyl)diphenylmethanol (T118) was synthesized and afterward chlorinated (T118-Cl) as described for T124. Without further purification and characterization T118-Cl (2.9 g, 9.9 mmol) was immediately reacted with 2-aminopyrimidine (2.36 g, 24.8 mmol) according to general method C. T125 was obtained as a white powder after recrystallization from ethanol (1.46 g, 42%); Mp 183.1˚C; 1H NMR (CDCl3) δ: 8.09 (s, 1H, phenyl-CH3), 6.43 (t, 1H, J = 4.8 Hz, pyrimidine-H5), 7.05 (d, 1H, J = 7.1 Hz), 7.08 (d, 1H, J = 7.7 Hz), 7.14–7.29 (m, 14H), 8.05 (s, 2H); HRMS (ESI) m/z calculated 352.1814 [M + H]+; found 352.1808 [M + H]+; Anal. calc'd for C22H12N2O: C, 82.02; H, 6.02; N, 11.96; found: C, 81.79; H, 5.93; N, 11.86.

-N-[Diphenyl][2-trifluoromethoxy][phenyl][methyl]-1,3-thiazol-2-amine (T126): Diphenyl[2-(trifluoromethoxy)phenyl]methanol (T119) was synthesized from 1-bromo-2-(trifluoromethoxy)benzene (3.69 mL, 6.03 g, 25 mmol) according to general method A. Without further purification and characterization T119 was chlorinated according to method B to afford 1-(chlorodiphenylmethyl)-2-(trifluoromethoxy)benzene (T119-Cl). Without further purification and characterization T119-Cl (g, 14.38 mmol) was immediately reacted with 2-aminopyrimidine (3.6 g, 35.94 mmol) according to general method C. T126 was obtained as an off-white powder after recrystallization from ethanol (2.25 g, 36.7%); Mp 178.8˚C; 1H NMR (CDCl3) δ: 8.04 (d, 1H, J = 3.6 Hz, thiazole-H4), 6.82 (s, 1H, N-H), 6.98 (d, 1H, J = 3.6 Hz, thiazole-H3), 7.10–7.29 (m, 13H), 7.61 (dd, 1H, J = 8.1 Hz, J' = 1.4 Hz); HRMS (ESI) m/z calculated 427.1092 [M + H]+; found 427.1087 [M + H]+; Anal. calc'd for C22H12F3N2O: C, 64.78; H, 4.02; N, 6.57; S, 7.52; found: C, 64.64; H, 3.89; N, 6.56; S, 8.1.

-N-[Diphenyl][2-(trifluoromethoxy)][phenyl][methyl][pyrimidin-2-amine (T127): Diphenyl[2-(trifluoromethoxy)phenyl]methanol (T119) was synthesized and afterward chlorinated (T119-Cl) as described for T126. Without further purification and characterization T119-Cl (5.2 g, 14.38 mmol) and 2-aminopyrimidine (3.42 g, 35.94 mmol) were reacted according to general method C. T127 was obtained as a white powder after recrystallization from ethanol (1.2 g, 19.8%); Mp 135˚C; 1H NMR (CDCl3) δ: 8.04 (d, 1H, J = 4.8 Hz, thiazole-H4), 7.15–7.20 (m, 3H), 7.24–7.29 (m, 9H), 7.39 (dd, 1H, J = 8.2 Hz, J' = 7.4 Hz), 7.42 (d, 1H, J' = 7 Hz, J = 1.7 Hz), 7.51 (dd, 1H, J = 8 Hz, J' = 1.7 Hz), 8.87 (s, 2H); pyridine-H3 and -H5); HRMS (ESI) m/z calculated 347.1480 [M + H]+; found 422.1471 [M + H]+; Anal. calc'd for C24H17F3N2O: C, 68.48; H, 4.31; N, 9.97; found: C, 68.3; H, 4.58; N, 9.99.

-N-[2-Fluorophenyl][diphenylmethyl][pyrimidin-2-amine (T128): (2-Fluorophenyl)diphenylmethanol (T36) was synthesized from bromobenzene (2.63 mL, 3.93 g, 25 mmol) and 2-fluorobenzophenone (4.22 mL, 5.0 g, 25 mmol) according to general method A (yellowish solid, 6.5 g, 93.41%). Spectroscopic data were in accordance with literature (Wulf et al., 2000). In a next step T36 was chlorinated according to method B to afford 1-(chlorodiphenylmethyl)-2-fluorobenzene (T36-Cl).
Without further purification and characterization, T36-Cl (2.75 g, 25.7 mmol) was obtained as a slightly yellowish powder after recrystallization from ethanol (1.73 g, 47.3%). Mp 161.1°C; 1H NMR (DMSO-\(\text{d}_6\)): \(\delta\) 6.53 (t, 1H, \(J = 4.8 \text{ Hz}\), pyrimidine-H4), 7.16 (t, 2H, \(J = 7.2 \text{ Hz}\)), 7.22–7.28 (m, 7H), 7.30–7.32 (m, 4H), 7.35 (s\(_{\text{broad}}\), 1H), 7.87 (s, 1H, N-H), 8.10 (d, 2H, \(J = 4.3 \text{ Hz}\), pyrimidine-H3 and -H5); HRMS (ESI) m/z: calcld 377.0879 [M+H]+, found 377.0861 [M+H]+; Anal. calcld for C\(_{23}\)H\(_{18}\)F\(_3\)N\(_2\)S: C, 67.3; H, 4.17; N, 6.82; S, 7.81; found: C, 67.03; H, 4.28; N, 7.94.

