HIV-1 Integrase Strand Transfer Inhibitors with Reduced Susceptibility to Drug Resistant Mutant Integrases

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Supporting Information

ABSTRACT: HIV integrase (IN) strand transfer inhibitors (INSTIs) are among the newest anti-AIDS drugs; however, mutant forms of IN can confer resistance. We developed nontoxic naphthyridine-containing INSTIs that retain low nanomolar IC50 values against HIV-1 variants harboring all of the major INSTI-resistant mutations. We found by analyzing crystal structures of inhibitors bound to the IN from the prototype foamy virus (PFV) that the most successful inhibitors show striking mimicry of the bound viral DNA prior to 3′-processing and the bound host DNA prior to strand transfer. Using this concept of “bi-substrate mimicry,” we developed a new broadly effective inhibitor that not only mimics aspects of both the bound target and viral DNA but also more completely fills the space they would normally occupy. Maximizing shape complementarity and recapitulating structural components encompassing both of the IN DNA substrates could serve as a guiding principle for the development of new INSTIs.

Inhibiting enzymes that modify DNA or RNA substrates has the potential to be used in the treatment of a wide range of diseases. Polynucleotide transferases are important targets for drug development; however, the catalytic centers of these enzymes are often extended to accommodate their large substrates. It has been difficult to design potent, specific, and selective small molecule inhibitors. Fortunately, this has not been an intractable problem, as exemplified by the development of drugs that act as interfacial inhibitors of HIV-1 integrase (IN), one of three viral enzymes that are essential for viral replication. IN inserts viral DNA into the host genome through two sequential reactions. The first reaction, termed “3′-processing” (3′-P), involves the cleavage of the 3′-dinucleotides from viral DNA; the second reaction “strand transfer” (ST) involves the subsequent insertion of the processed ends of the viral DNA into host DNA. These reactions take place in the context of a stable nucleoprotein complex containing a multimer of IN assembled on viral DNA ends, referred to as the intasome. Divalent metal cofactors are essential for IN-mediated catalysis, and chelation of the active site Mg2+ ions is a key component of the successful integrase strand transfer inhibitors (INSTIs), which represent the newest class of anti-AIDS drugs. However, in infected patients, the HIV-1 genome diversifies rapidly due to an accumulation of mutations that arise during viral replication. Consequently, the emergence of resistance is a limitation for all anti-HIV therapeutics, including INSTIs, and resistant forms of the virus have been isolated from patients who received raltegravir (RAL, 1) or elvitegravir (EVG, 2), the first two INSTIs approved by the FDA for the treatment of HIV/AIDS. More recently, dolutegravir (DTG, 3) has come to market as a second generation INSTI. DTG appears to be considerably less prone to virological failure than RAL and EVG. However, viruses that carry IN resistance mutations can be selected by growing the virus in the presence of DTG, and mutations that confer cross-resistance to all three INSTIs have been identified.

A primary objective of continued INSTI development is to discover compounds with minimal toxicity that retain good effectiveness against existing resistant mutants. Until now, efforts to create new drugs that retain broad efficacy against the...
resistant forms of IN have largely been empirical. This is in spite of the availability of “cocrystal” structures of the leading first and second-generation INSTIs bound to the intasome formed by the othologous enzyme from prototype foamy virus (PFV) in a complex with metal cofactors and the cognate viral DNA substrate (referred to as the “intasome”).\(^1\)\(^8\)\(^–\)\(^2\)\(^1\) Although these advances elucidated the structural basis for INSTI function, they have not yet led to principles that can guide the design of the next generation INSTIs, which would retain effectiveness against the known resistant IN mutants.\(^6\)\(^,\)\(^2\)\(^2\)

In our current work, we empirically varied the structure of our recently disclosed 1-hydroxy-2-oxo-1,8-naphthyridine-containing INSTIs (4) to improve their ability to potently block in cell culture, the replication of a panel of HIV-1 based vectors that carry all of the major INSTI-resistant IN mutants.\(^2\)\(^3\)\(^,\)\(^2\)\(^4\) We also obtained “cocrystal” structures of the PFV intasome with the best inhibitors and found that when they bind to IN, they show striking mimicry of the binding of viral DNA in its initial state, prior to the cleavage of the dinucleotide in the 3′-P reaction. The inhibitors also mimic features of target DNA in their binding to the intasome active site.\(^2\)\(^5\) Our findings suggest that employing aspects of molecular mimicry of viral and host DNA may be useful in the design of improved INSTIs. Importantly, these principles may also be applicable to the broader class of enzymes that have DNA or RNA substrates.

