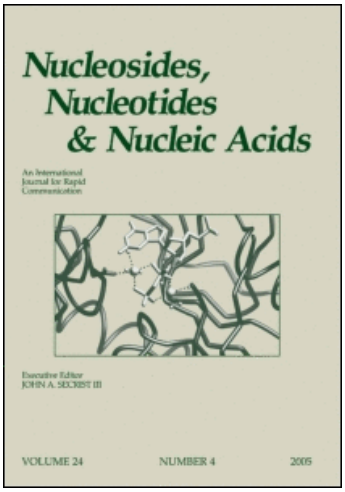

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2-5A LIGANDS—A NEW CONCEPT FOR THE TREATMENT OF PROSTATE CANCER

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□ *Several potent prostate specific membrane antigen (PSMA) inhibitors have been described recently. We generated a PSMA-specific 2-5A ligand called RBI 1033 by linking 2-5A to the N-acetylaspartylglutamate (NAAG)-based inhibitor ZJ-24. We measured the inhibitory activity of RBI 1033 to the folate hydrolase activity of PSMA. Amazingly, we found that compared to ZJ-24 (IC50 = 53.9 nM), RBI 1033 was more than 10 times more potent (IC50 = 4.78 nM) as a folate hydrolase inhibitor, while SMCC 2-5A lacking the ZJ-24 part, did not show much activity (IC50 = 1974 nM). Also, RBI 1033's affinity to PSMA was found to be 10 times higher than ZJ-24 itself.*

Keywords PSMA; RBI 1033; inhibitory activitty

INTRODUCTION

Ridgeway Biosystems, Inc. is developing novel drugs for targeting cancer and infectious diseases by harnessing the powerful endoribonuclease RNase L. RNase L is activated by 2′,5′-linked oligoadenylates collectively referred to as 2-5A. To date the only well-established biochemical function of 2-5A is activation of RNase L. It was found that 2-5A activation of RNase L leads to RNA damage-mediated apoptosis in metastatic prostate tumor cell lines.[1]

In this investigation we linked a stabilized 2-5A analog to a ligand, which specifically recognizes prostate specific membrane antigen (PSMA), thereby directing 2-5A directly into prostate cancer cells.

PSMA is a unique membrane bound glycoprotein, which is highly prostate specific and greatly overexpressed on prostate cancer cells and on tumor vascular endothelium of virtually all solid carcinomas and sarcomas,

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but not in the vasculature of normal tissues.[2–10] PSMA is a type II integral cell-surface membrane protein that is not secreted.[11] PSMA, like other cell surface receptors, undergoes internalization, thereby mediating the internalization of a putative ligand. This makes PSMA an ideal target for the treatment of cancer in particular prostate cancer.

RESULTS AND DISCUSSION

We previously have shown that RNase L activation by 2-5A leads to apoptosis and therefore to the killing of prostate tumor cells.[1] However, so far delivery to the target organ has not been addressed. It was shown that small ligands based on the structure of NAAG show very high binding affinity to the NAALADase domain of prostate specific membrane antigen (PSMA).[12–15] Structure activity relationship (SAR) studies revealed that a urea containing at least one glutamate residue plus a second residue bearing a carboxyl group in addition to another group (SR orCO_2H) represents the minimum requirement to achieve effective NAALADase inhibition.[16] Based on these findings several simple di-peptide ligands were identified with activities against NAALADase in the low nanomolar range.[12] Such ligands formed the basis of the novel 2-5A ligand presented here.

Synthesis of the 2-5A Ligand RBI1033

RBI 1033 was prepared by linking an amino modified 2-5A moiety to the thiol group of the dipeptidyl ligand Cys-C(*O*)-Glu using the bifunctional linker SMCC (Pierce, WI, USA). The urea-based ligand Cys-C(*O*)-Glu was prepared following published procedures.[12]