-\(N\)\{[3-Chlorophenyl]diphenylmethyl\}-1,3-thiazol-2-amine (T129): (3-Chlorophenyl)diphenylmethanol (T2) was synthesized from 1-bromo-3-chlorobenzene (2.94 mL, 4.79 g, 25 mmol) according to general method A (bromobenzene (2.84 mL, 4.24 g, 25 mmol) and 2-aminopyrimidine (2.1 g, 21.95 mmol) were reacted according to general method C. T130 was obtained as a white powder after recrystallization from ethanol (1.73 g, 47.3%). Mp 161.1°C; 1H NMR (DMSO-\(\text{d}_6\)): \(\delta\) 6.53 (t, 1H, \(J = 4.8 \text{ Hz}\), thiazole-H4), 7.16 (t, 2H, \(J = 7.2 \text{ Hz}\)), 7.22–7.28 (m, 7H), 7.30–7.32 (m, 4H), 7.35 (s\(_{\text{broad}}\), 1H), 7.87 (s, 1H, N-H), 8.10 (d, 2H, \(J = 4.3 \text{ Hz}\), pyrimidine-H3 and -H5); HRMS (ESI) m/z: calcld 377.0879 [M+H]+, found 377.0861 [M+H]+; Anal. calcld for C\(_{23}\)H\(_{18}\)F\(_3\)N\(_2\)S: C, 67.3; H, 4.17; N, 6.82; S, 7.81; found: C, 67.03; H, 4.28; N, 7.94.

-\(N\)\{[3-Chlorophenyl]diphenylmethyl\}-1,3-thiazol-2-amine (T130): (3-Chlorophenyl)diphenylmethanol (T2) was synthesized and afterward chlorinated (T2-Cl) as described for T129. Without further purification and characterization, T2-Cl (2.75 g, 8.78 mmol) and 2-aminopyrimidine (2.2 g, 21.95 mmol) were reacted according to general method A. T130 was then chlorinated according to method B to afford 1-(chlorodiphenylmethyl)-2-aminopyrimidine (T131): (3-Chlorophenyl)diphenylmethanol (T2) was synthesized and afterward chlorinated (T2-Cl) as described for T130. Without further purification and characterization reacted with 2-aminopyrimidine (2.2 g, 21.95 mmol) according to general method A. T131 was then chlorinated according to method B to afford 1-(chlorodiphenylmethyl)-2-aminopyrimidine (T132): (2-Bromophenyl)diphenylmethanol (T116-Cl) was synthesized and afterward chlorinated (T116-Cl) as described for T131. T116-Cl (4.5 g, 12.5 mmol) was immediately without further purification and characterization reacted with 2-aminoypyrimidine (1.2 g, 12.6 mmol) according to general method C. T132 was obtained as an off-white to yellowish powder after recrystallization from ethanol (850 mg, 16%). Mp 158.7°C; 1H NMR (DMSO-\(\text{d}_6\)): 6.57 (t, 1H, \(J = 4.5 \text{ Hz}\), pyrimidine-H4), 7.10 (s, 1H), 7.14–7.32 (m, 13H), 7.49 (d, 2H, \(J = 8.5 \text{ Hz}\), pyrimidine-H3 and -H5), 8.10 (s, 1H, N-H); HRMS (ESI) m/z: calcld 416.0762 [M+H]+, 418.0742, 419.0776; found 416.0758 [M+H]+, 418.0728, 419.0752; Anal. calcld for C\(_{23}\)H\(_{18}\)BrN\(_2\): C, 66.16; H, 4.23; N, 9.74.

-\(N\)\{[2-Bromophenyl]diphenylmethyl\}-1,3-thiazol-2-amine (T132): (2-Bromophenyl)diphenylmethanol (T116-Cl) was synthesized and afterward chlorinated (T116-Cl) as described for T131. T116-Cl (4.5 g, 12.5 mmol) was immediately without further purification and characterization reacted with 2-aminoypyrimidine (1.2 g, 12.6 mmol) according to general method C. T132 was obtained as an off-white to yellowish powder after recrystallization from ethanol (850 mg, 16%). Mp 158.7°C; 1H NMR (DMSO-\(\text{d}_6\)): 6.57 (t, 1H, \(J = 4.5 \text{ Hz}\), pyrimidine-H4), 7.10 (s, 1H), 7.14–7.32 (m, 13H), 7.49 (d, 2H, \(J = 8.5 \text{ Hz}\), pyrimidine-H3 and -H5), 8.10 (s, 1H, N-H); HRMS (ESI) m/z: calcld 416.0762 [M+H]+, 418.0742, 419.0776; found 416.0758 [M+H]+, 418.0728, 419.0752; Anal. calcld for C\(_{23}\)H\(_{18}\)BrN\(_2\): C, 66.16; H, 4.23; N, 9.74.
Without further purification and characterization T135-Cl was immediately reacted with 2-aminothiazole (1.5 g, 15 mmol) according to general method C. T136 was obtained as a slightly yellowish powder (silica column cyclohexane/ethylacetate (8/2); 80 mg, 2.8%): Mp 160.7°C; 1H NMR (DMSO-d6): δ: 6.58 (dt, 1H, 3J = 7.9 Hz, 4J = 1.2 Hz), 6.93 (t, 1H, 3J = 7.5 Hz), 7.05 (s (broad), 1H), 7.19–7.32 (m, 11H), 7.44 (d, 1H, 3J = 8 Hz), 7.82, (d, 1H, 3J = 7.8 Hz), 8.1 (s (broad), 2H, pyrimidine-H3 and -H5); MS (ESI) m/z calc 469.4 [M + H]+; found 469.0213 [M + H]+.

