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Structural analysis of inhibition mechanisms of Aurintricarboxylic Acid on SARS-CoV polymerase and other proteins

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Abstract

We recently published experimental results that indicated Aurintricarboxylic Acid (ATA) could selectively inhibit SARS-CoV replication inside host cells by greater than 1000 times. This inhibition suggested that ATA could be developed as potent anti-viral drug. Here, to extend our experimental observation, we have incorporated protein structural studies (with positive/negative controls) to investigate the potential binding modes/sites of ATA onto RNA-dependent RNA polymerase (RdRp) from SARS-CoV and other pathogenic positive-strand RNA-viruses, as well as other proteins in SARS-CoV based on the fact that ATA binds to Ca2+-activated neutral protease (m-calpain), the protein tyrosine phosphatase (PTP) and HIV integrase which have existing crystal structures. Eight regions with homologous 3D-conformation were derived for 10 proteins of interest. One of the region, Rbinding (754–766 in SARS-CoV’s RdRp), located in the palm sub-domain mainly constituted of anti-parallel H9252-strand-turn-H9252-strand hairpin structures that covers two of the three RdRp catalytic sites (Asp 760, Asp761), was also predicted by molecular docking method (based on free energy of binding ΔG) to be important binding motif recognized by ATA. The existence of this strictly conserved region that incorporated catalytic residues, coupled with the homologous ATA binding pockets and their consistent ΔG values, suggested strongly ATA may be involved in an analogous inhibition mechanism of SARS-COV’s RdRp in concomitant to the case in m-calpain, PTP and HIV integrase.

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1. Introduction

Severe Acute Respiratory Syndrome (SARS) is an infectious disease that appeared in November 2002 (Drosten et al., 2003; Marra et al., 2003; Rota et al., 2003), and has imperilled the health of human population in more than 30 nations during its outbreak. It has claimed over 770 lives and infected more than 8050 people (~9.6% death rate) around the globe. On May 15, 2003, the primary etiological agent for SARS that belongs to the coronavirus family was found to fulfil Koch’s postulate through experimental infection of cynomolgus macaques (Macaca fascicularis). By May 18, 2004, the last human case of infection was successfully contained in China. The detailed chronicles for the discovery of SARS CoronaVirus (SARS-CoV) can be found in websites (James, 2003).

Despite almost two years of intensive SARS-CoV research around the world, the key question regarding the original reservoir of SARS-CoV remains widely speculative and unanswered. This raises the concerns that another outbreak could be imminent. This concern, coupled with the fact that preventive vaccine projects underway now (Marshall and Enserink, 2004) are expected to take years to be developed with possible adverse side effects (Glansbeek et al., 2002; Marshall and Enserink, 2004), the anti-viral agents such as an effective protein inhibitor must be studied exhaustively to define alternative options. In the past, some studies have...
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reported potent inhibitors that block key proteins from corona-

viruses, including those from SARS-CoV (e.g. 3C-Like pro-
tease, S proteins) which critically involved in the viral entry

machinery (Anand et al., 2003; Bermejo Martin et al., 2003;

Chou et al., 2003; Koren et al., 2003; Pace et al., 2004;

Takeda-Shitaka et al., 2004; Wu et al., 2004; Zhang and Yap,

2004). The successful inhibitory binding of these anti-viral

agents to the targeted proteins will disrupt the normal viral

cell cycle and therefore render the viral infection to the human

host cell unsuccessful (Anand et al., 2003; Bermejo Martin

et al., 2003; Chou et al., 2003; Koren et al., 2003; Pace et al.,

2004; Takeda-Shitaka et al., 2004; Wu et al., 2004; Zhang

and Yap, 2004). In contrast to the widely studied proteins men-
tioned above, there are limited documentations reporting the

inhibition of the RNA-dependent RNA polymerase (RdRp)

from any coronaviruses by any potent inhibitor despite the

fact that this attractive protein target is crucial for the

overall viral RNA replication inside the host cells (Dhandi

et al., 2002; Sarnisky, 2004). Recently, we have shown by

experiment, that Aurintricarboxylic Acid (ATA) could selec-

tively inhibit SARS-CoV replication inside host cells with

their viral mRNA transcripts production 1000-fold less than

that in the untreated control (He et al., 2004). This inhibi-
tion of viral replication is possibly related to SARS-CoV’s

