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Intracellular Kinase Inhibitors Selected from Combinatorial Libraries of Designed Ankyrin Repeat Proteins*[S]

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The specific intracellular inhibition of protein activity at the protein level allows the determination of protein function in the cellular context. We demonstrate here the use of designed ankyrin repeat proteins as tailor-made intracellular kinase inhibitors. The target was aminoglycoside phosphotransferase (3′)-IIIa (APH), which mediates resistance to aminoglycoside antibiotics in pathogenic bacteria and shares structural homology with eukaryotic protein kinases. Combining a selection and screening approach, we isolated 198 potential APH inhibitors from highly diverse combinatorial libraries of designed ankyrin repeat proteins. A detailed analysis of several inhibitors revealed that they bind APH with high specificity and with affinities down to the subnanomolar range. *In vitro*, the most potent inhibitors showed complete enzyme inhibition, and *in vivo*, a phenotype comparable with the gene knockout was observed, fully restoring antibiotic sensitivity in resistant bacteria. These results underline the great potential of designed ankyrin repeat proteins for modulation of intracellular protein function.

In the postgenomic era, the functional analysis of the proteins encoded in the genomes is the rate-limiting step in understanding cellular processes. Most approaches in use today do not target the protein of interest directly but prevent its synthesis, either by total gene knockout (*e.g.* by homologous recombination), by antisense technologies, or by RNA interference (RNAi)[1] (1, 2). Although these technologies have generated remarkable insights into some intracellular protein functions, they suffer from a number of limitations. Total gene knockouts are often difficult to achieve and might even be lethal to the studied organism. Furthermore, the effects can be compromised by compensating changes in the regulation of other genes. The effect of antisense technologies is often not very strong. In a number of cases, even an 80% reduction of mRNA has no effect on the phenotype caused by the protein (3). Furthermore, the interpretation of results obtained from RNAi can be complicated by the up-regulation of other genes, limitation in specificity, and induction of the interferon response (4, 5). Finally, all methods that prevent the synthesis of the entire protein only allow the description of a loss-of-function phenotype.

The use of molecules acting at the protein level is a complementary approach. This approach has the additional advantage that single functions, domains or posttranslational modifications of a protein may be targeted, without removing the whole protein. Inhibitors of proteins can also be used in subsequent studies of the target protein *in vitro* and might then even be used as first lead compounds in the drug discovery process or at least simulate the effect of a future small molecule drug. Both constrained peptides (6–9) and single-chain antibody fragments (scFv (10)) have been applied for *in vivo* protein inhibition. In most cases, it is rather difficult to obtain high-affinity peptides to a target protein due to their high flexibility and the resulting loss of peptide chain entropy upon binding, limiting the free energy of binding, and thus the generation of high-affinity antibody fragments to a given target has been pursued more often (11). However, the drawback of antibodies is that their functionality is compromised in the reducing environment inside the cell, since the stabilizing intramolecular disulfide bonds are not formed (12). Whereas a number of antibody frameworks have been reported that are stable in the reducing environment (10, 13–15), the stability in the reducing state still does not reach that of other proteins. The solution to this problem is to use alternative binding proteins without disulfide bonds. Such alternative scaffolds have been developed (16–18), but only one intracellular selection for binding molecules with the yeast two-hybrid system has been published (19); no intracellular inhibitors have been reported to date.

We previously reported the generation of designed ankyrin repeat (AR) proteins as high-affinity binders with a great potential for intracellular applications (20, 21). Next to antibodies, repeat proteins are the most abundant class of binding molecules found in nature (22–24). They often act as intracellular enzyme modulators (*e.g.* as inhibitors of kinases) (25). Unlike antibodies, AR proteins do not contain disulfide bonds and are found in all cellular compartments, mediating protein-protein interactions with affinities in the nanomolar to picomolar range. AR proteins are built of consecutive homologous repeats, which assemble to form elongated domains with a continuous hydrophobic core. The number of repeats found in natural AR proteins varies from 2 to over 30 (26, 27). We used

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[1] The abbreviations used are: RNAi, RNA interference; AR, ankyrin repeat; EPK, eukaryotic protein kinase; APH, aminoglycoside phosphotransferase (3′)-IIIa; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MBP, maltose-binding protein; IMAC, immobilized metal-ion affinity chromatography; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; MIC, minimal inhibitory concentration.

this modular architecture to generate designed AR protein libraries. By structure and sequence consensus analyses, we engineered an AR module of 33 amino acids with fixed framework residues and randomized potential interaction residues (28). This module was used as building block to create combinatorial libraries of designed AR proteins of different repeat numbers (Fig. 1). Members of these libraries are very well expressed, monomeric in solution, highly soluble, and stable (28). By x-ray crystallography, the expected AR domain fold could be verified (29). Specific binders to maltose-binding protein (MBP) of *Escherichia coli* and two human mitogen-activated protein kinases (JNK2 and p38) have been selected with dissociation constants in the low nanomolar range (20).

Here, we report the first tailor-made enzyme inhibitors based on designed AR proteins. Using a combination of *in vitro* selection and *in vivo* screening, we isolated designed AR proteins that specifically bind and inhibit aminoglycoside phosphotransferase type (3′)-IIIa (APH). APH is a bacterial kinase conferring resistance to aminoglycoside antibiotics (30) and can serve as a model system, since it shows structural homology to eukaryotic protein kinases (EPKs). We show that the selected AR proteins inhibit APH both *in vitro* and *in vivo*. Based on these results, the use of designed AR proteins as specific kinase inhibitors and as general intracellularly active tools is discussed. We describe elsewhere (31) the crystal structure of one selected APH-inhibiting AR protein in complex with APH and report the details of the inhibition mechanism of this particular inhibitor.

MATERIALS AND METHODS

Molecular Biology—Unless stated otherwise, all experiments were performed according to the protocols of Ref. 32. Enzymes and buffers were from New England Biolabs (Beverly, MA) or Fermentas (Vilnius, Lithuania). Oligonucleotides were from Microsynth (Balgach, Switzerland).

Antigen Cloning and Production—The APH gene was amplified from the plasmid pETSACG1 (a kind gift from Prof. G. Wright (33, 34) with the oligonucleotides APHf (5′-GCCGGCCATGGGTACCGCTAAAATGAGAATA-3′) and APHr (5′-GCATAAGCTTTCATTAAAACAATTCATCCA-3′). The PCR product was purified (QIAquick; QIAgen, Hilden, Germany), cut with NcoI/HindIII, and ligated into pAT223 (GenBank™ accession number AY327138), which is a pQE30 (QIAgen, Hilden, Germany) derivative, yielding pAT223_APH. The expression from this vector yields a fusion protein with an N-terminal avi tag for *in vivo* biotinylation, phage λ protein D (pD) as fusion protein, a His_6 tag for immobilized metal ion affinity chromatography (IMAC) purification, and APH as target molecule (avi-pD-His_6-APH).

