Screening

DOI: 10.1002/ange.200601695

Small-Molecule Screening Made Simple for a Difficult Target with a Signaling Nucleic Acid Aptamer that Reports on Deaminase Activity**

Nadine H. Elowe, Razvan Nutiu, Abdellah Allali-Hassani, Jonathan D. Cechetto, Donald W. Hughes, Yingfu Li, and Eric D. Brown**

Conventional approaches to small-molecule screening are currently being challenged by the availability of vast amounts of genomic sequence information and a plethora of potential targets. This challenge has precipitated a need for new screening paradigms that emphasize simplicity, capacity, and parallelization. Herein we report the application of signaling DNA-aptamer technology for the development and execution of a high-throughput screen for an otherwise problematic target, adenosine deaminase (ADA). The approach employed a signaling DNA aptamer that reports on adenosine concentration over the course of the enzymatic reaction. The assay was extremely robust in a screen of more than 44000 molecules and revealed a new competitive inhibitor of the deaminase. Nucleic acid aptamers have proven worthy as a routinely selectable species for a wide variety of small molecules and so this proof-of-principle work has broad

[*] N. H. Elowe,[+] Dr. A. Allali-Hassani,[#] Dr. J. D. Cechetto, Dr. Y. Li, Dr. E. D. Brown
Department of Biochemistry and Biomedical Sciences
McMaster University
Hamilton, ON L8N 3Z5 (Canada)
Fax: (+1) 905-525-9033
E-mail: liying@mcmaster.ca
ebrown@mcmaster.ca

R. Nutiu, Dr. D. W. Hughes, Dr. Y. Li
Department of Chemistry
McMaster University
Hamilton, ON L8N 3Z5 (Canada)
Fax: (+1) 905-522-9033

[+] Current address:
Novartis Institutes for BioMedical Research, Diabetes and Metabolism
100 Technology Square
Cambridge, MA 02139 (USA)

[#] Current address:
Structural Genomics Consortium
University of Toronto
100 College Street, Floor 3A, Room 351
Toronto, ON M5G 1L5, (Canada)

[**] We thank Jan Blanchard for her help with mass spectrometry and comments on the manuscript. This work was supported by Ontario Research and Development Challenge Fund, Natural Sciences and Engineering Research Council, and MDS Sciex. E.D.B. and Y.L. hold Canada Research Chairs.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

applicability and potential for the development of enzymatic assays suitable for a chemical genomic approaches.

Aptamers are single-stranded DNA and RNA molecules derived from random libraries that are capable of binding to diverse biological targets, such as small-molecule metabolites, peptides, hormones, or proteins, with high affinity and specificity. Aptamers have found diverse utility ranging from research[1] to diagnostic and therapeutic applications.[2,3] Nucleic acid aptamers were first shown to have promise as a displaceable ligand in compound screening in which a panel of a dozen naphthalensulfonic acids were tested for their capacity to compete a ^{32}P-labeled aptamer from platelet-derived growth factor.[4] An interesting property of nucleic acid aptamers is that they are known to undergo conformational changes upon target binding.[5] This characteristic is emerging as exploitable for the design of signaling probes that report on the interaction of aptamers with their targets and thus, signaling aptamers have genuine potential in assay development and small-molecule screening.[6,7] Recently, we reported a proof-of-concept study in which the enzyme activity of alkaline phosphatase was monitored in real time by using a signaling DNA aptamer with a higher affinity for adenosine than for AMP.[8] The assay employed a "structure-switching" aptamer in which adenosine binding induced a state of high fluorescence through release of a quenching group. The assay was amenable to screening in a 96-well plate and was sensitive to known inhibitors of alkaline phosphatase. Thus, signaling nucleic acid systems have begun to show real promise as reporter systems that can be tailored with relative ease to a variety of assays. In the work reported herein, we have rigorously tested this potential in an automated, high-throughput screen (HTS) of ADA against a small-molecule library of more than 44000 compounds.