RdRp. However, more extensive experiments will be needed
to prove that ATA does bind to RdRp and inhibit the viral

replication. We searched the appearance of term “ATA” in

MedLine and found out that ATA is tightly correlated to poly-

merase activity (Akiyama et al., 1977; Givens and Manly,

1976; Swennen et al., 1978; Swennen et al., 1979). Addi-
tionally, when ATA was compared with interferons (IFN)

alpha (α) and beta (β), which is the current standard treat-

ment for SARS patients, ATA is reported to be approximately

10 times more potent than IFN alpha and 100 times more than

interferon beta at their highest concentrations designed in our

previous experiment. All these observations suggested that

further exploration on ATA might be scientifically a reward-

ing task, and this chemical compound (ATA) can possibly

be developed into an effective anti-SARS drug with high

selectivity. Here, to extend our experimental observations,
Fig. 1. (Continued).
we have incorporated multiple bioinformatic tools to inves-
tigate the structural relationships between ATA and RdRps
of SARS-CoV and other pathogenic positive-strand RNA
viruses (with positive controls). Such a study attempts to
reveal the potential binding modes/sites of ATA onto RdRps
from these viruses. The positive controls used constituted
of three known ATA’s protein targets: (1) the Ca²⁺-activated
neutral protease (m-calpain) (Posner et al., 1995), (2) the
protein tyrosine phosphatase (PTP) of Yersinia pestis (Laang
et al., 2003; Sun et al., 2003) and (3) HIV integrase (Cushman
and Sherman, 1992), which have existing 3D crystal struc-
tures. Eventually, current studies attempt to elucidate the
plausible conformations for the inhibition mode of ATA, and
hence provide clues to rational anti-SARS drug design based
on ATA.

2. Materials and methods

In current study, the protein interactions with small
molecule, ATA, were made up of theoretically-determined
(six structures) and experimentally-determined (13
structures) structures as stated the following. The three-
dimensional theoretical models for RNA-dependent RNA
polymerase (RdRp, ID = 1O5S) (Xu et al., 2003), spike
protein subunit 1 (S1, ID = 1Q4Z) (Spiga et al., 2003; Zhang
and Yap, 2004), spike protein subunit 2 (S2, ID = 1Q4Y)
(Spiga et al., 2003; Zhang and Yap, 2004) of SARS-CoV,
the 3-dimensional crystal structures for nucleocapsid protein
(N protein, ID = 1SSK) (Huang et al., 2004), non-structural
protein 9 (Nsp9, ID = 1Q28) (Egloff et al., 2004), molecular
docking to determine the best conformation in term of lowest
Gibbs free energy and shape complementary was performed
using Autodock 3.0 (Morris et al., 1998). The visualisation
of the 3-dimensional structural data was generated by
Rasmol (Bernstein, 2000).

3. Results

3.1. Binding motifs

Protein 3D structural alignments were performed on 10
amino acid (mainly RdRps) sequences of interest, namely:
(1) Hepatitis C virus RNA-dependent RNA polymerase;
(2) m-calpain; (3) bacteriophage φ6 RNA-dependent
RNA polymerase; (4) Rabbit hemorrhagic disease virus
RNA-dependent RNA polymerase; (5) SARS-CoV RNA-
dependent RNA polymerase; (6) Yersinia pestis phosphatase
yoph; (7) Poliovirus RNA-dependent RNA polymerase; (8)
Bovine viral diarrhea virus RNA-dependent RNA poly-
merase; (9) Norwalk virus RNA-dependent RNA poly-
merase; (10) HIV Type 1 RNA-dependent RNA polymerase.
Regions with homologous 3D-conformation were identified
together with their conserved secondary structures (under-
neath their amino acid alignment in Fig. 1). In total, there
are eight structurally conserved motif blocks (CMBs) with
each block extending at least eight amino acid residues. The
secondary structures for all CMBs include six α-helices and
two β-strands regions. The exact positions of all CMBs in
each protein are provided in Fig. 1.

The molecular docking method based on free energy of lig-
and binding, ΔG, between ATA and all proteins (Figs. 2 and 3)
revealed that ATA binds favorably to one structurally con-
served region (Rbind) among all proteins (Fig. 1 in red
box). As shown in Fig. 4, the corresponding Rbind region
in SARS-CoV’s polymerase (Ser754–Tyr766) overlapped with
one CMB. Rbind is located in the palm sub-domain and con-
stituted mainly of anti-parallel β-strand-turn β-strand hairpin
structures. This conserved region is similar to the majority
of the remaining nine proteins in term of their secondary
structures. Surprising, this Rbind region also contains a
highly conserved ‘XSDD’ amino acid motif that is espe-
cially prominent among viral RdRps, of which two of the
highly conserved aspartic acid (D) residues form the cat-
ylytic center important for polymerase activity (Xu et al.,
2003).