Protein Production and Purification—The biotinylated proteins avi-pD-His_6-APH and avi-pD-His_6 were produced according to protocols from Avidity (Denver, CO) and QIAgen by *in vivo* biotinylation in *E. coli* XL1-Blue (Stratagene, La Jolla, CA), which was co-transformed with pBirAcm (Avidity) and pAT223 or pAT223_APH, respectively. Successful biotinylation of the IMAC purified proteins was confirmed by detection of the biotin tag on immobilized proteins using ELISA and blotting procedures with a streptavidin-alkaline phosphatase conjugate (Roche Applied Science) as detection reagent.

Nonbiotinylated wild-type APH used for the *in vitro* kinetic assays was expressed from pETSACG1 (33, 34) in BL21 cells (Stratagene) and purified over a Q-Sepharose column (Amersham Biosciences), followed by gel filtration (Superdex 75; Amersham Biosciences) as described (35) with some modifications (for details, see Supplemental Data). The correct size of APH was confirmed by SDS-PAGE, mass spectrometry, and multi-angle static light scattering.

For protein production of the AR proteins, the corresponding genes were isolated from pQIA (see below) and inserted into pQE30 to avoid co-purification of APH at a later stage. The proteins were expressed and purified by IMAC as described (29).

In Vitro Selection with Ribosome Display—The PCR-amplified N2C and N3C AR protein libraries (20) were transcribed, and selections were performed by ribosome display as described (36) on avi-pD-His_6-APH immobilized over its biotinylated avi tag (for details, see Supplemental Data). The number of total reverse transcription (RT)-PCR cycles after each selection round was reduced from round to round from 40 to 35 to 30, adjusting to the yield due to enrichment of binders.

In Vivo Selection by Replica Plating—For the *in vivo* selection, the vector pQIA was constructed with β-lactamase and APH dicistronically arranged under the control of the β-lactamase promoter, as well as an expression cassette for AR proteins under the control of an IPTG-inducible T5-promoter (Fig. 3*A*). For details, see Supplemental Data. The pools of binders selected by ribosome display were cloned into pQIA using the restriction enzymes BamHI and HindIII, transformed into *E. coli* (XL1-Blue) and plated on LB agar plates, containing 1% glucose, 50 μg/ml ampicillin, and 100 μM IPTG, and incubated overnight at 37 °C. The colonies were replica-plated on selective plates containing 1% glucose, 50 μg/ml ampicillin, 500 μg/ml kanamycin A, and 100 μM IPTG using Accutran replica plater devices (Schleicher & Schuell), incubated for 2 h at 4 °C and finally incubated overnight at 37 °C. Colonies whose replica failed to grow on the selective plates were taken up in LB and replica-spotted again on selective plates to make sure no false positives were selected due to incomplete replica plating. By this procedure, 198 kanamycin A-sensitive colonies were identified. All of these clones were ranked for their kanamycin A sensitivity by spotting them on selection plates with kanamycin A at concentrations of 50, 100, 250, and 500 μg/ml. The plasmids of the most potent inhibitors were isolated and sequenced using standard DNA sequencing.

Determining the Minimal Inhibitory Concentrations (MICs)—Cultures of *E. coli* (XL1-Blue) harboring pQIA, encoding an AR protein, were grown in LB with 1% glucose and 50 μg/ml ampicillin (LB-Glc-ampicillin) at 37 °C overnight. The cultures were diluted 1:30 into 5 ml of the same medium and shaken at 37 °C until an A_{600} of 0.3 was reached. The cultures were then diluted 1:1000 (~2.5×10^5 colony-forming units/ml) into deep well plates (Abgene, Epsom, Surrey, UK) containing 1 ml of the different selection media: LB-Glc-ampicillin-IPTG (100 μM) plus kanamycin A (0, 1, 5, 10, 25, 50, 100, 150, 250, 500 or 1000 μg/ml) and as a control LB-Glc-ampicillin with 1000 μg/ml kanamycin A (without IPTG) and shaken at 37 °C for 24 h. Growth was monitored by following the A_{600}. MIC end points were determined to be the concentrations of aminoglycoside antibiotic where no visible growth was obtained.

Analytical Size Exclusion Chromatography—Gel filtration experiments were done on a SMART system (Amersham Biosciences) using a Superdex 75 PC 3.2/30 column or a Superdex 200 PC 3.2/30 column (Amersham Biosciences) at 20 or 37 °C. The void volume was experimentally determined by using blue dextran. Samples of AR_1, AR_3a, and AR_B (20 μl) were loaded at a concentration of 25 μM on the Superdex 200 column and were run at 60 μl/min in TBS_{150}, pH 7.5 (50 mM Tris HCl pH 7.5, 150 mM NaCl), at 20 °C. Samples of APH and APH in complex with the AR protein AR_3a (20 μl) were loaded at a concentration of 10 μM on the Superdex 75 column and were run at 60 μl/min in $HEPES_{200}$ (50 mM HEPES, pH 7.5, 100 mM NaCl, 40 mM KCl, 60 mM $MgCl_2$) at 37 °C. Cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and β-amylase (200 kDa) were used as molecular mass standards.

ELISA—To determine the specificity of the interaction between the selected AR proteins and APH, ELISA experiments were performed as described (13). Biotinylated proteins (avi-pD-His_6-APH, avi-pD-His_6, avi-pD-JNK2-His_6, and avi-pD-p38-His_6, final concentration 100 nM) were immobilized in neutravidin-coated ELISA plates blocked with bovine serum albumin (BSA). AR proteins (200 nM) were added for 30 min, and subsequently, the plates were washed five times. A first antibody against the RGS-His_6 tag of the AR protein (QIAgen; detects only the RGS-His_6 tag of the AR protein but not the His_6 tag of the antigen), followed by a secondary antibody/alkaline phosphatase conjugate (Pierce), was used for detection. After the addition of substrate (*p*-nitrophenylphosphate; Fluka) and incubation at 37 °C for 45 min, the A_{405} was measured in a plate reader. All incubation steps, including the antibody or AR protein incubations, were carried out in TBS_{150} with 0.5% BSA and 0.05% Tween 20, whereas washing was done with TBS_{150} containing only Tween 20.

