DOI: 10.1002/anie.201004437

Hybrid Drugs

Combining Independent Drug Classes into Superior, Synergistically Acting Hybrid Molecules**

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Increasing the potency of synthesized drugs has been a stepwise process accomplished by progressively modifying the chemical scaffold of a single parent lead compound. To date, there has been no basis for thinking that the combination of pharmacological effects of independently acting drugs could be achieved beyond mere simultaneous administration. We reasoned that if the target molecule of two independent classes of drugs was the same, chemical synthesis of a hybrid compound where these drugs presented moieties within one molecule might yield synergistic effects; that is, a new quality might emerge that would be more than the sum of the single-moiety compounds. Such multifunctional hybrid compounds that assign different functions to its different moieties to achieve a synergistic pharmacodynamic effect have successful predecessors in nature: for example, bleomycin is a natural compound with three different moieties acting in concert to cleave DNA.[1]

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[**] The major support for this work was from a grant of the Volkswagen-Stiftung to C.K., T.S., D.W., and H.S. (I/82 649); further funding was provided by EU-FP6 (cNEUPRO), EU-FP7 (PRIORITY) grants to C.K. and a DFG-GRK1033 grant to J.K. and C.K.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004437.

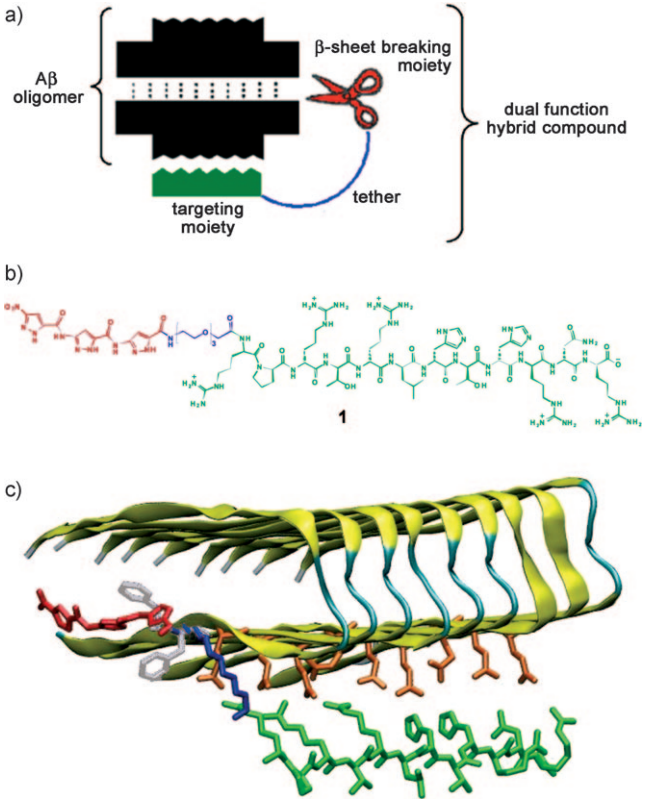

Figure 1. Design, function, and modeling of hybrid compounds. a) Hybrid compound, composed of a recognition moiety docking on the target (here: Aβ oligomers) linked by a flexible tether to the functional moiety, a β-sheet breaker. b) Chemical structure of lead hybrid compound Trimer-TEG-D3 (**1**). c) Model of the Aβ/**1** complex. The side chains of the glutamates (orange) in adjacent Aβ monomers (yellow) are located in close spatial proximity and are contacted by the basic residues of the D3 peptide (green). The aminopyrazole trimer (red) is bound to the Aβ backbone and interacts with the two phenylalanines (F19, F20; gray). The color coding of the individual parts of **1** is according to (a) and (b).

Herein we describe the design and chemical synthesis of hybrid compounds that target the disassembly of neurotoxic Aβ oligomers. Cross-β-sheeted Aβ oligomers or amyloids are a hallmark of Alzheimer's disease (AD). Misprocessing of an amyloidogenic fragment Aβ derived from proteolytic processing of amyloid precursor protein is critical in a cascade of events that starts with the oligomerization of Aβ and, ultimately, ends in neuronal death in the central nervous system.[2] To date, rationally designed small-molecule β-sheet breakers, although highly efficient in preventing or disassembling β-sheet structures in cell-free in vitro systems, have failed to show convincing effects in vivo owing to their highly unspecific binding.

