A strategy for designing allosteric modulators of transcription factor dimerization

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Transcription factors (TFs) are fundamental in the regulation of gene expression in the development and differentiation of cells. They may act as oncogenes and when overexpressed in tumors become plausible targets for the design of antitumor agents. Homodimerization or heterodimerization of TFs are required for DNA binding and the association interface between subunits, for the design of allosteric modulators, appears as a privileged structure for the pharmacophore-based computational strategy. Based on this strategy, a set of compounds were earlier identified as potential suppressors of OLIG2 dimerization and found to inhibit tumor growth in a mouse glioblastoma cell line and in a whole-animal study. To investigate whether the antitumor activity is due to the predicted mechanism of action, we undertook a study of OLIG2 dimerization using fluorescence cross-correlation spectroscopy (FCCS) of live HEK cells transfected with 2 spectrally different OLIG2 clones. The selected compounds showed an effect with potency, which correlated with the earlier observed antitumor activity. The OLIG2 proteins showed change in diffusion time under compound treatment in line with dissociation from DNA. The data suggest a general approach of drug discovery based on the design of allosteric modulators of protein–protein interaction.

Significance

Transcription factors in the bHLH family are potentially relevant for tumor growth. Activation requires homodimerization or heterodimerization. Thus, the dimerization step is a likely significant drug target. The oligodendrocyte transcription factor 2 (OLIG2) is overexpressed in gliomas. Here, we developed a fluorescence cross-correlation spectroscopy protocol to examine 10 compounds selected using a pharmacophore-based computational strategy targeting OLIG2 dimerization. We showed that the potency to interact with OLIG2 dimerization in live cells correlates with carcinostatic efficacy. The data indicate a promising approach toward drug development targeting transcription factor overactivity and protein–protein interaction more generally.

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dashed line shows the average RCA value in untreated cells. The IC50 values for OLIG2 homodimerization in live HEK cells were determined (\(0.09\)). Statistical analysis was performed against untreated (*\(P < 0.01\)). Blue dashed line shows the average RCA value in untreated cells.

The field of protein–protein interactions (PPIs) is still a largely uncharted territory for drug design. It has been estimated that the interactome is composed of several hundred thousand PPIs, yet, only a small fraction has still been explored therapeutically.

**Fig. 1.** Inhibitory effect of test compounds on OLIG2 dimerization in live HEK cells. Comparison of RCA in live HEK cells. RCA, \(N_C/N_G\), was calculated from FCCS data. \(N_C\) and \(N_G\) denote the cross-correlated number and number of OLIG2-eGFP, respectively. Average ± SD in RCA value for negative control (NC) (0.05 ± 0.04), untreated (UN) (0.28 ± 0.07), 1 \(\mu\)M compound #1 (129407: 0.14 ± 0.04), #2 (691240: 0.11 ± 0.06), #3 (50467: 0.08 ± 0.03), #4 (157532: 0.2 ± 0.08), #5 (130815: 0.16 ± 0.06), #6 (219903: 0.13 ± 0.04), #7 (10486: 0.16 ± 0.07), #8 (57144: 0.12 ± 0.04), #9 (13103: 0.19 ± 0.04), #10 (92959: 0.23 ± 0.07). Statistical analysis was performed against untreated. (\(P < 0.01\)).

**Fig. 2.** Correlation analysis of OLIG2 dimerization against IC50 tumorigenic cell viability. Linear regression analysis (red dashed line) gave 0.62 Pearson’s correlation coefficient based on average values, indicating strong correlation. Blue dashed line shows the average RCA value in untreated cells. Average ± SD in RCA value for negative control (NC) (0.05 ± 0.04), untreated (UN) (0.28 ± 0.07), 1 \(\mu\)M compound #1 (129407: 0.14 ± 0.04), #2 (691240: 0.11 ± 0.06), #3 (50467: 0.08 ± 0.03), #4 (157532: 0.2 ± 0.08), #5 (130815: 0.16 ± 0.06), #6 (219903: 0.13 ± 0.04), #7 (10486: 0.16 ± 0.07), #8 (57144: 0.12 ± 0.04), #9 (13103: 0.19 ± 0.04), #10 (92959: 0.24 ± 0.09). Statistical analysis was performed against untreated.

The DNA-binding fraction decreases by compound treatment, due to OLIG2 dimer dissociation.

**Discussion**

The field of protein–protein interactions (PPIs) is still a largely uncharted territory for drug design. It has been estimated that the interactome is composed of several hundred thousand PPIs, yet, only a small fraction has still been explored therapeutically.

**Fig. 3.** Dose-response of active compounds. The three most active compounds, which inhibited OLIG2 dimerization effectively were selected for dose-response on dimerization in HEK cells expressing OLIG2-eGFP and OLIG2-Tomato. All compounds showed dose-dependent inhibitory effect for OLIG2 dimerization. Average ± SD in RCA value for #2 (691240) (Left), #3 (50467) (Center), and #8 (57144) (Right). Red solid line denotes curve fitting for determining IC50 value in each compound.
Studies in silico has defined potential small molecular weight drug candidates (20). Experimentally, in vitro assays are cumbersome since they require significant amounts of pure protein and are not suitable where interactions lead to conformational change in signal transduction (21). Live cell assays with fluorescence-based methodology have been introduced as fluorescence lifetime imaging microscopy (FLIM)-Förster resonance energy transfer (FRET) (22) but FRET is limited to cases where distance between interacting molecules is short and spatially aligned (23). FCCS does not show any of these limitations.