Surface Plasmon Resonance—SPR was measured using a BIAcore3000 instrument (BIAcore, Uppsala, Sweden) at 20 °C. The running buffer was 20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.005% Tween 20. An SA chip (BIAcore) was used with 600 RU biotinylated avi-pD-His_6-APH immobilized. The interactions were measured at a flow of 60 μl/min with 5-min buffer flow, 2-min injection of APH-binding AR protein in varying concentrations (1–200 nM), and an off-rate measurement of 40 min with buffer flow. The signal of an uncoated reference cell was subtracted from the measurements. The kinetic data of the interaction were evaluated using BIAevaluation 3.0 (BIAcore), and global fits were used to determine K_D. The affinities of clones AR_3a and AR_3b were confirmed by competition BIAcore (Supplemental Data).

In Vitro Inhibition Studies—APH activity assays were performed essentially as described (35) with small modifications to facilitate inhibition studies. Briefly, the kinase buffer we used (50 mM HEPES, pH 7.5, 160 mM NaCl, 40 mM KCl, 10 mM $MgCl_2$, 0.025% BSA, 5 units of pyruvate kinase/lactate dehydrogenase, 2.5 mM phosphoenolpyruvate, 0.12 mg/ml NADH, 1 mM ATP) contains HEPES instead of Tris and additionally 160 mM NaCl, 0.025% BSA, and a lower concentration of NADH (0.12 instead of 0.5 mg/ml). APH was present at a concentration of 200 nM, whereas the AR proteins were present at 10 μM for the kanamycin A studies. This mixture (990 μl) was preincubated for 15 min, and the assay was initiated by the addition of 10 μl of kanamycin A solution (K4000; 10 μM final concentration; Sigma). Both the preincubation and the assay were carried out at 37 °C. For the studies with amikacin, both the preincubation and the assay were carried out at 20 °C in the presence of 2 μM AR proteins, and the reaction was started with 10 μl of amikacin solution (A1774; 740 μM final concentration; Sigma). The initial rates were normalized for background activity by subtracting the activity monitored in the absence of substrate.

RESULTS

To identify APH inhibitors, we applied a two-step approach of *in vitro* selection and *in vivo* screening. First, we used ribosome display to select APH binders from highly diverse combinatorial AR protein libraries. The resulting pools of binders were subsequently screened for inhibitors by replica plating. As APH mediates bacterial resistance to aminoglycoside antibiotics, inhibitors of the enzyme give rise to a kanamycin-sensitive phenotype.

Selection for APH Binders with Ribosome Display—The AR protein libraries used here (20) were termed N2C and N3C. The digit describes the number of designed and randomized AR modules between the N- and C-terminal capping repeats in the library members (Fig. 1). Three rounds of ribosome display selection were carried out for both the N2C and the N3C AR protein libraries in parallel. Selections were performed using purified target protein. For this purpose, APH was expressed as a fusion to bacteriophage λ pD (37) with an N-terminal avi tag for *in vivo* biotinylation (38) and a His_6 tag in the linker for IMAC purification (avi-pD-His_6-APH). This target protein and the same construct without the kinase (avi-pD-His_6) were expressed in soluble and biotinylated form in *E. coli* by using the expression plasmids pAT223_APH and pAT223, respectively. For ribosome-display selections, purified biotinylated APH was immobilized on neutravidin-coated wells blocked with BSA. To eliminate BSA-, neutravidin-, and avi-pD-His_6-binding library members, a prepanning step on correspondingly immobilized avi-pD-His_6 was applied, where neutravidin and BSA were also present. In the actual panning step, binders to APH were thus selected. The relative amounts of ribosomal complexes binding to the target protein can be estimated by monitoring the yield from RT-PCR on agarose gels. We observed a significant increase of RT-PCR yield when comparing the results from the first and the second selection round (Fig. 2). To our knowledge, such pronounced enrichment after only one round of ribosome display selection has not been reported for any kind of naive library (scFv, cDNA, or random sequence). This might indicate that AR protein libraries and ribosome display are a very favorable combination. In the third selection round, the selected pools of binders were analyzed for their specificity, comparing the RT-PCR yields after selection against APH or the unrelated MBP (Fig. 2). High specificity to APH could be monitored by comparing the agarose gel band intensities of these RT-PCR products (Fig. 2). In summary, we observed a rapid enrichment of binders and generated pools of specific binders in only three rounds of ribosome-display selection.

Screening for APH Inhibitors by Replica Plating—The pools of specific binders were subjected to an *in vivo* screen, based on replica plating, to identify those APH-binding AR proteins that are also inhibitors. For this purpose, the selected pools of binders were cloned into the selection vector pQIA (Fig. 3*A*). This vector carries an expression cassette for the selected APH binders under the control of an IPTG-inducible T5 promoter.