The amino-functionalized 2-5A moiety (RBI 1024) was synthesized by means of solid-phase synthesis using a Polygen 10 DNA synthesizer. Thereby a 5 umol slider was filled with 3′-Phtalimidyl-Amino Modifier C3 CPG (Glen Research, VA, USA). The first three couplings were performed using 5′-O-DMT-3′-O-TBDMS-N-Bz-adenosince 2′-CED-phosphoramidte (ChemGenes, MA, USA) and the last coupling using a TMT-on phosphorylation reagent, which was synthesized using published procedures (Guzaev and Manoharan, 2001). Beaucage Reagent was used as sulfurization reagent. The free amino-functionalized 2-5A moiety was isolated by (1) detritylation on the column: (2) cleavage from the support and base deprotection using NH_4OH/EtOH 3:1 and; (3) desilylation using a cocktail made up of N-methyl-pyrrolidinone, triethylamine, TEA x 3HF (1.5/0.75/1.0 v/v/v).

The amino-functionalized 2-5A moiety (RBI 1024) was conjugated to the ligand Cys-C(*O*)-Glu using the bifunctional linker ulfosuccinimidyl-4-(N-maleimidomethyl)-1-carboxylate (SMCC, Pierce, WI, USA) (see Figure 1). After RP-HPLC purification RBI 1032 was coupled to the ligand Cys-C(O)-Glu. The final product was purified by HPLC and its mass confirmed by mass spectrometry using MALDI.

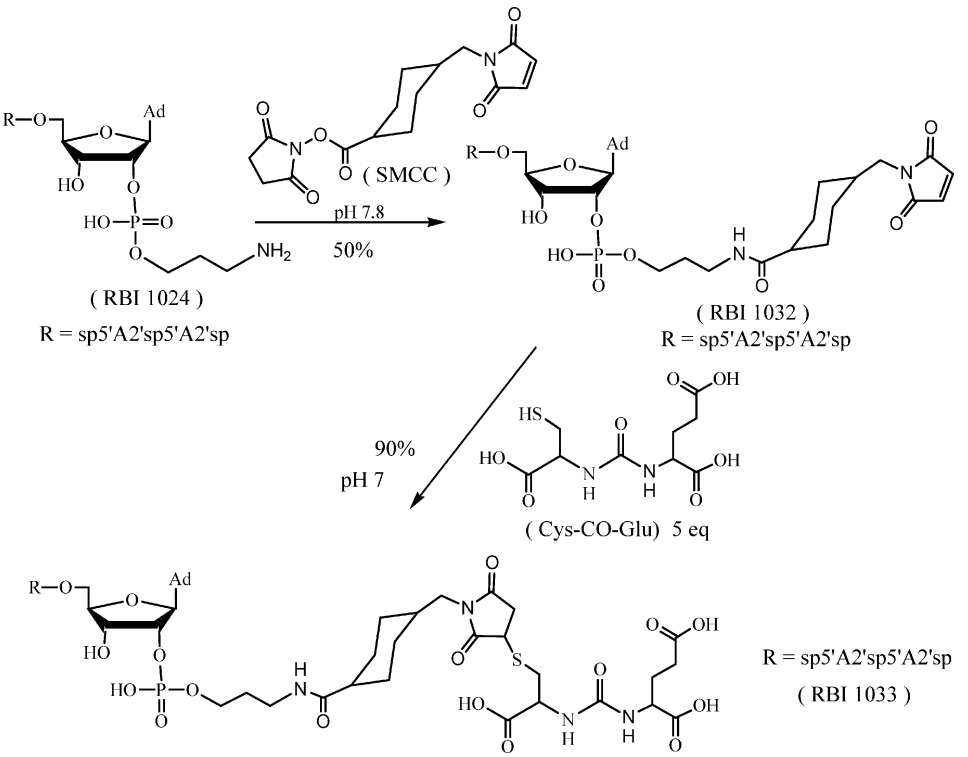

FIGURE 1 Synthesis of 2-5A ligand RBI 1033. The amino-modified 2-5A moiety RBI 1024 was linked to the urea-based dipeptidyl peptide Cys-C(*O*)-Glu using the bifunctional linker SMCC.