ADA is a key enzyme in purine metabolism, catalyzing the irreversible deamination of adenosine/deoxyadenosine to inosine/deoxyinosine. ADA is a ubiquitous enzyme best known for its role in certain types of severe combined immunodeficiency diseases.[9] The enzyme has both cytosolic and extracelluar forms in which the latter is associated with CD26, which is strongly upregulated in T-cell activation.[10] Increasingly, extracellular adenosine is recognized to have a role in attenuating immunity and inflammation, and thus inhibitors of ADA may have real potential for clinical applications.[11] Despite growing interest in this target, ADA does not have a simple, homogenous, and sensitive assay amenable to HTS. Common methods measure ammonia produced[12] or the change in adenosine concentration by monitoring absorbance at 265 nm.[13] The former is not easily automated and the short-wavelength detection of the latter is prone to interference from the intrinsic absorbance of screening compounds. A long-wavelength coupled assay system has also been described,[14] but it is inherently cumbersome owing to the need for three coupling enzymes. Thus, none of the assays described to date could be considered ideal for small-molecule screening.

To develop a screening assay for ADA, we employed a fluorescence-signaling aptamer with high affinity for adenosine and virtually no affinity for inosine (Figure 1 A).[15,16] The fluorescence signal in this system is derived from a fluorescein

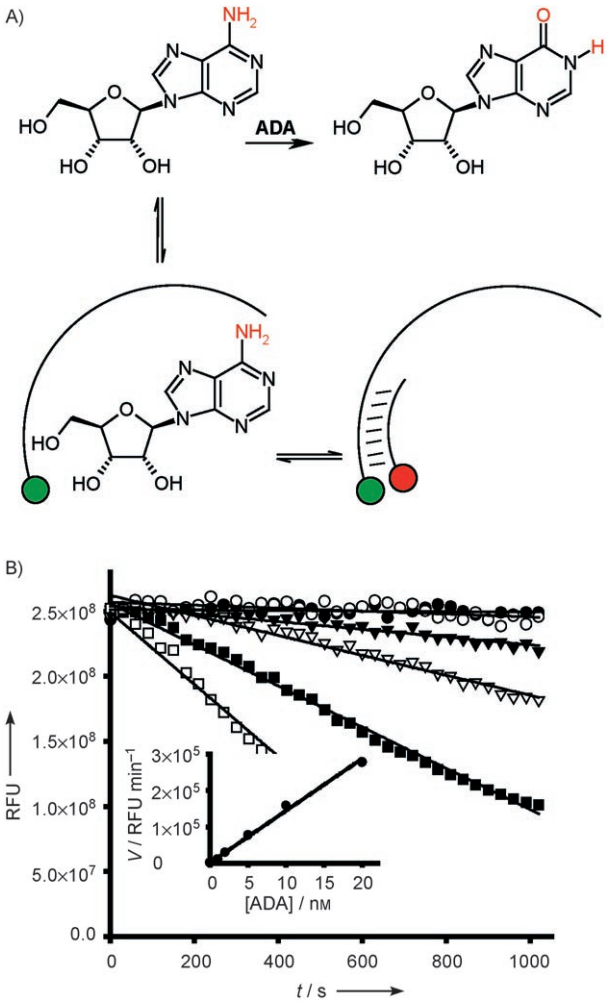


Figure 1. A) Fluorescence-signaling aptamer assay for screening. The fluorescein-labeled aptamer, depicted as an arc with a green-filled circle (Fluorescein-5′-TCACTGACCTGGGGGAGTATTGCGGAG-GAAGGT), complexes with either adenosine or a quenching oligonucleotide (5′-CCCAGGTCAGTG-Dabcyl; arc with red-filled circle) resulting in the exclusion of the other. The fluorescein-labeled aptamer has little affinity for inosine. Thus as adenosine is transformed to inosine by ADA, the aptamer becomes available to complex with the quenching group and reports on the progress of the reaction with decreasing fluorescence over time. Inhibition of ADA maintains high adenosine concentration and the highly fluorescent adenosine–aptamer complex is therefore formed. B) Assay linearity with time and enzyme concentration. Assays were run with varying enzyme concentration (● No ADA; ○ 1 nM ADA; ▼ 2 nM ADA; ▽ 5 nM ADA; ■ 10 nM ADA; □ 20 nM ADA) and with 500 μM adenosine, 20 nM aptamer, 40 nM QDNA, 5 mM $MgCl_2$, 300 mM NaCl in 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer solution at pH 8.0. The reaction was initiated with the addition of an adenosine and DNA mixture and monitored over time, with excitation at 485 nm and measuring emission at 530 nm. RFU = relative fluorescence units. V = reaction velocity.