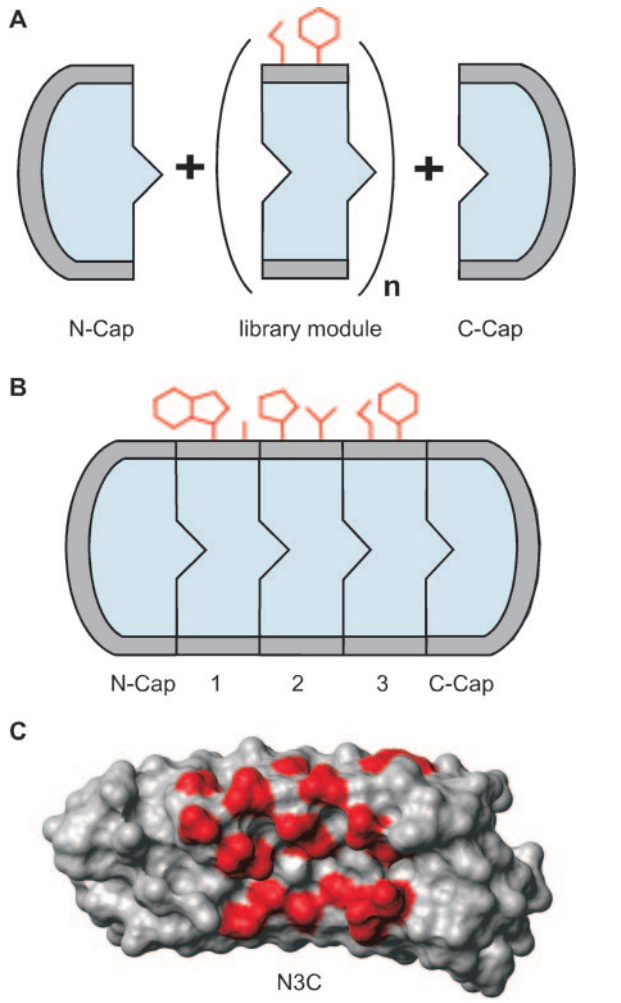

FIG. 1. **Designed AR protein libraries.** *A*, AR protein libraries are made from building blocks of designed self-compatible library modules with randomized positions (*red*), for potential target interaction and capping modules, which shield the hydrophobic core (*blue*). A varying number of library modules are genetically fused between an N- and a C-terminal capping repeat (*N-cap* and *C-cap*), yielding AR protein libraries of a specific size. *B*, an N3C library consists of proteins made from three random library modules, flanked by an N- and a C-terminal capping repeat. *C*, space-filling representation of an unselected N3C library member. The constant regions are *colored* in *gray*, and the library positions are depicted in *red*. The large potential interaction surface spans one side of the molecule (Protein Data Bank code 1MJ0) (29). For further details, see Refs. 21 and 28.

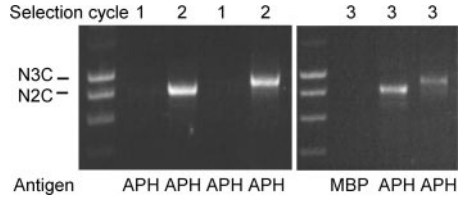

FIG. 2. **Enrichment and specificity of ribosome display selection rounds.** The outcome of ribosome-display selection rounds was monitored at the level of RT-PCR product yield by agarose gel electrophoresis. The RT-PCR yield of the first and the second selection round were compared for both the N2C and N3C library. In the third round, the specificities of the selected pools of the N2C and the N3C library were checked by panning against APH and an unrelated protein, MBP. All experiments were done in duplicates, which were combined for the agarose gel. For the specificity check of panning on the control protein MBP, both N2C and N3C duplicates were combined.

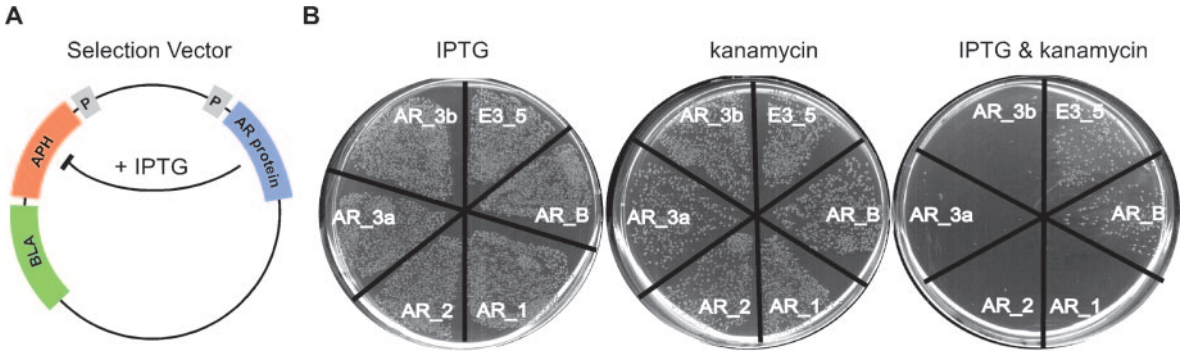

FIG. 3. **Phenotype of the selected clones.** *A*, the selection vector pQIA, carrying the β-lactamase (*BLA*) and APH gene for ampicillin and kanamycin resistance under a constitutive promoter in a dicistronic arrangement and the IPTG-inducible AR protein gene. Induction of the AR protein can result in inhibition of APH, if the AR protein has inhibitory properties, yielding a kanamycin-sensitive phenotype. *B*, the plating results of four APH inhibitors (AR_1, AR_2, AR_3a, AR_3b), one APH binder (AR_B), and an unrelated AR protein (E3_5) are shown. *E. coli* XL1-Blue cells were transformed with pQIA, carrying the respective genes in the AR protein cassette and plated under different conditions: nonselective in the case of only IPTG (100 μM), where the AR protein is expressed but no kanamycin A is present or if only kanamycin A is present (200 μg/ml) but the AR protein is not expressed; selective where the combination of IPTG and kanamycin A creates an environment where inhibitors repress growth.

pQIA also carries β-lactamase and APH as dicistronic genes under the control of the constitutively active β-lactamase promoter. Hence, this vector confers antibiotic resistance to ampicillin and kanamycin, of which the kanamycin resistance can be reverted, if an APH-inhibiting AR protein is expressed upon IPTG induction (Fig. 3*A*). As a control, we tested *E. coli* transformed with a pQIA variant harboring the unselected AR protein E3_5 (29) in the expression cassette. These cells were able to grow on plates with kanamycin A at concentrations of >1000 μg/ml, regardless of the presence of IPTG.

The pools of binders were cloned into pQIA and plated under nonselective conditions (ampicillin, IPTG, no kanamycin A). About 1000 colonies from the N3C pool and 2000 colonies from the N2C pool were subsequently replica-plated under selective conditions (ampicillin, IPTG, kanamycin A (500 μg/ml)). By this procedure, 129 N3C and 69 N2C clones were identified that showed a kanamycin-sensitive phenotype.

The identified clones were ranked for their *in vivo* inhibition efficiency by spotting them on selective plates with different kanamycin A concentrations, ranging from 10 to 500 μg/ml, and the most potent clones were sequenced. The sequencing revealed that variants of one potent inhibitor (AR_3 family) dominated the N3C inhibitor population, but also other sequences had been selected. Four different APH inhibitors (named AR_1, AR_2, AR_3a, and AR_3b), with different *in vivo* performances, were chosen for further characterization (for sequences see Supplemental Data). Whereas AR_1 was selected from the N2C library, AR_2, AR_3a, and AR_3b came from the N3C library. AR_3a and AR_3b differ by three point mutations at the DNA level, causing two amino acid mutations. We also studied the properties of clone AR_B, an N2C molecule that binds APH but fails to inhibit the target enzyme. As a further control, the unselected AR protein E3_5 (28, 29) was tested in parallel. The characterization of these AR proteins included expression tests, determination of minimal inhibitory concentrations (MICs), ELISA, BIAcore, and gel filtration studies as well as *in vitro* enzyme inhibition experiments.