3.2. Binding energy

The free energy of ligand binding (final intermolecular
energy + torsional free energy), ΔG, between ATA and
all proteins were compared (Table 1). For the proteins
that were documented to be inhibited by ATA molecule
were assigned as positive controls (HIV integrase, yoph
and m-calpain), their estimated free energies of binding
were −11.88 kcal/mol, −7.79 kcal/mol and −7.67 kcal/mol,
Fig. 2. Calculated structure (using Autodock) for the interaction of ATA with ypoH. YpoH is a protein tyrosine phosphatase which is essential for virulence in the Yersinia pestis. It is known that the functionality of this protein is inhibited strongly by ATA molecule (Liang et al., 2003). This figure shows the 10 most possible confirmations of ypoH-ATA complexes. The border residues that have contact with ATA are highlighted in red. This region is structurally conserved between ypoH, m-calpain and SARS-CoV RdRps, and constituted mainly of anti-parallel β-strand-turn-β-strand hairpin structures. Below, the two-dimensional chemical structure for ATA (C\textsubscript{22}H\textsubscript{14}O\textsubscript{9}) was shown.

Fig. 3. Calculated structure (using Autodock) for the interaction of ATA with m-calpain. The neutral protease (calpain) is a class of cytosolic enzyme that is activated during apoptosis. It is known to be inhibited strongly by ATA molecule (Posner et al., 1995). This figure shows the 10 most possible confirmations of m-calpain-ATA complexes. The border residues that have contact with ATA are highlighted in red. This region is structurally conserved between ypoH, m-calpain and SARS-CoV RdRps, and constituted mainly of anti-parallel β-strand-turn-β-strand hairpin structures.

Fig. 4. Calculated structure (using Autodock) for the interaction of ATA with RdRp of SARS-CoV. This figure shows the 10 most possible confirmations of RdRp-ATA complexes. The structurally conserved residues between ypoH, m-calpain and SARS-CoV RdRp are colored red, and constituted mainly of anti-parallel β-strand-turn-β-strand hairpin structures.

respectively. Any estimated free energy around/lower than $\Delta G = -7.67$ kcal/mol will potentially suggest similar inhibitory binding mechanism by ATA molecule, if the corresponded binding motif incorporates catalytic sides of that specific protein. When we studied the binding of ATA onto various RNA dependent RNA polymerases (RdRps) from other genomes, the free energy of binding for most RdRps were significantly higher than $\Delta G = -7.67$ kcal/mol, and therefore suggested a lower strength of inhibition upon binding by ATA molecule (Bovine viral diarrhea virus, Dsrna bacteriophage, Feline calicivirus, Hepatitis C virus, HIV, Poliovirus and Rabbit hemorrhagic disease virus). Only the RdRps from SARS-CoV ($\Delta G = -7.68$) and Norwalk virus ($\Delta G = -14.92$) were estimated to have a lower $\Delta G$ ($\geq 7.67$ kcal/mol). This potentiated the possibility that ATA molecule could inhibit strongly the polymerase activity of these two RdRps as in HIV integrase, yph and m-calpain.

4. Discussion

In summary, we first identified the homologous amino acid regions shared by 10 proteins (CMBs) using 3D structural alignments. Of these 10 proteins studied, three proteins were documented to be strongly inhibited by ATA molecules. In parallel, we employed the molecular docking method (based on free energy of ligand binding) to predict the most energy favorable binding conformation of ATA onto these 10 proteins and eventually determined one binding pocket ($R_{binding}$).
RdRp includes two strictly conserved aspartate residues (D) recognized by ATA, that also overlapped with CMBs. This binding pocket (R\textsubscript{bind}), denoted to be 754–766 in SARS-CoV’s RdRp, not only shared a highly conserved secondary structure with other RdRps, but also incorporates two of the three predicted RdRp catalytic residues (Asp 760, Asp761) because ATA does not bind to the active domains of 3CL main protease (Yang et al., 2003).

As reported in Section 3, there are altogether eight structurally conserved motif blocks (CMBs) and their secondary structures include six α-helices and two β-strands regions. Among these structurally conserved regions, we subsequently identified that there was one common region recognized by ATA. The binding of ATA to this region also fulfilled the lowest free energy of ligand binding. By using this free energy of ligand binding, we were able to quantify the binding strength between a macromolecule and a ligand and therefore inhibits the metal ion chelation process crucial for the functionality of this protein during viral replication. In contrast, although we found out that ATA binds to 3CL main protease at a much stronger binding strength (Table 1), we do not anticipate a similar inhibitory binding because ATA does not bind to the active domains of 3CL main protease (Yang et al., 2003).