N-[(2-Iodophenyl)diphenylmethyl]-pyrimidin-2-amine (T137): (2-Iodophenyl)diphenyl methanol (T135) was synthesized and afterward chlorinated (T135-Cl) as described for T135. In a next step T135-Cl (2.4 g, 6 mmol) was immediately with further purification and characterization reacted with 2-aminopyrimidine (1.48 g, 15.56 mmol) according to general method C. T136 was obtained as a slightly sized from phenylmagnesium bromide (1.89 g, 15.3 mmol) and methoxybenzophenone (5.0 g, 23.5 mmol) according to general method A and recrystallized from ethanol as a white powder (0.6 g, 5.2%): Mp 81°C; 1H NMR (DMSO-d6): δ: 3.39 (s, 6H, -OCH3), 5.37 (s, 1H, -OH), 6.87–6.89 (m, 3H, 6H), 7.18–7.22 (m, 2H), 7.26–7.36 (m, 11H), 7.80 (s, 1H, N-H), 8.09 (d, 2H, 3J = 3.5 Hz, pyrimidine-H3 and -H5), 8.19 (d, 2H, 3J = 4.8 Hz); HRMS (ESI) m/z calc 372.1268 [M + H]+, found 372.1261 [M + H]+.

N-[(4-Chlorophenyl)diphenylmethyl]-4-(trifluoromethoxy)aniline (T150) was synthesized from T3-Cl (1.6 g, 5 mmol) and 4-(trifluoromethoxy)aniline (2.01 mL, 2.66 g, 15 mmol) according to general method C and recrystallized from ethanol as an off-white powder (1.18 g, 72.4%): Mp 174.2°C; Lit. 174–175°C (Dahlbom and Ekstrand, 1944). Spectroscopic data were in accordance with literature (Zunszain et al., 2002).

T136 was obtained as an off-white powder (1.5 g, 66%) according to general method C. T137 was obtained as a white powder after recrystallization from ethanol (2.3 g, 12%): Mp 117.8°C; 1H NMR (DMSO-d6): δ: 6.33 (t, 1H, 3J = 2.1 Hz), 6.99–7.04 (m, 5H), 7.10 (t, 1H, 3J = 1.9 Hz), 7.34–7.43 (m, 9H), 7.66 (d, 1H, 3J = 1.6 Hz); HRMS (ESI) m/z calc 69.04528 [C3H5N2]+, found: 69.0432 [C3H5N2]+, 277.0784 [C19H14Cl]+, found 277.0757 [C19H14Cl]+; Anal. calc. for C19H14ClN2: C, 76.63; H, 4.97; N, 5.17; found: C, 76.13; H, 4.92; N, 5.13.

Bis(2-methoxyphenyl)(phenyl)methanol (T165) was synthesized from phenylmagnesium bromide (1.89 g, 15.3 mmol) and 2-aminothiazole (1.05 g, 10.54 mmol) according to general method B to afford 1-chloro-4-(2-iodophenyl)diphenylmethanol (T135) was synthesized and afterward chlorinated (T135-Cl) as described for T135. Without further purification and characterization T1-Cl (15.7 g, 50 mmol) was reacted with 2-aminopyrimidine (11.88 g, 128 mmol) according to general method C. T145 was obtained as a white powder after recrystallization from ethanol (2.3 g, 12%): Mp 117.8°C; 1H NMR (DMSO-d6): δ: 6.51–6.54 (m, 3H, 3J = 1.6 Hz); HRMS (ESI) m/z calc 454.1180 [M + H]+, 178.0474 [C12H17F3NO]+, found 454.1156 [M + H]+, 178.0461 [C12H17F3NO]+; Anal. calc. for C12H17F3NO: C, 68.8; H, 4.22; N, 3.09; found: C, 68.52; H, 4.09; N, 3.05.

N-[(4-Chlorophenyl)diphenylmethyl]-1H-pyrazole (T142): (3-Chlorophenyl)diphenylmethanol (T2) was synthesized and afterward chlorinated (T143) as described for T129. Without further purification and characterization T2-Cl (3.3 g, 10.54 mmol) and pyrazole (1.77 g, 26 mmol) were reacted according to general method C. T142 was obtained as a white powder after recrystallization from ethanol (3.1 g, 85.3%): Mp 127.3°C; 1H NMR (DMSO-d6): δ: 6.33 (t, 1H, 3J = 2.1 Hz), 6.99–7.04 (m, 5H), 7.10 (t, 1H, 3J = 1.9 Hz), 7.34–7.43 (m, 9H), 7.66 (d, 1H, 3J = 1.6 Hz); HRMS (ESI) m/z calc 454.1180 [M + H]+, 178.0474 [C12H17F3NO]+, found 454.1156 [M + H]+, 178.0461 [C12H17F3NO]+; Anal. calc. for C12H17F3NO: C, 68.8; H, 4.22; N, 3.09; found: C, 68.52; H, 4.09; N, 3.05.