We reasoned that lack of in vivo efficiency of small β-sheet breakers might be overcome by the addition of a molecular recognition unit that would direct the β-sheet breaking moiety to its target molecule. Molecular recognition is a key property of polypeptides that can be identified in evolutionary algorithms, for example in phage display systems comprising iterative cycles of panning and selection of peptides. We identified D3, a D-enantiomeric dodecapeptide, as a potent Aβ oligomer binder in a mirror-image phage display,[3,4] that is able to modulate Aβ aggregation, plaque load, and neuroinflammation processes in the brains of transgenic (APPswe/PS1ΔE9) mice.[3,5]

In our hybrid compound (Figure 1), we would thus combine two entirely different drug development strategies: evolutionary selection from a peptide library together with rationally designed small molecules. As rationally designed β-sheet breakers, we chose aminopyrazoles (APs) that possess a specific donor–acceptor–donor (DAD) sequence of hydrogen bond donors and acceptors, that are perfectly complementary to that of a β sheet,[6] binding selectively to the backbone of misfolded peptides residing in the β-sheet conformation,[7] and able to disassemble preformed Aβ fibrils in vitro.[8]

Comparative modeling and molecular dynamics simulations were used to investigate the interaction of Aβ[9] of AP linked to D3 with different linkers and to a pentalysine peptide (KKKKKG) as charge control for D3. The AP moiety of **1** was modeled to interact with the diaromatic motif F19/F20, and the D3 part was placed in spatial vicinity of a negatively charged surface patch of the Aβ protofibril to maximize electrostatic complementarity (see the Supporting Information, Figure S1). These simulations revealed that after 10 ns, the multiple interactions formed between D3 peptide and the E22 residues remained stable (Figure 1c; Supporting Information, Figure S2) whilst only one salt bridge was preserved between the pentalysine moiety and the Aβ fibril (Supporting Information, Figure S2D), thus providing an explanation for the enhanced activity of D3 compared to other substances (Figure 2a; Supporting Information, Figure S4B).

When added to cells that continuously secrete Aβ oligomer (7A2 cells[10]), the hybrid compound with the triethylen-