group present at the 5′-end of the DNA aptamer and determined by the ratio of two different structural states, the adenosine-bound and free forms of the aptamer. Only the latter is capable of complexing with antisense DNA containing a dabcyl quenching group (QDNA). As adenosine is converted to inosine, the QDNA-bound form of the aptamer

predominates, resulting in a decrease in fluorescence. Real-time monitoring of the adenosine-dependent fluorescent signal provided a convenient and homogeneous assay of ADA activity that was optimized in this work for linearity with time and enzyme concentration (Figure 1B). An enzyme concentration of 10 nM fell in the linear range of the latter and had a robust signal for HTS. For the sensitive detection of competitive inhibitors,[17] we chose a substrate concentration equal to the K_m for adenosine (500 μM; data not shown). Work with a known inhibitor of ADA, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), confirmed that the assay conditions faithfully reported on the activity and inhibition of adenosine deaminase. Dose–response relationships for EHNA were virtually superimposable by using either the signaling aptamer assay or a HPLC method that measures adenosine turnover directly (Figure 2).

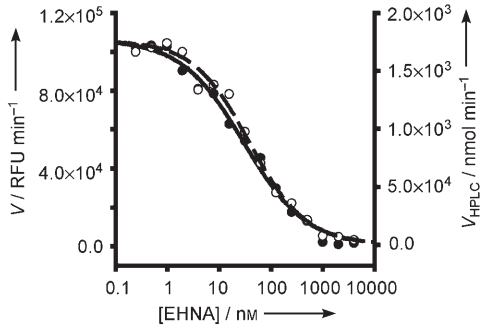


Figure 2. Dose–response analysis for the known inhibitor EHNA determined by using the fluorescence-signaling aptamer assay and a direct determination of adenosine turnover by HPLC. Data for the signaling aptamer method (——) and the HPLC determination (– – –) were fit to the dose–response equation described in the Experimental Section, and IC_{50} values of 30 ± 5 and 39 ± 7 nM, respectively, were obtained. Reactions contained 10 nM ADA, 500 μM adenosine, and varying EHNA concentrations (4% final DMSO v/v). The signaling aptamer assay also included 20 nM fluorescent aptamer, 40 nM QDNA, 5 mM $MgCl_2$, and 300 mM NaCl in 25 mM HEPES buffer solution at pH 8.0, whereas the reactions prepared for analysis by HPLC were in 25 mM HEPES buffer at pH 8.0 and 100 mM KCl. These were quenched with 0.2 N HCl, 3 min after initiation with adenosine and ultrafiltered through a membrane with a molecular-weight cutoff at 3000 g mol^{-1} and the filtrate analyzed. Inosine and residual adenosine, as detected at 250 nm, were separated by HPLC on a reverse-phase column, over a buffer solution gradient from 100% 30 mM KH_2PO_4 to 30% acetonitrile/water (1:1 v/v) over 9 mL at a flow rate of 1 mL min^{-1}.