Bis(2-methoxyphenyl)(phenyl)methanol (T165) was synthesized from magnesium turnings (0.6 g, 25 mmol), 1-bromo-2-methoxybenzene (4.0 mL, 5.99 g, 32 mmol) and 2-methoxybenzophenone (5.0 g, 23.5 mmol) according to general method A and recrystallized from ethanol as a white powder (1.2 g, 15.8%): Mp 115.8°C; Lit. 115°C (Baeyer, 1907); 1H NMR (DMSO-d6): δ: 3.57 (s, 1H, -OH), 6.87 (dt, 2H, 3J = 7.8 Hz, 4J = 0.7 Hz), 6.98 (d, 2H, 3J = 7.9 Hz), 7.03 (dd, 2H, 3J = 7.8 Hz).
2H, \( ^3J = 7.8 \text{ Hz} \), \( ^3J = 1.5 \text{ Hz} \), 7.15–7.17 (m, 3H), 7.20–7.21 (m, 2H), 7.23–7.27 (m, 2H); MS (ESI) m/z calcd 343.38 [M + Na\(^+\)], 303.14 [M - OH\(^-\)]\(^+\), found 343.1 [M + Na\(^+\)], 303.1 [M - OH\(^-\)]\(^+\); Anal. calcd for C\(_2\)H\(_2\)O\(_2\)S: C, 78.73; H, 6.29; found: C, 78.14; H, 6.97.

\(-\) bis(2-methoxyphenyl)[phenyl]methyl]-1,3-thiazol-2-amine (T166): In a first-step T165 was chlorinated according to general method B (T165-Cl). The crude T165-Cl (530 mg, 1.56 mmol) was immediately reacted with 2-aminothiazole (392 mg, 3.9 mmol) according to the manufacturer’s protocol. Human Kv7.2 and Kv7.3 in PTLN or pcDNA-3 was provided by Bernhard Attali (Weizmann Institute of Science, Rehovot, Israel).

\(\text{N1E-115} \) neuroblastoma cells (expressing mKv1.1, HERG) in HEK-293 cells (Craig January, University of Wisconsin-Madison); \(\text{nNav}1.4 \) in HEK-293 cells (Frank Lehmann-Horn, University of Ulm, Germany) and Cav1.2 in HEK-293 cells (Frant Hofmann, Munich, Germany). Cells stably expressing mKv1.1, mKv3.1, hKCa1.5, and mKv3.1 have been previously described (Grismer et al., 1994); N1E-115 neuroblastoma cells (expressing Nav1.2) were obtained from ATCC. Rat Nav1.5 in pSP64T was provided by Roland G. Kallen (University of Pennsylvania), inserted into pcDNA-3.1(+) as described (Sanckaranarayanan et al., 2009), and transiently transfected into COS-7 cells together with eGFP-C1 with Fugene-6 (Roche) according to the manufacturer’s protocol. Human Kv7.2 and Kv7.3 in PTLE or pcDNA-3 was provided by Bernhard Attali (Weizmann Institute of Science, Rehovot, Israel).

**Electrophysiology**

Junctional conductance measurements were performed on N2A neuroblastoma cells transiently transfected with cDNAs corresponding to individual connexins or on mouse primary lens epithelial cells isolated from postnatal day 6. Dissociation of lens epithelial was performed as described previously (White et al., 2007). Junctional conductance was measured between cell pairs using the dual whole-cell voltage-clamp technique with Axopatch 1D patch-clamp amplifiers (Molecular Devices, CA) at room temperature. Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200 msec hyperpolarizing pulses from the holding potential of 0 mV to −20 mV were applied to one cell to establish a transjunctional voltage gradient \( (V_j) \), and junctional current was measured in the second cell (held at 0 mV). The solution bathing the cells contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, 2 mM pyruvate, and 1 mM BaCl\(_2\), pH 7.4. Patch electrodes had resistances of 3–5 MΩ when filled with internal solution containing 130 mM CsCl, 10 mM EGTA, 0.5 mM CaCl\(_2\), 3 mM MgATP, 2 mM Na\(_2\)ATP, and 10 mM HEPES, pH 7.2. Macroscopic recordings were filtered at 0.2–0.5 kHz and sampled at 1–2 kHz. Data were acquired using pClamp software (Axon Instruments) and plotted using Origin 6.0 software (OriginLab Corp, Northampton, MA). Drugs were applied with a gravity-fed perfusion system. Solution exchanges were complete within 10–20 s. All compounds were applied to Cx50 expressing N2A cells at an initial concentration of 10 μM. Compounds that reduced Cx50 junctional currents by >80% at 10 μM were then applied at lower concentrations ranging from 0.5 to 5 μM. Concentration-response curves for drug-induced uncoupling were typically determined by exposure of each cell pair to 0.5 or 1 μM, 5 and 10 μM of each drug. Concentrations of drugs \([D]\) that caused a half-maximal inhibition \( (IC_{50}) \) and the Hill coefficients \( (n_H) \) of concentration-response relationships were estimated by fitting the data to the equation: \( g_j/\% \text{ control} = 1/1 + ([D]/EC_{50})^{n_H} \) where \( g_j/\% \text{ control} \) is fraction of the conductance \( (g_j) \) in the absence and presence of the drug. Data are presented as means ± S.E.M.