In Vivo Performance (MIC)—The MICs of kanamycin A necessary to inhibit cell growth in liquid culture experiments were determined for the selected AR proteins. The plasmid pQIA with the nonbinding control AR protein E3_5 as insert mediated resistance to kanamycin A (MIC > 1000 μg/ml) regardless of the presence of IPTG, showing that the expression of an AR protein *per se* has neither an influence on kanamycin A resistance nor on bacterial growth. Clone AR_B showed the same phenotype as E3_5 (Fig. 3*B*). All AR proteins conferring kanamycin A sensitivity did not influence *E. coli* growth by themselves, even when induced, as long as no kanamycin A was present (Fig. 3*B*). In contrast, induction of the inhibitors stopped growth at different kanamycin A concentrations ranging from 10 to 150 μg/ml (Table I and Fig. 3*B*). Whereas clones AR_1 and AR_2 had MICs of 100 μg/ml and 150 μg/ml, respectively, clones AR_3a (25 μg/ml) and AR_3b (10 μg/ml) were more potent inhibitors (Table I). To put these results into perspective, we also evaluated the natural resistance of *E. coli* to kanamycin A in the absence of APH. The MIC of *E. coli* carrying a pQIA variant, where the APH gene had been removed, expressing AR_B or AR_3a was found to be 5 μg/ml each. Taken together, these results lead to the conclusion that the most potent clones AR_3a and AR_3b showed a phenotype comparable with the APH gene knockout.

Expression of the Selected AR Proteins—Since the selected AR proteins bind and inhibit APH inside the cell, we analyzed the expression of the selected clones in the selection vector pQIA under the expression conditions used for screening. Since the exact intracellular concentration of the AR proteins is difficult to determine, we performed a qualitative comparison of the selected clones by comparing the AR protein band intensities on Coomassie-stained SDS-PAGE. All AR proteins were expressed as soluble proteins regardless of the vector and the expression conditions (Fig. 4, *A* and *B*). The expression levels of clones AR_1, AR_3a, and AR_3b seem to be similar and around 2-fold higher than the one of clone AR_2. For *in vitro* studies, we recloned the AR proteins into an expression vector (pQE30) to avoid co-purification of APH. All AR proteins could be purified to homogeneity in a single IMAC step by means of their His_6 tag (Fig. 4*C*). Around 100 mg of purified AR protein was obtained from a standard 1-liter shake flask culture when expressed from the pQE30-derived vectors, without optimization of the expression and purification procedure. Even under selection conditions, where expression is not fully induced, the AR proteins show the most prominent bands on the gel (Fig. 4*B*). Since APH did not give rise to a pronounced band on the gel, the inhibitors must be present in large excess over APH in *E. coli* during screening.

Size Exclusion Chromatography of AR Proteins and Complexes—The selected AR proteins free or in complex with APH were analyzed by gel filtration experiments. Whereas most previously investigated AR proteins are monomers (20, 21), different oligomerization tendencies, depending on the individual protein, were detected for the AR proteins described here (Fig. 5*A*). AR_1 was monomeric, AR_B was present as monomer

TABLE I
Summary of ankyrin properties

Construct	MIC[a]	Expression level[b]	K_D[c]	k_{on}[c]	k_{off}[c]
	μg/ml		*nM*	$M^{-1}\ s^{-1}$	s^{-1}
No APH[d]	5				
E3_5[e]	>1000	+++			
AR_B[f]	>1000	+++	28.6 ± 16.2	$(1.4 \pm 0.5) \times 10^5$	$(4.1 \pm 0.8) \times 10^{-3}$
AR_1	100	+++	19.0 ± 10.0	$(1.2 \pm 0.4) \times 10^5$	$(2.2 \pm 0.4) \times 10^{-3}$
AR_2	150	+	8.1 ± 6.5	$(2.8 \pm 1.0) \times 10^5$	$(2.3 \pm 1.0) \times 10^{-3}$
AR_3a	25	+++	1.7 ± 1.3	$(1.6 \pm 0.6) \times 10^6$	$(2.7 \pm 0.9) \times 10^{-3}$
AR_3b	10	+++	0.5 ± 0.4	$(2.5 \pm 1.5) \times 10^6$	$(1.1 \pm 0.4) \times 10^{-3}$

[a] With kanamycin A.
[b] Estimated from corresponding band intensities from Coomassie-stained SDS-PAGE of whole cell extracts (Fig. 4*A*).
[c] Apparent values as described under "Materials and Methods," independently rounded.
[d] Control of *E. coli*, where no APH is present.
[e] Control of an unselected AR protein (28).
[f] Control of a selected binder, which did not show *in vivo* or *in vitro* inhibition.

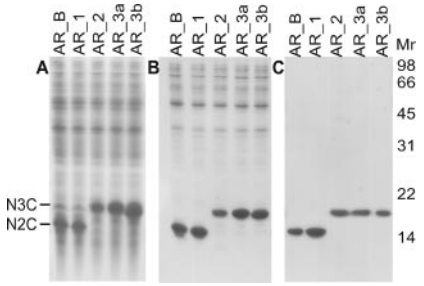

FIG. 4. **Expression and purification analysis of selected AR proteins.** The proteins were visualized on a 15% SDS-PAGE stained with Coomassie Brilliant Blue. *A*, expression of AR proteins under selection conditions: pQIA in *E. coli* XL1-Blue, carrying the respective genes in the AR protein cassette, induced with 100 μM IPTG. Cells were collected 4 h after induction and lysed in loading buffer. *B*, preparative expression of AR proteins with pQE30 (does not contain the APH gene) in *E. coli* XL1-Blue, induced with 500 μM IPTG. Cells were collected 4 h after induction and lysed in loading buffer. Crude extracts are shown. *C*, single step IMAC-purified AR proteins from the soluble *E. coli* fraction of the preparative expression. The size marker is indicated in kDa.

and trimer, and AR_2, AR_3a, and AR_3b appeared to be a mixture of monomer, dimer, and soluble multimer (see Fig. 5*A* for data of AR_B, AR_1, and AR_3a). Reapplying single peaks from AR_3a resulted in a similar elution pattern, indicating equilibrium between the oligomeric states. The exact molecular weight of the different species was verified for AR_1 and AR_B by static multi-angle light scattering (data not shown). Clone AR_1 (14.8 kDa) was determined to have an apparent molecular mass of 15.2 kDa, and clone AR_B (14.8 kDa) of 15.9 and 49.1 kDa. The molecular weight of the presumably monomeric and dimeric species of AR_3a and AR_3b could not be determined by static light scattering, since the peaks strongly overlapped. Since AR_1 and most other selected and unselected library members are monomers (20, 21), it seems likely that the oligomerization behavior is a consequence of the particular selected interaction residues of the individual AR proteins.