By using the assay described, we screened a diverse collection of 44400 commercial compounds in duplicate. Figure 3A shows a replicate plot of screening data in which the quality of the screen is evident in the high correspondence of duplicate determinations. More evidence of the quality of this screen is evident in Figure 3B, which shows a plot of the signal from high-control wells (no inhibitor added) and low-control wells (2 μM EHNA). We calculated a *Z*′-factor for the high and low controls of 0.51. This parameter is a statistical measure of the quality of the screening data in which a value of 0.5 or greater indicates a very workable signal window and

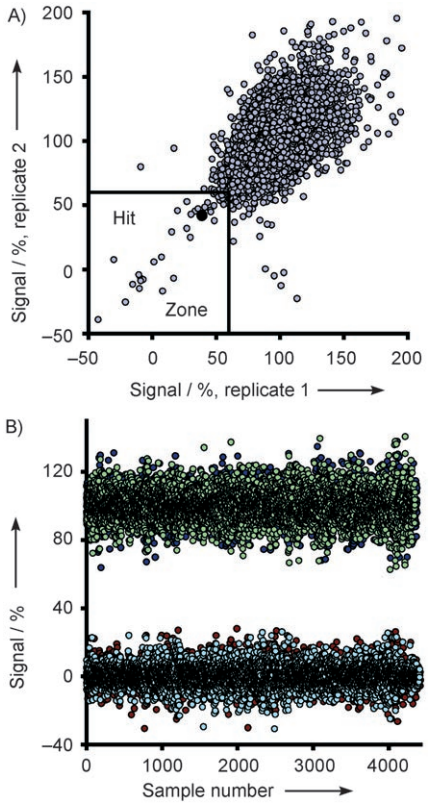
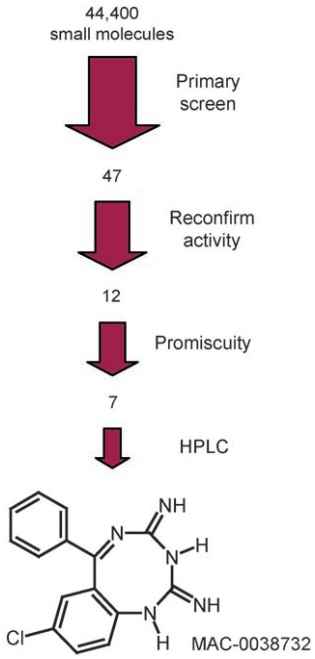


Figure 3. Primary screen of ADA. A) Plot of duplicate data from a screen of 44400 compounds (Maybridge plc, Cornwall, UK). Each 75-μL reaction contained 10 μM library compound (4% v/v final dimethyl sulfoxide content), 10 nM ADA, 500 μM adenosine, 20 nM aptamer, 40 nM QDNA, 25 mM HEPES buffer, pH 8.0, 5 mM $MgCl_2$ and 300 mM NaCl. The outlined hit zone was 3σ from the mean of the data and defined as 60% residual signal. Coordinates of the defined ADA inhibitor, MAC-0038732, are highlighted in black. B) Plot of the percent residual signal of the high-control data (dark-blue- and light-green-filled circles) and low-control data (burgundy- and light-blue-filled circles) of the first and second replicates, respectively, of the screen. The calculated Z′-factor for this screen was 0.51. Neat DMSO was added to wells containing high controls, whereas low controls contained 2 μM EHNA.

limited variation associated with the high and low control data.[18]

Some 47 screening "hits" were selected based on a hit zone three standard deviations below the mean of the data. These compounds were subsequently analyzed in a series of secondary assays as summarized in Scheme 1. Retesting of the hit compounds under primary screening conditions confirmed that 12 compounds were repeatable hits. The compound screening literature has few analyses that would make for an objective analysis of hit and confirmation rates.[19] Nevertheless, with a hit rate of about 0.1% (47 of 44400) of compounds screened and confirmation rate of 26% (12 of 47), we conclude, based on our own screening experience,[20–23] that the signaling aptamer assay proved to be quite tractable and well behaved in primary screening. The activity of these 12 compounds was further evaluated in the fluorescence-signaling aptamer assay by using additives that are commonly used to test for promiscuous or nonspecific inhibitors, such as



Scheme 1. Workflow resulting in the identification of MAC-0038732, a new inhibitor of adenosine deaminase.