Experiments on K\(^+\) and Na\(^+\) channels were performed with an EPC-10 amplifier (HEKA, Lambrecht/Pfalz, Germany) in the whole-cell configuration of the patch-clamp technique with a holding potential of −80 mV. Pipette resistances averaged 2.0 MΩ. Solutions of triarylmethanes in Ringer were freshly prepared before the experiments from 10 mM stock solutions in DMSO. The final DMSO concentration never exceeded 1%. For measurements of KCa2 and KCa3.1 currents we used an internal pipette solution containing (in mM): 145 K\(^+\) aspartate, 2 MgCl\(_2\), 10 HEPES, 10 K\(_2\)EGTA, and 5.96 (250 mM free Ca\(^{2+}\)) or 8.55 CaCl\(_2\) (1 mM free Ca\(^{2+}\)), pH 7.2, 290–310 mMosm. Free Ca\(^{2+}\) concentrations were calculated with MaxChelator assuming a temperature of 25°C, a pH of 7.2 and an ionic strength of 160 mM. To reduce currents from native chloride channels in COS-7 and HEK-293 cells, Na\(^+\) aspartate Ringer was used as an external solution (in mM): 160 Na\(^+\) aspartate, 4.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, pH 7.4, 290–310 mMosm. KCa2 and KCa3.1 currents were elicited by 200-ms voltage ramps from −120 to 40 mV applied every 10 s and the fold-increase of slope conductance at −80 mV by drug taken as a measure of channel activation.

KCa1.1 currents were elicited by 200-ms voltage steps from −80 to 60 mV applied every 10 s (1 μM free Ca\(^{2+}\)), and channel modulation measured as a change in mean current amplitude. Kv1.1, Kv1.3, Kv1.4, Kv1.5, Kv3.1, Kv3.2, and Kv4.2 currents were recorded in normal Ringer solution with a Ca\(^{2+}\)-free pipette.
solution containing (in mM): 145 KF, 10 HEPES, 10 EGTA, 2 MgCl₂, pH 7.2, 300 mOsm. Currents were elicited by 200-ms depolarizing pulses to 40 mV applied every 10 s. HERG (Kv11.1) currents were recorded with a KCl-based pipette solution (4 mM ATP) and with a two-step pulse from −80 mV first to 20 mV for 2 s and then to −50 mV for 2 s. The reduction of both peak and tail current by the drug was determined. Current from co-expressed Kv7.2/7.3 channels was elicited by depolarizing pulses from the holding potential (−80 mV) to +40 mV for 500 ms followed by hyperpolarization to −120 mV for 200 ms. Nav1.2 currents from N1E-115 cells and Nav1.4 currents from stably transfected HEK cells were recorded with 20 ms pulses from −80 to −10 mV every 10 s with a KCl-based pipette solution and normal Ringer as an external solution. Blockade of Na⁺ current was determined by measuring the reduction of peak maximum conductance.

RESULTS
SCREENING OF A “FOCUSED” LIBRARY FOR Cx50 INHIBITORS
To identify Cx50 inhibitors we first screened a small library of compounds containing known ion channel pharmacophores including the antihistamine astemizole, several psoralens and related heterocycles, benzothiazoles, triazines, and flavanoid glycosides as well as the antifungal agent clotrimazole. From this library we identified four novel low micromolar inhibitors of Cx50: Astemizole, rutin (a flavonoid glycoside), PAA-10 (an alkyl substituted dibenzazocinone), and clotrimazole (see Figure 1 for structures). All compounds produced significant inhibition of the Cx50 junctional current at a concentration of 10 μM (Figure 1). The inhibition of functional currents caused by clotrimazole, astemizole and PAA-10 was completely reversible upon washout. In contrast, the effects of rutin were only partially reversible. Of these four hits, the triarylmethane clotrimazole seemed the most drug-like and attractive compound to us. Astemizole is known to affect many other ion channels including the cardiac K⁺ channel HERG (Kv11.1; Suessbrich et al., 1996), a liability not generally encountered with triarylmethanes (Toyama et al., 2008). We also discarded rutin as a template since preliminary experiments showed that the rutin aglycon, quercetin, had no effect on Cx50 at 10 μM (data not shown) demonstrating that the sugar moiety is essential for connexin inhibition. The dibenzazocinone PAA-10 would of course also have been a possible lead but we preferred to perform structure activity relationship (SAR) studies around clotrimazole since our laboratory had a library of 80 triarylmethanes that were immediately available for an SAR analysis on Cx50. These compounds had been previously synthesized for an SAR study to determine the structural requirements for inhibition of the intermediate-conductance calcium-activated potassium channel KCa3.1 (a.k.a. IKCa1, SK4). By using the so-called selective optimization of side activities (SOSA) approach, which allows for the selective optimization of the side activity of an old drug (Wermuth, 2004), our group successfully designed a triarylmethane, TRAM-34 (T34), that selectively blocked KCa3.1 channels without affecting cytochrome P450-dependent enzymes, the main target of clotrimazole (Wulff et al., 2000). This previous work had demonstrated that it is possible to achieve selectivity for different targets by appropriately modifying the triarylmethane (TRAM) pharmacophore, which was another reason for us to choose clotrimazole as a template for our current study on Cx50.

Clotrimazole reversibly inhibited Cx50 expressed in N2A cells with an IC₅₀ of 5 μM and a Hill slope of ∼ 2.1 (Figure 2). At concentrations of 10 μM clotrimazole had no effect on channels built out of Cx32, Cx36, and Cx46 (Figure 2). GJ channel conductance in all cases was measured by using the dual whole-cell patch-clamp technique as described in the Section “Materials and Methods” (Srinivas et al., 2001; Srinivas and Spray, 2003; Cruikshank et al., 2004).