The complexes of the AR proteins with APH were also analyzed by gel filtration experiments. All complexes gave one distinct symmetric peak, which was shifted to higher molecular weight, compared with the one of APH alone, and which corresponded to the correct molecular weight of the complex. For example, the complex of APH and AR_3a eluted in a single peak at an apparent molecular mass of about 47 kDa (calculated molecular mass 49.6 kDa; Fig. 5*B*), although AR_3a alone eluted in various oligomeric states (Fig. 5*A*). The complexes showed no signs of oligomeric states, supporting the hypothesis that the selected binding interfaces of the AR proteins are responsible for and may be directly involved in the observed partial oligomerization of some AR proteins (Fig. 5*A*). Binding of the target thus abolishes oligomerization and leads to a defined 1:1 APH-AR protein complex. Indeed, co-purification of an APH mutant bound to AR_3a was used successfully for crystallization and structure determination of the complex as described elsewhere (31).

Binding Constants and Specificities of the Inhibitors—To investigate the binding properties of the selected AR proteins, the affinity and the specificity of the interaction were analyzed by ELISA and BIAcore studies. In an ELISA experiment with purified AR proteins of all analyzed clones, the binding to APH was compared with the binding to other proteins, such as pD, JNK2, p38, BSA, and neutravidin (Fig. 6). p38 and JNK2 are two EPKs (39) sharing structural homology to APH. The selected AR proteins bind specifically to APH but not to any of the other proteins (Fig. 6). As expected, the unselected AR protein E3_5 fails to bind any of the immobilized proteins. We conclude that specific APH-binding AR proteins were selected.

To determine the dissociation constants of the selected AR proteins to APH, kinetic surface plasmon resonance experiments were performed. The dissociation constants of all APH binders were found to be in the low nanomolar to subnanomolar range (Table I). K_D values of the two high-affinity inhibitors AR_3a and AR_3b were verified by competition BIAcore (40); for details, see Supplemental Data. The surface plasmon resonance data were fitted with the assumption that all species (monomer and dimer; see above) share the same binding properties or convert to monomers upon binding. If this is not the case, the K_D values given here reflect the average of all states.

In Vitro Inhibition of APH—To confirm that the *in vivo* effects of the selected AR proteins were indeed due to direct inhibition of the APH, *in vitro* enzyme assays were performed. Enzyme activity was monitored by coupling the release of ADP to a pyruvate kinase/lactate dehydrogenase reaction as described previously (35) with some modifications to adapt the assay for the inhibition studies (see "Materials and Methods"). The inhibition assay was set up to mimic the *in vivo* situation, where the AR protein concentration is higher than the one of APH and the kanamycin A concentration is low, since it has been suggested that kanamycin A import is the rate-limiting process (41). For all clones showing a kanamycin A-sensitive phenotype *in vivo*, enzyme inhibition was detected, whereas the control proteins E3_5 and AR_B showed almost no influence on APH activity (Fig. 7). Under these conditions (200 nM APH, 10 μM AR protein, kanamycin as substrate), the inhibition was not complete; a remaining activity, ranging from 6 to 52%, depending on the individual inhibitor, was found (Fig. 7*A*). The kinetic experiments with kanamycin A were, however, complicated by an apparent substrate inhibition at low micromolar concentrations (Fig. 7*C*). Substrate inhibition had previously been described at higher kanamycin A concen-

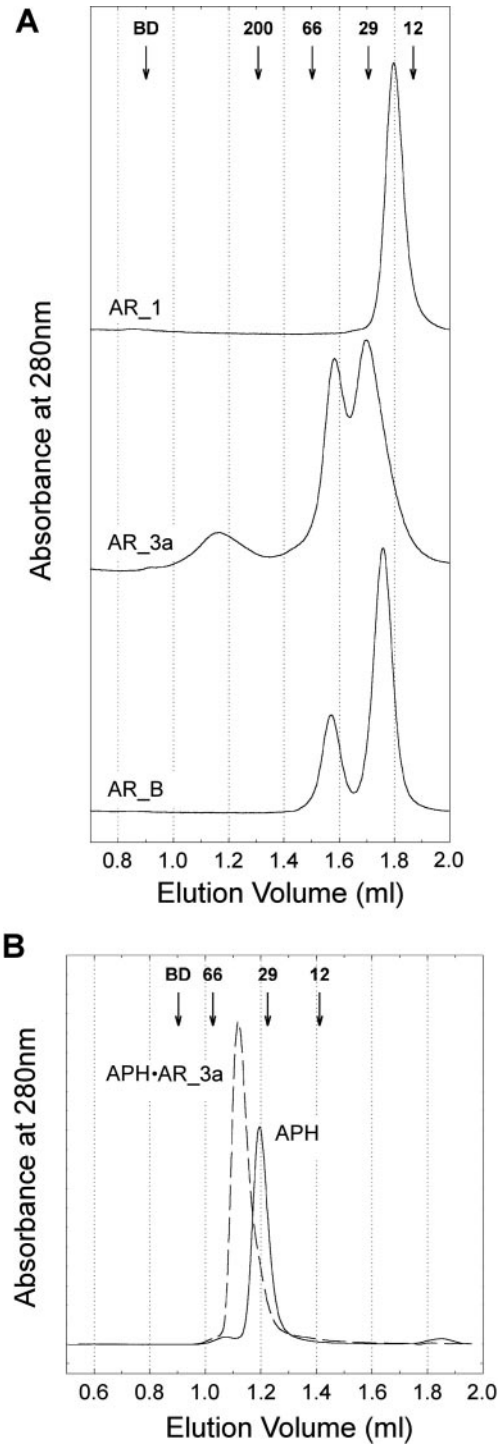

FIG. 5. **Size exclusion chromatography of selected AR proteins alone and in complex with APH.** The *arrows* indicate the elution volumes of the marker proteins (MW in kDa given above the arrow) and of blue dextran (*BD*). *A*, elution profiles of the AR protein clones AR_1, AR_3a, and AR_B from a Superdex 200 column. Clone AR_1 (14.8 kDa) eluted at an apparent molecular mass of 17 kDa; clone AR_3a (18.6 kDa) eluted at 400, 49, and 27 kDa; and clone AR_B (14.8 kDa) eluted at 52 and 20 kDa. *B*, elution profiles of APH and APH in complex with the AR protein AR_3a from a Superdex 75 column. The apparent molecular masses of the APH·AR_3a complex (49.5 kDa) and APH (30.9 kDa) are 47 and 34 kDa, respectively.