reducing agent 1,4-dithio-D,L-threitol (DTT), detergent (Triton X-100), and bovine serum albumin (BSA). Promiscuous electrophilic compounds are frequently sensitive to the inclusion of excess DTT or BSA in the reaction mixture,[20] whereas the attenuation of compound activity in the presence of detergent is often an indication of promiscuous inhibition through compound aggregation.[24,25] Of the confirmed 12 hits, the inhibitory activity of seven of the compounds was not significantly perturbed in the presence of any of the three agents tested (1 mM DTT, 0.01% (v/v) Triton X-100, and 0.1 mg mL^{-1} BSA). The remaining five compounds were discarded as they exhibited promiscuous behavior.

The remaining seven compounds were analyzed by using an HPLC assay as a direct test for inhibited ADA. HPLC analysis showed that one of these compounds, MAC-0038732 (Scheme 1), was indeed an inhibitor of adenosine deaminase. Interestingly, analysis by mass spectrometry revealed that the identity of the active molecule (297.1 Da) was not that predicted for the structure in the compound database (315 Da). Indeed, NMR spectroscopic analysis of the active molecule indicated a structure that was consistent with cyclization of the biguanidine functionality to form an imine (for details see the Supporting Information) with the loss of a water molecule. Steady-state kinetic analysis of the inhibition of ADA by MAC-0038732 revealed competitive inhibition with respect to adenosine and a K_i of 2 μM (data not shown). The six remaining compounds showed, through the HPLC assay, no inhibition. Careful examination of the signaling aptamer assay revealed that these compounds were falsely positive as a result of interference with the fluorescence signal. Two of these compounds were highly fluorescent and obscured the output of the signaling aptamer assay, whereas the remaining four compounds could quench the aptamer–adenosine fluorescence signal. Although these six compounds revealed a potential shortcoming in the signaling aptamer assay, namely interference with the fluorescent reporter system, we note herein that relatively few compounds were problematic (6 out of 44400) and that these false positives were easily identified in secondary screening.

The new inhibitor (MAC-0038732) of adenosine deaminase discovered in this work is a cyclized imine with an extraordinary eight-membered ring containing a biguanidine moiety. The compound has notable similarity to a deaminase inhibitor reported by Wang and Hosmane (seven-membered ring containing a biguanidine system),[26] which was 100-fold less potent (K_i of 200 μM) but was also a competitive inhibitor. Together, these molecules present an interesting case that large biguanidine-containing ring systems may merit further exploration for their capacity to inhibit ADA. Indeed, ADA inhibitors reported to date are largely nucleoside analogues such as EHNA.[27] MAC-0038732 and other non-nucleoside inhibitors[28] represent fresh approaches to this emerging target in inflammation and immunity.

In conclusion, we report the first use of an aptamer in high-throughput compound screening. Described is the use of a fluorescence-signaling aptamer system in a simple, automated, and homogeneous assay of an otherwise problematic target, adenosine deaminase. The small-molecule screen was robust and yielded a new competitive inhibitor of adenosine deaminase. The work has broad implications for emerging genome-scale screening paradigms in which the goal is to streamline assay development and implementation with universal assay methodologies. Although genome-scale assay parallelization and throughput have great potential for transforming small-molecule screening, these approaches await considerable technological advancements. This is particularly true in biochemical screens for inhibitory compounds in which methods are needed that are in harmony with proteomic approaches and simplify the establishment of assays amenable to automation and throughput. The ability to routinely select aptamers as assay reagents coupled with the performance of fluorescence-signaling aptamers, as demonstrated in the work described herein, suggests that nucleic acid aptamers have exciting potential for this purpose.