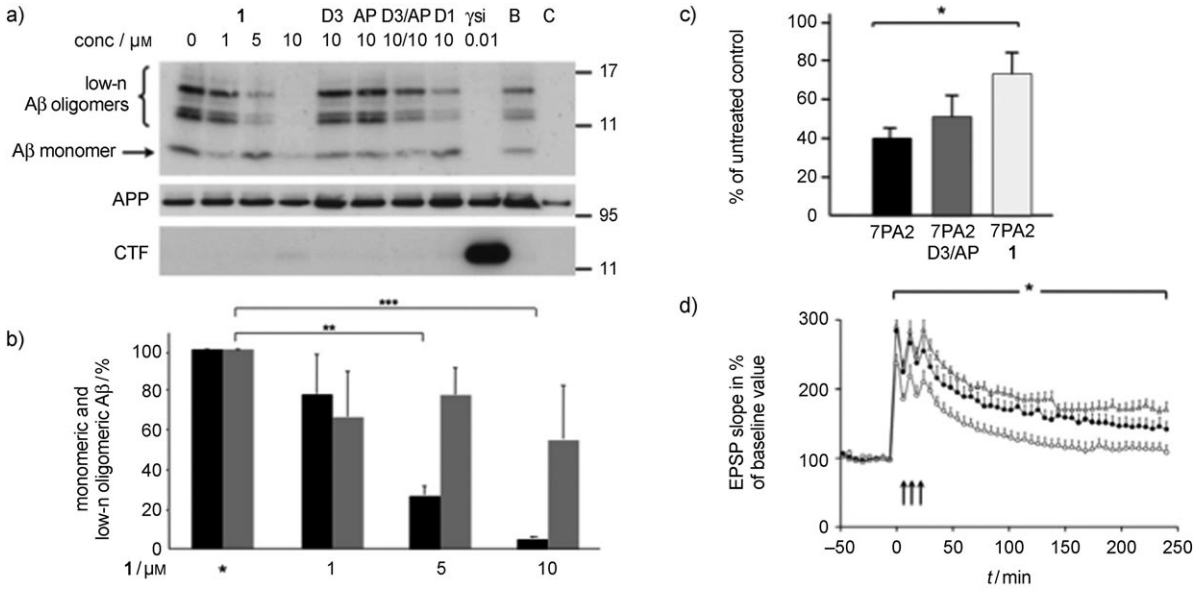

Figure 2. Biochemical and biological effects of hybrid compounds. a) Western blot of immunoprecipitated Aβ oligomers and monomers from 7PA2 cells that were treated with different substances. Addition of **1** led to a dose-dependent reduction of Aβ oligomers. Treatment with the D-peptide D3 or the aminopyrazole (AP) moiety alone, or as simultaneously applied substances (D3/AP) at 10 μM, did not show any Aβ oligomer reducing effects in this assay. Further control experiments included a hybrid compound consisting of another D-peptide (10 μM; D1), as well as γ-secretase inhibitor LY411575 (10 nM), 7PA2 cells treated with AP-dissolving substance DMSO at the same concentrations (B), and untransfected CHO cells not secreting Aβ oligomers (C). Expression of APP and APP-C-terminal fragments (CTF) as a control is shown in the lower panels, as indicated. b) Comparison of 5 independent experiments of 7PA2 cells treated with **1** at 1, 5, and 10 μM, showing clear effects on oligomeric Aβ (*** $p = 0.0007$, ** $p = 0.0082$), whereas monomeric Aβ was not significantly affected (oligomeric Aβ black bars, monomeric Aβ gray bars). c) 10 μM **1**, but not a composition of both single compounds at 10 μM each, reversed the effect of 7PA2 supernatant on mEPSC frequency in cultured cortical mouse neurons. Significant reversal of mEPSC suppression (* $p < 0.025$ by Student's test after Bonferroni correction). Bars represent mean ± SEM (see Supporting Information for details). d) Compound **1** prevented the Aβ-mediated decrease of LTP in acutely isolated hippocampal slices. Compared to control LTP (●), oligomeric Aβ (1–42) significantly reduced LTP (○), which was prevented by co-application of 10 μM **1** (△) * $p < 0.05$ analysis of variance with repeated measures; significance between control and Aβ and between Aβ and **1**/Aβ.

glycol (TEG) linker, termed Trimer-TEG-D3 (**1**), inhibited Aβ oligomerization in a dose-dependent manner (Figure 2a,b). Remarkably, single compounds or the combination of both single-compound moieties administered at same concentrations did not produce any effect in this assay, thus demonstrating the dramatic synergistic action of both moieties within the composite compound that in effect leads to a new function (Figure 2a). Upon administration of **1**, only the decrease of the Aβ oligomer fraction was significant compared to the Aβ monomer fraction, and no increase of C-terminal APP fragments was observed, suggesting that **1** neither decreased Aβ monomer generation nor inhibited γ secretase (Figure 2a). Even very high concentrations of each single compound or their combination did not lead to a reduction in Aβ oligomers (Supporting Information, Figure S3), and **1** revealed cytotoxicty only at concentrations of more than 100 μM (Supporting Information, Figure S4).

To demonstrate the specificity of the D3 moiety in targeting Aβ oligomers, a hybrid with a D-peptide that recognizes a different epitope of Aβ, termed D1, was also tested.[4] With this compound, only an insignificantly weak inhibition of Aβ oligomerization was observed (Figure 2a). Moreover, as predicted from molecular modeling, the AP–pentalysine hybrid had no effect on Aβ oligomer assembly (Supporting Information, Figure S5B).

Our concept of synergistic hybrid compounds predicts a critical importance of linker length between moieties. Structural and energetic considerations suggest that a shorter linker should favor a tight interaction owing to the smaller entropic loss upon binding, whereas longer linkers should be unfavorable. When we synthesized hybrids with no spacer, a shorter γ-aminobutyric acid (GABA) spacer, or a longer $(TEG)_2$ spacer (Supporting Information, Figure S5A), we verified this relation in that only no spacer or a five-atom linker (GABA) showed markedly increased Aβ oligomer inhibition (Supporting Information, Figure S5B,C).

Synaptic pathology is a key biological effect of Aβ oligomers, which should be reversed by **1**. Compound **1** blocked the Aβ-induced decrease in mEPSC frequency (mEPSC = miniature excitatory post-synaptic current) that is mediated by AMPA receptors[11] in cultured cortical neurons (Figure 2c), but it did not affect the mean mEPSC amplitude. The effect was achieved with 10 μM **1** but not with the single compounds or a combination of both single compounds (Figure 2c). Similarly, Aβ-oligomer-induced impairment of long-term potentiation (LTP),[12] a form of synaptic plasticity in acute hippocampal slices, could be reversed by coapplication of 10 μM **1** (Figure 2d). These results demonstrated that **1** prevented Aβ-induced synaptotoxicity in two independent assays.

Multimer growth to amyloid fibrils should be inhibited by increased binding of the two moieties of **1** to Aβ oligomers. Analytical ultracentrifugation of synthetic Aβ in vitro[13] showed an equal distribution across all multimeric fractions (Figure 3a, upper panel). When preincubated with **1**, Aβ partitioned to high-molecular-weight fractions at the expense of oligomeric fractions (Figure 3a, middle panel), as evidence that direct binding of **1** to Aβ oligomers influenced Aβ oligomer assembly. As Aβ thioflavin T (ThT) fluorescence