RNase L Activation, PSMA Binding, and PSMA Activity Assays

The 2-5A ligand was tested on its ability to activate RNase L in a previously developed fluorescence resonance energy transfer (FRET) assay, which is now being used routinely in our lab for screening novel 2-5A analogs on activity.[17] Thereby, the 2-5A analog was added to a cell-free system containing purified RNase L and a cleavable 36-nucleotide RNA substrate labeled with fluorescein at the 5′- and with black hole quencher 1 at the 3′-end. EC_{50} (concentration of activator to give 50% maximum activation) was determined for the 2-5A ligand and found to be about 10 nM (see Figure 2). Therefore, the activity is somewhat reduced in comparison to natural 2-5A. However, the activity is similar to other 2'-tailed 2-5A analogs such as 2-5A antisense. Despite this decreased activity in vitro, such tailed 2-5A analogs show enhanced activity in vivo due to their increased resistance towards 2',5' ribonucleases.[18,19]

Binding of the novel 2-5A ligand RBI 1033 was compared to the methyl thioether of the parent ligand, MeS-Cys-C(O)-Glu (ZJ-24) and RBI 1032 (2-5A devote of the ligand moiety, see Figure 1) toward the active dimeric form of soluble recombinant hPSMA (see Figure 3). The binding assay

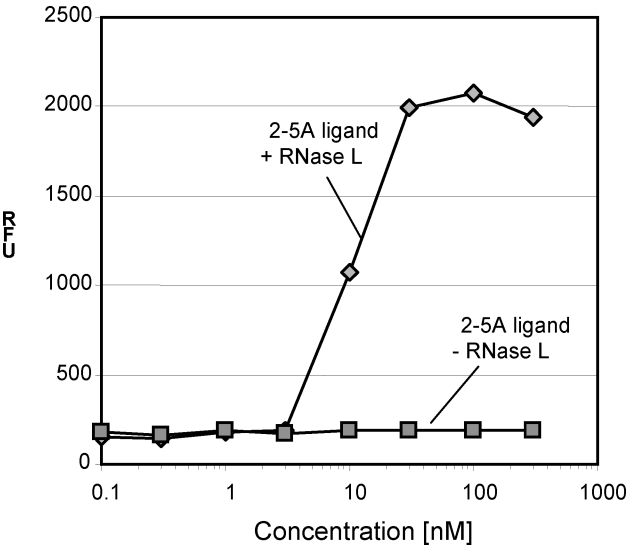

FIGURE 2 RNase L activation assay. RBI 1033's ability to activate RNase L in the presence of a fluorescence resonance energy transfer (FRET) labeled RNA substrate. 2-5A ligand activates RNase L with an EC_{50} of 10 nM.

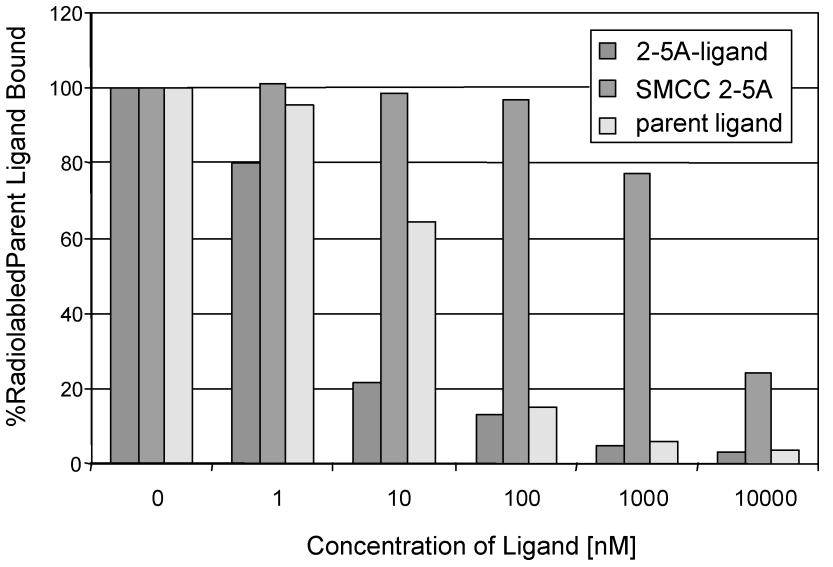

FIGURE 3 PSMA binding assay. Ability of a novel compound to compete for binding with radiolabeled ligand. 2-5A ligand binds to PSMA at lower concentrations (EC_{50} = 1.5 nM) than parent ligand ZJ-24 (EC_{50} = 15.3 nM).