PROBING OF THE TRIARYLMETHANE PHARMACOPHORE FOR Cx50 INHIBITION
Using clotrimazole as a template we explored the SAR of the triphenylmethane scaffold according to the synthetic strategy shown in Figure 3. In a Grignard reaction mono-substituted benzophenones and bromobenzenes were reacted in anhydrous diethyl ether to yield the corresponding triphenylmethanols. These alcohols were then either ammonolyzed, cyanated with copper cyanide or chlorinated using acetyl chloride. The triphenylmethane chlorides were further reacted in a nucleophilic substitution to give the respective triphenylmethane derivatives (further details on exact conditions and quantities are given in the see Materials and Methods). We first substituted the imidazole ring of clotrimazole with several other heterocycles, differently substituted carbocycles or aliphatic functional groups while keeping the 2-chlorophenylidiphenyl methane basic structure (Figure 3). Except for T44 (pyrrol), T69 (2-aminopyridine), T89 (4-methyl-2-phenylimidazole) and the bicyclic T71 (phthalimide) and T103 (2-aminobenzothiazole), most of the heterocyclic derivatives blocked Cx50 in the low micromolar range. Spacer linked carbocycles (T102, T104, T106, T107, T109, T150) in contrast showed no effect on Cx50 at concentrations of 10 μM with the exception of T106, which was found to be a weak blocker with an IC₅₀ 10 μM (Figure 4). We next tested the heterocyclic substituted triarylmethanes for selectivity over KCa3.1 (Figure 4). As previously reported (Wulff et al., 2000), clotrimazole and T34 (= TRAM-34) are nanomolar KCa3.1 blockers, that exhibit IC₅₀ values of 70 and 20 nM, respectively, and are therefore not useful as Cx50 inhibitors. In contrast, the aminothiazole and aminopyrimidine substituted T66 and T68 were found to be 15- to 200-fold less potent on KCa3.1 and T66 even exhibited a moderate three-fold selectivity for Cx50 over KCa3.1.

Since the alcohol T3, which is the first-step intermediate for the heterocyclic substituted triarylmethanes, was also found to reduce Cx50 currents with an IC₅₀ of 2 μM we further synthesized and tested several triarylmethane alcohols, amines, nitriles, and ureas on Cx50 (Table A1 in Appendix). While several of the alcohols including the p-chloro substituted T1, the m-chlorosubstituted T2 as well as the non-substituted triphenylmethanol and triphenylamine (T162) exhibited IC₅₀ values for Cx50 in the 1–2 μM range (Table A1 in Appendix), all these compounds lacked selectivity over KCa3.1 and were further found to inhibit other connexins like Cx43, Cx46 at similar concentrations as Cx50 (data not shown). In addition, the inhibition produced by these compounds was often enhanced by a prior application of the compounds. We therefore did not study these compounds further and instead...
concentrated our synthetic efforts on the heterocyclic substituted triarylmethanes and explored the substitution position of the chlorine atom on one of the phenyl rings by moving it from the ortho- to the meta- or para-position or completely removing it (Figure 5).

All four imidazole ring containing compounds (T97, clotrimazole, T143, T144) but only the o-chloro and m-chloro substituted pyrazole derivatives (T34 and T142) inhibited Cx50 channels with IC50s of 5–8 μM. In the 2-aminothiazole and 2-aminopyrimidine...
series only the o-chloro substituted T66 and T68 were active, while the other regio-isomers or the unsubstituted analogs showed no effect at 10 μM. Because the imidazole and pyrazole-substituted triarylmethanes were poorly selective for Cx50 over KCa3.1, we studied the effect of modifications of the o-chloro substituent in the 2-aminothiazole and 2-aminopyrimidine series. Specifically, we replaced the o-chloro substituent with other halogens (F, Br, I), the more lipophilic CF3 or OCF3 groups or electron-donating methyl or methoxy groups. All 14 compounds exhibited IC50 values in the low micromolar range (Figure 6) with the methoxy-substituted T122 (IC50 1.2 μM) and the iodo-substituted T136 (IC50 2.4 μM) being the most potent (Figure 6).

T122 AND T136 ARE SELECTIVE FOR CX50
The effects of T122 and T136 on Cx50 junctional channels were further characterized using a five-point dose response curve (Figure 7A). Non-linear least-squares fit of the individual data points to the Hill equation (see Materials and Methods) yielded IC50 values of 1.2 and 2.4 μM for the inhibition of Cx50 GJ channels by T122 and T136, respectively. In both cases, the Hill coefficients were ≈ 2 (1.6 for T122, 1.7 for T136), indicating that binding of two TRAM molecules was required to inhibit Cx50 GJ channels. Both compounds exhibited high selectivity for Cx50 channels over other connexin subtypes. The effects of T122 and T136 on GJ channels formed by several other connexins, including Cx26, Cx32, Cx40, Cx43, and Cx46 are illustrated in Figure 7B. At a concentration of 10 μM, sufficient to cause near-maximal decreases in Cx50 junctional current, T122 and T136 did not significantly inhibit Cx26, Cx32, Cx46, or Cx43 GJ channel currents. The reduction of junction conductance was less than 20% in each of these cases. These results demonstrate that inhibition of Cx50 GJ channels by T122 and T136 is highly connexin-selective.