The malignancy of gliomas is a challenge for new drug design. OLIG2 is an obvious target and an intriguing possibility is to introduce inhibition of its dimerization—a crucial step for DNA binding. It is generally known that experimental inhibition of protein–protein interaction is difficult due to commonly wide interaction areas and absence of grooves or peaks that give specificity. By molecular modeling it was possible to approximate areas of interaction (in absence of crystal structures) and using computational pharmacophore-based design to predict the compounds that would be possible inhibitors of dimerization leading to antitumor activity. A graphical approach was used in the simulation (Fig. 4). The approach led to selection of molecular candidates, some of which were active in cultures of cells expressing OLIG2. The present data indicate that indeed, cross-correlation analysis revealed inhibition of dimerization. Moreover, the FCCS data correlated with tumor growth inhibition (Fig. 2). The data also suggest that the cross-correlation analysis in live cells could be used to guide development of substances with known mechanism of action in the glioma family of tumors.

Given the medical need, there have been previous attempts to develop inhibition of OLIG2 activity. Peptides “stapled” developed to match the dimer interaction surface were found inactive, possibly due to limited access to the cell interior (24). A recent compound CT-179 has been reported active in a bioassay (25), but its mechanism of action is unclear. In more general terms, the data suggest a general approach of drug discovery based upon the design of allosteric modulators of PPI.

Materials and Methods

Chemicals and Expression Vector Constructs. The selection of test compounds (SI Appendix, Fig. S4) was based on in silico modeling with a pharmacophore-based computational strategy. A schematic of the docking of compound 3 (50467) and two OLIG2 molecules is shown in Fig. 4.

The FFT vector constructs were eGFP-fused (OLIG2-eGFP) or Tomato-fused (OLIG2-Tomato). The OLIG2 expression plasmid, pGEM-OLIG2 was kindly provided by Koichi Tabu, Tokyo Medical and Dental University, Tokyo, Japan (26). The OLIG2 region was amplified with PCR using forward primers with XhoI (eGFP) or KpnI (Tomato) restriction sites, and reverse primers with AgeI restriction site using 2× Phusion Master Mix with GC Buffer (Thermo Fisher Scientific). The N1 vectors encoding eGFP or Tomato and PCR-amplified fragments of OLIG2 were digested with XhoI-AgeI or KpnI-AgeI concurrently. The linear N1 vector of eGFP or Tomato and digested OLIG2 fragment were ligated by Instant Sticky-end Ligase Master Mix (NEB).

Cell Culture and Transient Transfection. HEK cells were purchased from ATCC and were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C in Dulbecco’s Modified Eagle Medium (Gibco Life Technologies) supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin (Gibco); final concentration 100 U/mL penicillin and 100 μg/mL streptomycin.

One day before the transfection, HEK cells were split into Lab-Tek 8-well Chambered Coverglass (Thermo Fisher Scientific) with 1.0 × 10⁶ cells/mL in each chamber. HEK cells on an 8-well chamber were transfected with 100 ng of total plasmid DNA (50 ng of OLIG2-eGFP and OLIG2-Tomato; 50 ng of peGFP-N1 and pTomato-N1 as negative controls) and 0.2 μL of Lipofectamine 2000 (Thermo Fisher Scientific). After the transfection, HEK cells were cultured for 24 h. Analysis of a test sample is shown in SI Appendix, Fig. S1.

The test compounds were diluted with phenol-free medium, FluoroBrite DMEM (Gibco), for treatment of transfected HEK cells for 1 h at 37 °C. The analysis of a test sample is shown in SI Appendix, Fig. S1.

Laser Scanning Microscopy Imaging and Cellular FCCS Measurements. Laser scanning microscopy (LSM) imaging and FCCS measurements were performed using an LSM510 META-Confocor3 (Carl Zeiss) equipped with a 488-nm Ar-ion laser, 543 nm He-Ne laser, and a water immersion objective (C-Apochromat, 40×, 1.2 N.A., Corr, Carl Zeiss), and avalanche photodiode detectors (APDs). eGFP and Tomato were excited using the 488-nm laser and 543-nm laser, respectively. The pinhole size was adjusted to 80 μm. The fluorescence of eGFP and Tomato was split by NFT 545. The fluorescence signal of eGFP and Tomato passed through BPS05-530 (eGFP) and BPE15-680 (Tomato) filter, respectively. FCCS measurements, over the cell nucleus, were carried out 10 times for a duration of 20 s each.

Software and Statistical Analysis. Statistical analysis was performed by 2-sided t test on Microsoft Excel. P < 0.01 was considered to be statistically significant. Linear regression analysis and dose–response curve fitting were performed using OriginPro 2018. The theoretical curve for fast component of diffusion was drawn by parameters for actual measurement except for fractional percentage of the second component (fraction 2 set to 0) on OriginPro 2018.

Data Availability. Raw data used to generate the figures are available from the corresponding author, L.T., upon request.

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