Received: April 29, 2006
Published online: July 20, 2006

Keywords: adenosine deaminase · assays · DNA structures · inhibitors · small-molecule screening

[1] R. R. Breaker, *Nature* **2004**, *432*, 838.
[2] S. E. Osborne, I. Matsumura, A. D. Ellington, *Curr. Opin. Chem. Biol.* **1997**, *1*, 5.
[3] S. M. Nimjee, C. P. Rusconi, B. A. Sullenger, *Annu. Rev. Med.* **2005**, *56*, 555.
[4] L. S. Green, C. Bell, N. Janjic, *Biotechniques* **2001**, *30*, 1094.
[5] T. Hermann, D. J. Patel, *Science* **2000**, *287*, 820.
[6] J. S. Hartig, S. H. Najafi-Shoushtari, I. Grune, A. Yan, A. D. Ellington, M. Famulok, *Nat. Biotechnol.* **2002**, *20*, 717.

[7] J. Srinivasan, S. T. Cload, N. Hamaguchi, J. Kurz, S. Keene, M. Kurz, R. M. Boomer, J. Blanchard, D. Epstein, C. Wilson, J. L. Diener, *Chem. Biol.* **2004**, *11*, 499.
[8] R. Nutiu, J. M. Yu, Y. Li, *ChemBioChem* **2004**, *5*, 1139.
[9] M. B. Aldrich, M. R. Blackburn, R. E. Kellems, *Biochem. Biophys. Res. Commun.* **2000**, *272*, 311.
[10] J. Kameoka, T. Tanaka, Y. Nojima, S. F. Schlossman, C. Morimoto, *Science* **1993**, *261*, 466.
[11] J. Linden, *Annu. Rev. Pharmacol. Toxicol.* **2001**, *41*, 775.
[12] G. Giusti, B. Galanti, *Adenosine Deaminase: Colorimetric Method*, *Vol. 4*, 3rd ed., Verlag Chemie, Weinheim, **1984**.
[13] F. Heinz, *Adenosine Deaminase:UV-Method*, *Vol. 4*, 3rd ed., Verlag Chemie, Weinheim, **1984**.
[14] H. M. Oosthuizen, J. P. Ungerer, S. H. Bissbort, *Clin. Chem.* **1993**, *39*, 2182.
[15] D. E. Huizenga, J. W. Szostak, *Biochemistry* **1995**, *34*, 656.
[16] R. Nutiu, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 4771.
[17] R. A. Copeland, *Anal. Biochem.* **2003**, *320*, 1.
[18] J. H. Zhang, T. D. Chung, K. R. Oldenburg, *J. Biomol. Screening* **1999**, *4*, 67.
[19] J. H. Zhang, X. Wu, M. A. Sills, *J. Biomol. Screening* **2005**, *10*, 695.
[20] J. E. Blanchard, N. H. Elowe, C. Huitema, P. D. Fortin, J. D. Cechetto, L. D. Eltis, E. D. Brown, *Chem. Biol.* **2004**, *11*, 1445.
[21] N. H. Elowe, J. E. Blanchard, J. D. Cechetto, E. D. Brown, *J. Biomol. Screening* **2005**, *10*, 653.
[22] X. Li, M. Zolli-Juran, J. D. Cechetto, D. M. Daigle, G. D. Wright, E. D. Brown, *Chem. Biol.* **2004**, *11*, 1423.
[23] M. Zolli-Juran, J. D. Cechetto, R. Hartlen, D. M. Daigle, E. D. Brown, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2493.
[24] S. L. McGovern, E. Caselli, N. Grigorieff, B. K. Shoichet, *J. Med. Chem.* **2002**, *45*, 1712.
[25] A. J. Ryan, N. M. Gray, P. N. Lowe, C. W. Chung, *J. Med. Chem.* **2003**, *46*, 3448.
[26] L. Wang, R. S. Hosmane, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2893.
[27] G. Bastian, M. Bessodes, R. P. Panzica, E. Abushanab, S. F. Chen, J. D. Stoeckler, R. E. Parks, Jr., *J. Med. Chem.* **1981**, *24*, 1383.
[28] T. Terasaka, T. Kinoshita, M. Kuno, I. Nakanishi, *J. Am. Chem. Soc.* **2004**, *126*, 34.