SELECTIVITY OVER Kv, KCa, AND Nav CHANNELS
In order to more broadly evaluate the selectivity of T122 and T136 we further determined their effect on a panel of 12 potassium and sodium channels from various gene families (Table 1). While both compounds exerted practically no effect on the neuronal Nav1.2 and the skeletal muscle Nav1.4 channel or Kv channels from the Kv4, Kv7, Kv11, or KCa2 (SK) family, they exhibited only moderate selectivity over Kv1, Kv2, Kv3-family channels, Kv11.1 (hERG), and the calcium-activated K+ channels KCa1.1 and KCa3.1. T122 in particular reduced Kv1.1 currents by 65% at 10 μM, while T136 was found to have an IC50 of 1.3 μM for KCa3.1.

T122 AND T136 INHIBIT COUPLING IN THE LENS
Cx50 is strongly expressed in the lens in both the epithelium and in fibers. In the epithelium, the functional contribution of Cx50 to epithelial cell coupling is highest during the first postnatal week (~70–75% of total coupling on average) with the remainder being contributed by Cx43. Therefore, we determined whether T122 and T136 (10 μM) also inhibited coupling
FIGURE 4 | Table showing the structures and IC$_{50}$ values for Cx50 and KCa3.1 inhibition of heterocyclic substituted triarylmethanes. Concentrations of triarylmethanes that caused a half-maximal inhibition (IC$_{50}$) values were obtained by fitting the data to the Hill equation, as described in the methods. Means of current inhibition and SD were determined by application of two or three concentrations of each triarylmethane to multiple cells (n ranging from 3 to 8 per concentration). The SD values are not shown for clarity; SD values typically ranged between 5 and 15%.

provided by Cx50 in epithelial cells (Figure 8). The effect of T122 and T136 on junctional currents between epithelial cells isolated from mouse lenses on postnatal day 6 is shown in Figure 8A. Both compounds strongly reduced junctional currents, an effect that was reversible on washout of the drug. The reduction of junctional currents caused by T122 and T136 ranged from 65 to 87% of the initial conductance (means ± SEM are 67 ± 9%, n = 7 for T122; and 64 ± 8%, n = 6 for T136). These values were similar to the reduction produced by quinine, which also selectively inhibits Cx50, but not Cx43 GJ channels (Figure 8B).

DISCUSSION
A major reason for the poorly developed pharmacology of GJ channel modulators is the intercellular location of these channels, which makes it extremely difficult to design high-throughput
were determined by application of two or three concentrations of each triarylmethane to multiple cells (n ranging from 3 to 8 per concentration). The SD values are not shown for clarity; SD values typically ranged between 5 and 15%.

### Table 1 | Selectivity over other ion channels.

| Channel       | T122 (10 μM) | T136 (10 μM) |
|---------------|--------------|--------------|
| Kv1.1         | 65 ± 10% block (n=5) | 28 ± 9% block (n=5) |
| Kv1.3         | 52 ± 2% block (n=3)  | 18 ± 4% block (n=3)  |
| Kv2.1         | 28 ± 4% block (n=3)  | 28 ± 4% block (n=3)  |
| Kv3.1         | 32 ± 12% block (n=5) | 37 ± 16% block (n=4) |
| Kv4.2         | 16 ± 4% block (n=3)  | 15 ± 3% block (n=3)  |
| Kv7.2/7.3     | No effect (n=3)  | No effect (n=2)  |
| Kv11.1 (hERG) | 42 ± 3% block (n=3) | 27 ± 4% block (n=3) |
| KCa2.3        | No effect (n=3)  | No effect (n=3)  |
| KCa3.1        | IC50 = 10.2 ± 0.7 μM | IC50 = 1.3 ± 0.2 μM |
| KCa1.1 (BK)   | 43 ± 4% (n=3)  | 20 ± 2% (n=3)  |
| Navi1.2       | 11 ± 1% block (n=3) | 9 ± 0.5% block (n=3) |
| Navi1.4       | No effect (n=3) | No effect (n=3) |

Percentage of current inhibition (mean ± SD) by 10 μM T122 and T136 for a panel of cloned Kv, KCa, or Nav channels. (For recording conditions and pulse protocols see Materials and Methods).

In our search for Cx50 inhibitors we therefore decided to screen a small library containing known ion channel modulators using conventional dual whole-cell voltage-clamp. Our library was enriched in so-called “privileged” structures which are small molecule pharmacophores that are able to bind to multiple targets and which are therefore highly likely to exert biological effects (Evans et al., 1988; Horton et al., 2003). By appropriately decorating such “privileged” scaffolds their potency and selectivity can often be directed toward a single target with relatively high affinity. If the template for such an SAR study is an “old” drug, the approach is also called SOSA approach (selective optimization of the side activity of an old drug) as suggested by Wermuth (2004). Using this approach we found four structurally very different compounds able to inhibit Cx50 channels in the low micromolar range including the triarylmethane (TRAM) clotrimazole (IC50 5 μM; **Figure 1**). Using clotrimazole as a template, we have tested a series of previously known or newly synthesized differently substituted TRAMs for their effects on Cx50 channels and identified several compounds inhibiting Cx50 in the low micromolar range including T122, which exhibits an IC50 of 1.2 μM for Cx50 and excellent selectivity over other connexins. Analyzing at the structural requirements for selective Cx50 inhibition we find that the TRAM pharmacophore should contain a heteroaromatic ring system in R1 position. Smaller, less bulky substitutions with functional groups such as OH, NH, or H also result in potent Cx50 blockers (**Table A1** in Appendix), however, these types of compounds lack selectivity over other connexins and are therefore not useful as pharmacological tools. In addition to a
heteroaromatic substituent in R1 position, the second requirement is that one of the phenyl rings of the triphenylmethane should be substituted preferably in ortho-position (Figure 5), whereby it seems to be of little consequence whether the substituent is electron withdrawing (CF3 in T72) or donating (OCH3 in T122), as long as it is lipophilic. Lastly, the heteroaromatic ring system, which can be directly attached or linked by a one-atom spacer (nitrogen or sulfur), should contain at least one hydrogen-bond accepting heteroatom, which might be directly interacting with a hydrogen-bond donor in the connexin protein (e.g., see clotrimazole with its imidazole ring versus the inactive T44 and T109). It also seems that steric bulk is a limiting factor for potency, because the bulkier bicyclic derivatives T103 and T71 are ineffective while the smaller T06 and T91 inhibit Cx50.