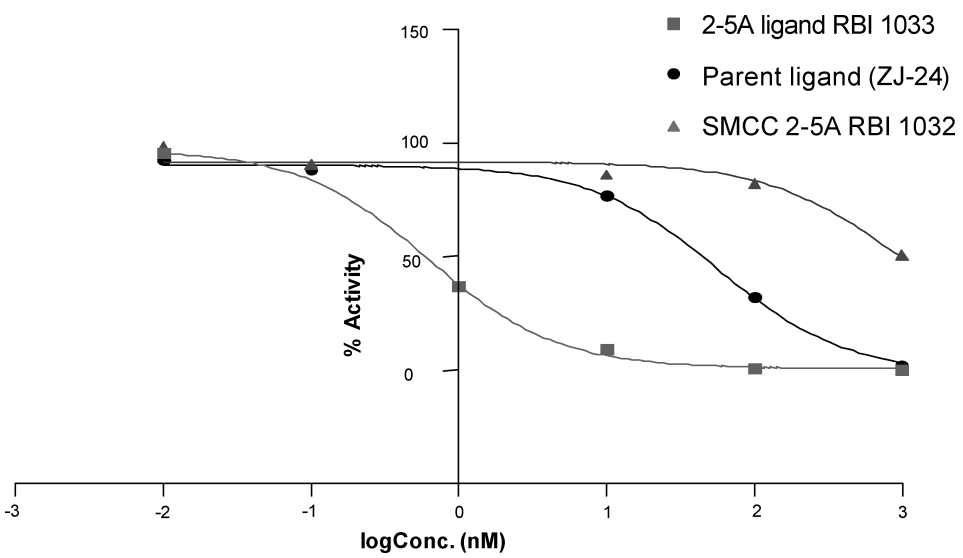

FIGURE 4 PSMA activity assay: Ability of hPSMA to cleave/hydrolyze a polyglutamte substrate under presence of a ligand at different concentrations. 2-5A ligand inhibits PSMA at lower concentrations ($EC_{50} = 0.62$ nM) than parent ligand ZJ-24 ($EC_{50} = 56.7$ nM).

examines the ability of a novel compound to compete for binding with radio-labeled ligand ZJ-24.[20] Thereby, different final concentrations of the inhibitor are incubated in the presence of ZJ-24* and the concentration determined, which is required to inhibit 50% of binding. The ligand concentration reducing binding by 50% is considered the EC_{50} of the ligand analog.

We determined the ability of hPSMA to cleave/hydrolyze a polyglutamte substrate under the presence of a ligand at different concentrations.[21,22] (see Figure 4). Briefly, recombinant PSMA was incubated with ligand ZJ-24, 2-5A ligand RBI 1033, or RBI 1032. The polyglutamate substrate MTXGlu2 (5 nMol) was added and the amount of MTX formed was determined by HPLC.

From the data presented here, we can conclude that the 2-5A ligand RBI 1033 is superior in binding and inhibiting PSMA than the parent ligand itself. The EC50 for binding was found to be 1.5 nM for RBI 1033 versus 15.3 nM for the parent ligand 6, while the inhibitory activity of RBI 1033 was 0.62 nM in comparison to 56.7 nM for the parent ligand. To verify that this increased activity is not due to non-specific binding of the 2-5A or SMCC part of the molecule, we determined the binding and inhibitory activity of RBI 1032 to PSMA as well. However, it was found that the SMCC 2-5A alone bound only weakly to PSMA with an EC50 of 3.5 mM (Figure 3) and the inhibitory activity was only in the millimolar range.

CONCLUSION

By chemically linking a 2-5A moiety to a ligand moiety we were able to create a 2-5A ligand with molecular weights below 1500 Dalton, which binds to PSMA with extremely high affinities and it is believed to become internalized into the cancer cell together with the protein. In comparison to mAb strategies currently in clinical trials for imaging and therapy[23–25] these molecules are very small and much easier and cheaper to make.

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