Furthermore, we also predicted the ATA binding regions for Ca\textsuperscript{2+}-activated neutral protease of Rattus norvegicus (m-calpain), the protein tyrosine phosphatase (PTP) of Yersinia pestis and HIV integrase of Human immunodeficiency virus 1, of which all were experimentally proven to be inhibited by ATA and contain existing crystal structures. However, to the best of our knowledge, there are no literatures attempting to explain explicitly how ATA inhibits the proteins mentioned above. Surprisingly, we performed molecular docking analysis and predicted that ATA binds to the region of these three proteins (Fig. 1) that have correspondence to R\textsubscript{bind}, in SARS-CoV and other viral RdRPs. This region (Fig. 1 in box) was identified to be highly conserved among viral RdRps, in term of their 3D structures and secondary structures. In essence, ATA selectively binds to the β-strand-turn β-strand hairpin structures. Such a strong conservation signifies that this region might be evolutionarily crucial and important for the function of the proteins. Therefore, it remains plausible that ATA inhibits the function of SARS-CoV’s RdRp by bind-

Table 1

| Targeted protein | Type of structure used | Estimated free energy of binding in complex with ATA (kcal/mol) |
|------------------|------------------------|---------------------------------------------------------------|
| RNA dependent RNA polymerase | Crystal structure | −5.82 |
| RdRp (Bovine viral diarrhea virus) | Crystal structure | −5.87 |
| RdRp (Feline calicivirus) | Theoretical model | −5.83 |
| RdRp (Hepatitis C virus) | Crystal structure | −5.8 |
| RdRp (HIV) | Crystal structure | −5.82 |
| RdRp (Norwalk virus) | Crystal structure | −14.92 |
| RdRp (Poliovirus) | Crystal structure | −5.83 |
| RdRp (Rabbit hemorrhagic disease virus) | Crystal structure | −5.94 |
| RdRp (SARS-CoV) | Theoretical model | −7.68 |

Protein known to be inhibited by ATA\textsuperscript{a}

- YopH\textsuperscript{b}
- HIV integrase\textsuperscript{c}
- m-Calpain\textsuperscript{d}
- N protein
- Nsp 9
- S1 (Spiga et al., 2003)
- S1 (Zhang and Yap, 2004)
- S2 (Zhang and Yap, 2004)
- N protein
- Main protease (3CL)
- HIV integrase
- Ca\textsuperscript{2+}

\textsuperscript{a} Cushman and Sherman (1992), Liang et al. (2003), Posner et al. (1995).
ing to the homologous region as in m-calpain, PTP and HIV integrase.

Finally, we had also analyzed the binding of ATA onto viral RdRps from the standpoint of free energy of ligand binding (ΔG). The results of ΔG between ATA and all viral RdRps, as well as the three proteins known to be readily inhibited by ATA molecules (Table 1), suggested that ATA binds to RdRp from SARS-CoV at the equivalent binding strength similar to yohop and m-calpain. On the other hand, ATA was predicted to bind to the remaining viral RdRps (except for Norwalk virus) at a much lower binding strength whereas ATA binds to RdRp from Norwalk virus at a significantly higher binding strength (approx. two times). If our hypothesis that ATA inhibits the functionality of RdRps by binding to its catalytic sites is indeed true, these numerical representations of ΔG would likely represent how well ATA will inhibit the viral replication inside virus-infected cell culture. To prove our hypothesis, we had also carried out some experimental study on how well ATA interrupts the viral replication in SARS-infected cell culture and demonstrated successfully that the viral load (e.g. HCV, West Nile virus and Feline calicivirus, etc.), with the addition of ATA at the highest concentration, corresponds to the ranking of the estimated free energy of ligand binding (experimental data to be published).

5. Conclusions

The following conclusions can be drawn from this structural study on the binding of ATA to proteins from SARS-CoV and other positive-strand RNA viruses:

1. ATA could bind to SARS-CoV’s RdRp and other SARS-CoV’s proteins. The strongest binding was predicted to be for S1 protein. By binding to a protein, ATA could inhibit their activity if the ATA binding motifs incorporate catalytic domains of that specific protein, for example: ATA binds to the catalytic sites of SARS-CoV’s RdRp and inhibit its function.

2. Derived from the inhibitory binding of ATA on SARS-CoV’s RdRp, ATA was also predicted to inhibit the poly-merase activity of RdRps from other RNA viruses in a weaker manner (Bovine viral diarrhea virus, DNA bacteriophage, Feline calicivirus, Hepatitis C virus, HIV, Poliovirus, Rabbit hemorrhagic disease virus). However, stronger inhibition (approx. two times) was predicted for the RdRp from Norwalk virus (experimental data to be published).

3. This inhibitory binding mechanism, especially for RdRps, could serve to explain why mRNA transcripts were decreased by >1000 times when SARS-infected cell culture was treated with ATA in our previous study. It could also explicate why the inhibition on the replication on other RNA viruses are milder when virus-infected cells was treated with ATA (experimental data to be published).

4. ATA could serve as a template for rational anti-SARS drug design, targeting the replication machinery possibly over large groups of positive-strand RNA viruses.

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