Since our compounds were derived from clotrimazole, which inhibits KCa3.1 channels with an IC50 of 70 nM, we also tested the more active compounds for selectivity over KCa3.1. For nanomolar KCa3.1 inhibition Wulff et al. (2000) previously proposed a propeller-shaped pharmacophore consisting of the triphenyl moiety with an o-halogen on one of the phenyl rings and an unsubstituted, polar π-electron-rich heterocycle of limited size such as pyrazole, tetrazole, or an even smaller nitrile group in R1 position. A similar KCa3.1-inhibiting TRAM pharmacophore was described by McNaughton-Smith et al. (2008) as exemplified by ICA-17043 (Senicapoc®), which contains a carboxamide moiety in R1 position and entered clinical trials for sickle cell anemia. Considering this knowledge about the TRAM pharmacophore for KCa3.1 inhibition in our current Cx50-focused SAR study, we could achieve a drop in potency on KCa3.1 of more...
than 1000-fold by inserting a one-atom linker between the triphenylmethylene scaffold and the heterocycle in R1 position. This insertion, which results in a “kink” in the perfect propeller shape of clotrimazole or TRAM-34 shifted selectivity toward Cx50 [clotrimazole (IC_{50} of 70 nM for KCa3.1 and 5 μM Cx50) versus T122 (IC_{50} of 10 μM for KCa3.1 and 1.2 μM for Cx50)]. At concentrations of 10 μM the two most potent compounds, T122 and T136, showed excellent selectivity over other tested connexin channels including channels built out of Cx43 and Cx46, which are also expressed in the lens (inhibition < 18%). In addition, T122 and T136 showed very good selectivity over sodium channels (<11%) and moderate selectivity over most of the tested potassium channels (18–43%), except for T122, which blocked Kv1.1 by 65%. Both compounds also inhibited Cx50 mediated coupling in primary lens epithelial cells. Thus, compared to the existing pharmacological agents such as quinine and 2-APB, which exhibit poor selectivity for Cx50 channels, T122 and T136 are likely better compounds for studying the role of Cx50 in lens development and the maintenance of lens transparency.

Taken together, we here identified the triphenylmethane scaffold as a new pharmacophore for Cx50 inhibition and synthesized two new compounds that inhibit cloned and native Cx50 channels in the low micromolar range. Even more importantly, we were able to develop a connexin subtype specific inhibitor starting with a lead compound that exhibited several fold higher selectivity for K\(^+\) channels over Cx50, indicating that application of a similar approach, i.e., identification of new lead compound(s) from existing ion channel libraries followed by selective optimization of their side activities, may lead to the development of specific blockers for other connexin channels such as Cx43 or Cx26.

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## APPENDIX

Table A1 | IC_{50} values for Cx50 inhibition for triarylmethane alcohols, amines, nitriles and ureas.

| Compound            | IC_{50} Cx50 (μM) | IC_{50} KCa3.1 (μM) |
|---------------------|-------------------|---------------------|
| T1                  | 2.8               | 0.53                |
| T2                  | 3.6               | 0.55                |
| T3                  | 2                 | 0.52                |
| T54                 | 3                 | 0.7                 |
| T154-OH             | No effect         | N/A                 |
| T117                | No effect         | N/A                 |
| T165                | No effect         | N/A                 |
| T43                 | 2                 | 0.75                |

(Continued)

| Compound            | IC_{50} Cx50 (μM) | IC_{50} KCa3.1 (μM) |
|---------------------|-------------------|---------------------|
| Triphenylmethanol  | 1                 | 0.5                 |
| T162                | 1.5               | 5.3                 |
| T41                 | 4                 | 1                   |
| T75                 | No effect         | 1.2                 |
| T95                 | 6                 | 5                   |
| T94                 | 7.3               | Not tested          |
| T39                 | 4                 | 0.06                |
| Triphenylmethane   | 0.9               | 3.7                 |

(Continued)
Table A1 | Continued

| Compound | IC<sub>50</sub> Cx50 (μM) | IC<sub>50</sub> KCa3.1 (μM) |
|----------|--------------------------|--------------------------|
| T51      | No effect 25             |                          |
| T24      | No effect 8              |                          |
| T74      | No effect 4              |                          |
| T52      | 10                       | >50                      |
| T53      | 10                       | >50                      |
| T50      | 10                       | >50                      |
| T160     | 4                        | 8.6                      |
| T9       | 4                        | 1.5                      |
| T35      | 10                       | 9                        |