trations (35). The measurements were thus repeated with amikacin, another aminoglycoside substrate of APH containing an additional hydroxybutyrate group, which does not show substrate inhibition and has a similar turnover rate (35) (Fig. 7, *C* and *D*). For this substrate, APH activity was reduced to 2–73%,

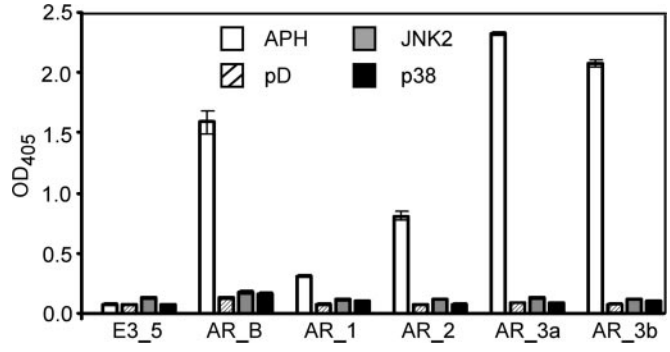

FIG. 6. **Binding specificities of the selected AR proteins.** The interaction of the selected AR proteins (AR_B, AR_1, AR_2, AR_3a, and AR_3b; all 200 nM) with immobilized APH was compared with the interaction with immobilized pD, p38, JNK2, neutravidin, and BSA. As a control, an unselected library member (E3_5; 200 nM) was included. The *error bars* show the standard deviation between three cells. The background binding of the detection antibodies was not subtracted.

depending on the respective AR inhibitor, whereas control proteins E3_5 and AR_B show almost no influence on activity (Fig. 7*B*). Thus, the overall *in vitro* inhibition of APH by the AR proteins using amikacin as substrate was similar to that observed when using kanamycin A (Fig. 7*A*), with almost complete inhibition for AR_3a and AR_3b (Fig. 7*B*). A more detailed characterization of AR_3a is given elsewhere, including precise titration analysis and structural insights in the inhibition mechanism (31).

The different inhibition efficiencies of the selected inhibitors seem to correlate well with the differences in K_D values and their inhibition performance *in vivo* (Table I). Nevertheless, it is worth commenting on the fact that residual activities were found at a concentration of AR proteins that is 10^3- to 10^4-fold above the K_D values determined by BIAcore analysis. Some of the APH·AR protein complexes may indeed possess a residual activity. However, there are other possible factors leading to this observation: (i) the presence of the substrates, ATP and aminoglycoside, acting as competitors for a distinct conformation of APH, which will raise K_I correspondingly above the K_D values; (ii) increased K_D values of the complexes as a result of different assay conditions of the kinetic measurements (*e.g.* increased temperature and different buffer composition); (iii) overestimation of the concentration of active AR proteins due to some degree of oligomerization (Fig. 5*A*).

One more difference should be noted: the inhibition of APH by AR_1 was less efficient with amikacin as a substrate (73% residual APH activity) than with kanamycin A (43% residual APH activity) under the assay conditions (Fig. 7, *A* and *B*). *In vivo* tests revealed that AR_1, in contrast to the other inhibitors, did not increase the amikacin sensitivity of *E. coli* harboring the appropriate plasmid but displayed the same phenotype as the noninhibitors E3_5 and AR_B (data not shown). These results suggest that amikacin can compete more efficiently than kanamycin A with the binding of AR_1 to APH.

In summary, all AR proteins tested showed direct inhibition of APH, although the inhibition was not complete except for AR_3b and AR_3a and might be substrate-specific in the case of AR_1.

DISCUSSION

Methods that are able to directly and specifically interfere with the function of a protein of interest intracellularly are necessary to validate results obtained from gene knockouts and especially from RNAi and antisense approaches. In addition, they give further insights into the target protein itself. The difficulty to create specific and intracellularly active binders and inhibitors to most proteins has, however, so far constituted