## RESULTS AND DISCUSSION

### Development of INSTIs That Retain Efficacy against RAL-resistant Mutant Forms of IN.

INSTIs selectively bind at the interface of IN and the viral DNA end following its 3′-P,\(^1\)\(^8\)\(^,\)\(^1\)\(^9\)\(^,\)\(^2\)\(^5\) INSTIs contain a triad of heteroatoms (shown in red in Figure 1) that chelate the essential pair of Mg\(^{2+}\) ions in the IN active site. INSTIs also typically include a halobenzyl ring that stacks against the base of the penultimate deoxycytidine near the processed 3′-end of the viral DNA (shown in blue in Figure 1).\(^3\)\(^,\)\(^2\)\(^6\) Our long-term goal is to develop small molecules that are active against IN mutants that are resistant to current INSTIs. Initially, our efforts were directed at the retention of efficacy against the Y143R and N155H mutants and the double mutant, Q148H/G140S.\(^1\)\(^3\)\(^–\)\(^1\)\(^6\) While these are associated with clinical HIV-1 resistance to RAL, there is a considerable overlap in the resistance profiles of RAL and EVG.\(^2\)\(^7\)\(^,\)\(^2\)\(^8\) Prior to the current work, we had performed extensive investigations on a core 1-hydroxy-2-oxo-1,8-naphthyridine platform.\(^3\)\(^,\)\(^2\)\(^4\) These efforts yielded compound 4a, with a ST inhibitory value of IC\(_{50}\) = 19 nM in an \textit{in vitro} IN assay (compound 4a, Table 1) and an antiviral potency equivalent to RAL (EC\(_{50}\) ≃ 1 nM) against HIV vectors carrying wild-type (WT) IN in single-round replication assays (Table 2). In similar assays, RAL shows a significant loss of antiviral efficacy when the HIV-1 vectors employed carried the drug resistance mutations Y143R, N155H, or Q148H/G140S mutants (EC\(_{50}\) ≃ 160 nM, 150 nM, and 1900 nM, respectively). In contrast, 4a shows effective retention of antiviral potency against both the Y143R and N155H variants (EC\(_{50}\) ≃ 2 nM and 5 nM, respectively, Table 2).\(^2\)\(^4\) These values are similar to those displayed by DTG; however, DTG is more potent than 4a against the Q148H/G140S double mutant (4a EC\(_{50}\) ≃ 35 nM; DTG EC\(_{50}\) ≃ 6 nM).\(^2\)\(^4\)

#### Overcoming Loss of Potency against the Integrase Double Mutant Q148H/G140S.

The extended tricyclic ring system of DTG permits its third ring (highlighted in green in Figure 1) to contact the β4-α2 loop in the active site of IN (highlighted in orange, Figure 2). Maintenance of interactions in this region has been suggested to be important for the binding of DTG, as well as for the binding of other second generation INSTIs.\(^2\)\(^0\)\(^,\)\(^2\)\(^9\)\(^–\)\(^3\)\(^1\) In order to determine the importance of these interactions, we modified 4a by adding substituents at its 6-position (highlighted in green in Figure 1). Our intent was to create hydrogen bonds with the protein or with bound water molecules proximal to the 6-position. Although there are a variety of constructs that could be used, the work reported herein is focused on simple linear methylene chains of increasing lengths with terminal hydroxyl groups (compounds 4b–4e, Table 1 and Supporting Information Scheme S1). The \textit{in vitro} ST inhibitory potencies were relatively independent of chain length in going from three methylenes (4b, IC\(_{50}\) value = 11 nM) to eight methylenes (4e, IC\(_{50}\) value = 5 nM). The most potent analogue (by a small margin) was 4c with IC\(_{50}\) = 2.7 nM; Table 1), which also displayed an antiviral inhibitory potency against the Q148H/G140S mutant (4c, EC\(_{50}\) ≃ 7 nM), which was similar to that of DTG (Table 2).