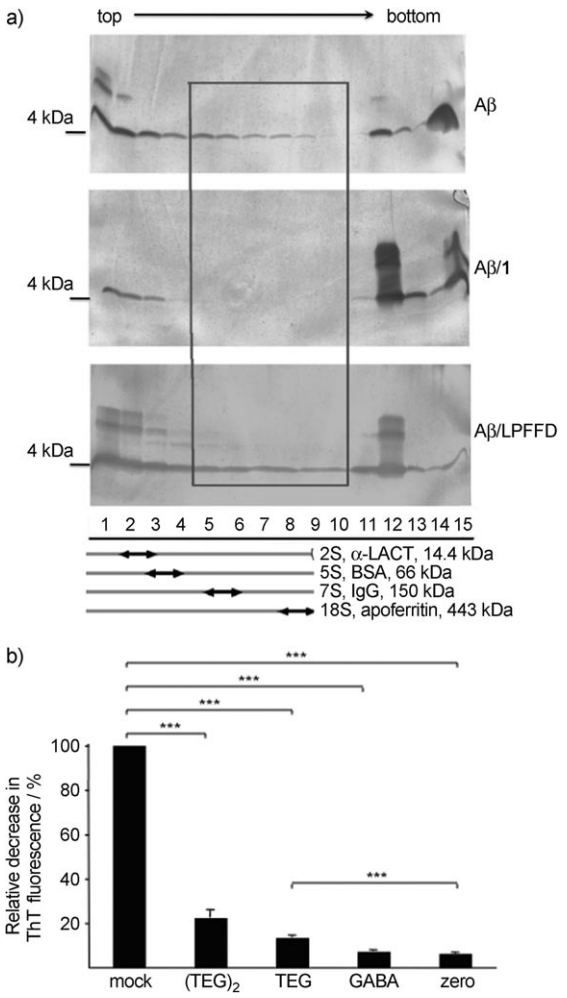

Figure 3. Biophysical characterization of the effect of hybrid compounds on Aβ assembly. a) Analysis of the effect of **1** on Aβ aggregation by analytical density gradient ultracentrifugation analyzed by SDS-PAGE and silver staining. Continuous fractions from light (left) to heavy (right) were loaded: monomeric Aβ in the leftmost fractions (lane 1), oligomeric Aβ in the fractions in the red box (lanes 5–10), and multimeric aggregated Aβ in fractions 12 and higher. Compared to untreated Aβ (125 μM; top panel), or control-treated Aβ (bottom panel), compound **1** (62.5 μM; middle panel) led to a shift of oligomeric fractions to high-molecular-weight, non-amyloid complexes. Calibration of fractions for sedimentation coefficients *S* (black double arrows) with α-lactalbumin (α-LACT), bovine serum albumin (BSA), immunoglobin G (IgG), and apoferritin. b) Inhibition of ThT-positive Aβ fibrillogenesis by **1** (TEG) and hybrid compounds with shorter (GABA) or longer ($(TEG)_2$) linker length or without linker (zero). (mean ± standard deviations of results, four separate runs, three replicates per run; *** $p \leq 0.001$ by Student's t-test). Clear anti-amyloid effects of all hybrid compounds are observed, with the zero linker being strongest.

(an indicator of amyloid fibril content) decreased upon application of **1** (Figure 3b), these higher fractions could not represent Aβ amyloid fibrils but rather irregularly structured high-molecular-weight complexes. Accordingly, transmission electron microscopy revealed the total lack of any remaining fibrillar structures in Aβ/**1** mixtures (Supporting Information, Figure S6), thus demonstrating that **1** inhibited Aβ oligomer assembly by driving Aβ aggregation

to nontoxic, non-amyloid complexes. As for native Aβ oligomer assembly inhibition in 7A2 cells (Supporting Information, Figure S5C), we also observed a structure–activity relationship of hybrid compounds in the cell-free ThT assay (Figure 3b), confirming that the zero-linker compound has strongest activity. Our biophysical analysis by three independent methods (density gradient centrifugation, ThT assay, and transmission electron microscopy), as well as our findings that monomeric Aβ is not elevated when Aβ oligomers are decreased (Figure 2b, 3a), lead us to conclude that rather than inhibiting Aβ oligomer assembly, hybrid compounds lead to incorrect, bioinactive, and non-amyloid Aβ misassembly, which could eventually be degraded more easily.

In conclusion, we demonstrated that chemical synthesis of two entirely different substance classes acting on the same target can be covalently linked to yield dramatic synergistic effects and lead to novel properties. Taking Aβ oligomers as an example, we also showed that two entirely different principles of drug development can be combined: polypeptides developed through mirror image phage display, which takes advantage of evolutionary algorithms to select for molecular recognition, and the rationally designed small-molecule β-sheet breakers. Our investigations may spark similar efforts with different target molecules and thereby greatly accelerate drug development.

Received: July 20, 2010
Published online: October 4, 2010

Keywords: aggregation · amyloid β peptides · drug design · molecular recognition · peptides

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