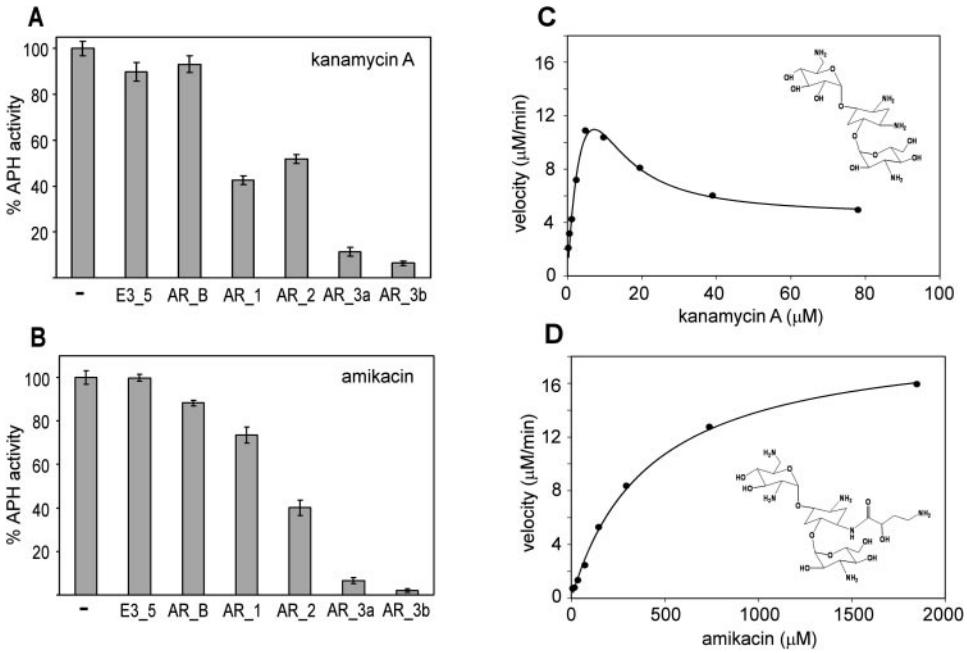

FIG. 7. **Inhibition properties of selected AR proteins and substrate-specific *in vitro* kinetics of APH.** *A* and *B*, the enzymatic activity of APH (200 nM) was determined with and without a large excess of AR protein present. *A*, with kanamycin A as substrate (10 μM) and AR proteins at a concentration of 10 μM, E3_5 and AR_B have no significant influence on APH activity, whereas the other AR proteins reduce the activity to 6–52%, depending on the inhibitor. *B*, with amikacin as substrate (740 μM) and in the presence of 2 μM AR proteins, the corresponding APH activity is reduced to 2–73%. Again, the control proteins E3_5 and AR_B show no significant influence on APH activity. For AR_3b, the inhibition is essentially complete. *C* and *D*, APH kinetics were measured at 37 °C using 50 nM purified monomeric APH, as described under "Materials and Methods," and two different substrates, kanamycin A (*C*) and amikacin (*D*). Initial velocities are plotted against substrate concentrations. *C*, when using kanamycin A as a substrate, a pronounced apparent partial substrate inhibition in the low micromolar range was observed. At high kanamycin A concentrations, k_{obs} reaches 1.64 s^{-1}. Although substrate inhibition of kanamycin A was previously reported (34), it seemed to be observed at lower substrate concentrations under our experimental conditions. *D*, the activity of APH followed Michaelis-Menten kinetics when using the substrate amikacin. The initial rates were fitted by nonlinear least squares fitting using the standard Michaelis-Menten equation. The determined K_m and k_{cat} values were 418 ± 29 μM and 6.56 ± 0.17 s^{-1}, respectively.

a bottleneck in elucidating the function of many proteins. By the rapid generation of selective high affinity intracellular enzyme inhibitors, we demonstrate here that designed AR proteins have the potential to solve this problem. The most potent inhibitors lead to a phenotype that is comparable with a gene knockout. This potency is achieved by a combination of factors; the selected AR proteins are free of disulfide bonds, they are able to fold well and are stable in the reducing intracellular environment, they are expressed in soluble form at high levels in the cytoplasm, and the interaction with the target enzyme is of high affinity and specificity. The combination of these factors leads to a clear advantage of AR proteins as intracellular inhibitors over antibodies, peptides, or other binding proteins reported so far.

Intracellular Protein Inhibitors—The approach to obtain intracellular protein inhibitors presented here, based on designed AR proteins, is very promising. Whereas high affinities are rather difficult to achieve with peptidic binders (except in the case of target proteins with pronounced binding pockets), the affinity and specificity of antibodies and designed AR proteins are good and comparable. The main drawback of antibodies for intracellular applications is that their stabilities rely on disulfide bonds, which cannot form in the reducing intracellular environment. Consequently, low levels of soluble functional protein and limited half-life of the antibody domains, due to aggregation, are the rule when applied in the cytoplasm (12). In this respect, the high intracellular expression level and stability of designed AR proteins is a clear advantage. It seems highly probable that these properties were responsible for the fact that we could identify not only a few individual but many different functional inhibitors, although the selection system described here selects for intracellular stability and enzyme inhibition at the same time. Several approaches have been described to generate antibodies that are active intracellularly (for details, see reviews (10, 15)). Although the inhibition of enzymes *in vitro* by antibodies has often been described (42–44), only a few of these inhibitors have been applied intracellularly (45).

Besides antibodies and AR proteins, other protein scaffolds have been used for the generation of enzyme inhibitors. Different high-affinity protease inhibitors have been generated based on protease-inhibitor scaffolds (54), and the function of β-lactamase inhibitory protein BLIP has been improved (46). These scaffolds mostly exploited naturally occurring enzyme inhibitors that were improved for affinity or specificity. All of these inhibitors contain disulfide bonds and were used for extracellular or periplasmatic applications.

In Vivo Inhibition of APH—The choice of APH as a model system was guided by several aspects of the enzyme. APH phosphorylates aminoglycoside antibiotics (*e.g.* streptomycin, kanamycin, or amikacin), mediating bacterial resistance to these antibiotics. This allowed the set-up of an efficient screening assay for the identification of inhibiting AR proteins (Fig. 3). Most importantly, APH displays high structural homology to EPKs (30), a class of enzymes involved in a wide range of diseases (see below). Finally, no satisfactory APH inhibitors are known to date that are active *in vivo*. Whereas different *in vitro* inhibitors of APH have been described in the literature (47, 48), they fail to inhibit bacterial growth efficiently. We describe here the first inhibitors that are fully functional *in vivo* and show a phenotype comparable with the APH gene knockout. Although the selected AR proteins are *per se* not suitable as antibiotic agents on their own, they could in the future accelerate the process of drug discovery by providing a detailed structure of the drug target in an inactive state. This

aspect is discussed elsewhere (31), where the crystal structure of the selected AR protein AR_3a in complex with an APH mutant is described.

Targeting Eukaryotic Protein Kinases—EPKs receive widespread interest, since they are key players in cellular signaling, making them targets for the treatment of a number of diseases, such as various cancers, asthma, and autoimmunity to name a few (49). In the case of EPKs, highly selective inhibitors are desired (for a recent review on the EPK inhibitor field see Ref. 50). However, the generation of specific EPK inhibitors is difficult, due to the large number of EPKs (about 518 in humans (49)), all sharing highly homologous active sites and using ATP as a co-factor. Most inhibition strategies involve targeting of the ATP binding pocket by small molecular weight drugs. This is not ideal, since this region of EPKs is highly conserved, and thus specificity is difficult to achieve and is further complicated by the high ATP level inside the cell (1 mM) that must be competed. Indeed, APH is inhibited by some EPK inhibitors, which on the one hand underlines the structural homology of APH and EPKs and on the other hand also demonstrates the poor specificity of these EPK inhibitors (51). Proteinaceous inhibitors could represent an attractive alternative, since they can interact via a large surface and, therefore, their binding is not restricted to grooves. This enables them to bind to the less conserved surface regions on the target protein while blocking its function.

It is noteworthy that many natural AR proteins are kinase inhibitors, such as for example the cell division kinase inhibitors $p16^{INK4a}$ and $p19^{INK4d}$ (52, 53). We have already successfully demonstrated the selection of mitogen-activated protein kinase binders from designed AR protein libraries (20). Again, the selected pools of binders might serve as starting points for inhibitor screens, in this case using an *in vitro* activity assay. Since mitogen-activated protein kinases are part of signaling networks, functional inhibition might not only be achieved by inhibition of the catalytic site, but also by simple binding, provided that the inhibitor blocks either the protein substrate or the activation of the kinase itself by an upstream kinase. Although our approach can be applied now as a discovery tool, therapeutic intracellular applications of designed AR proteins will have to await further maturation of gene therapy or protein uptake techniques.

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