### Table 1. Inhibitory Potencies of Compounds 4a–4f Determined Using an \textit{in Vitro} IN Assay\(^a\)

| no. | R | IC\(_{50}\) (μM) |
|-----|---|-------------|
| 4a  | H | 2.5 ± 0.3   |
| 4b  | (CH\(_2\))\(_3\)OH | 4.3 ± 0.6 |
| 4c  | (CH\(_2\))\(_2\)OH | 8.2 ± 1.2 |
| 4d  | (CH\(_2\))\(_3\)OH | 19 ± 1   |
| 4e  | (CH\(_2\))\(_2\)OH | 32 ± 6   |
| 4f  | (CH\(_2\))\(_2\)SO\(_2\)Ph | 0.0037 ± 0.0013 |

\(^a\)Assays were performed using a gel-based protocol with Mg\(^{2+}\) cofactor as described.\(^b\)\(^3\)\(^1\) Previously reported values.\(^2\)\(^4\)

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Table 2. Antiviral Potencies in Cells Infected with HIV-1 Vectors That Carry WT or Resistant IN Mutants

| no. | CC50 (μM) | EC50 (nM) | Y143R | N155H | G140S/Q148H | GI18R |
|-----|-----------|-----------|--------|-------|-------------|-------|
| 1   | > 250     | 4 ± 2     | 162 ± 16 (41) | 154 ± 33 (39) | 1900 ± 300 (475) | 36 ± 5 (9) |
| 2   | > 250     | 6.4 ± 0.8 | 7.9 ± 2.3 (12) | 90 ± 18 (14) | 5700 ± 1100 (891) | 21 ± 10 (3.3) |
| 3   | > 250     | 1.6 ± 0.9 | 4.3 ± 1.2 (2.7) | 3.6 ± 1.3 (2.3) | 5.8 ± 0.5 (3.6) | 13 ± 5 (8.1) |
| 4a  | > 250     | 1.1 ± 0.7 | 2.5 ± 0.6 (2.3) | 5.3 ± 2.3 (4.8) | 35 ± 9 (32) | 16 ± 5 (15) |
| 4b  | > 250     | 3.1 ± 2.0 | 2.8 ± 0.9 (0.9) | 8.5 ± 3.1 (2.7) | 13.4 ± 6.5 (4.3) | 10 ± 1.6 (3.2) |
| 4c  | > 250     | 1.3 ± 0.2 | 3.0 ± 0.5 (2.3) | 2.4 ± 0.8 (1.8) | 6.9 ± 2.3 (5.3) | 5.3 ± 1.6 (4.1) |
| 4d  | > 250     | 2.3 ± 0.6 | 2.1 ± 1.4 (0.91) | 2.7 ± 1.0 (1.2) | 9.4 ± 3.6 (4.1) | 6.4 ± 2.5 (2.8) |
| 4e  | > 250     | 3.7 ± 0.4 | 3.2 ± 0.8 (0.86) | 6.5 ± 2.7 (1.8) | 7.7 ± 1.9 (2.1) | 11 ± 1 (3.0) |
| 4f  | > 250     | 2 ± 0.1  | 0.6 ± 0.1 (0.3) | 2.1 ± 1.2 (1) | 5.2 ± 0.3 (3) | 11.4 ± 3.5 (6) |

EC50 (nM)cd

| no. | R263K | T66L | E92Q | H51Y | H51Y/R263K |
|-----|-------|------|------|------|------------|
| 1   | 5.7 ± 2.3 (1.4) | 2.8 ± 0.4 (0.7) | 30 ± 10 (7.5) | 3.4 ± 0.2 (0.2) | 6 ± 2.3 (1.5) |
| 2   | 10 ± 6 (1.6) | 66 ± 1 (10) | 154 ± 34 (24) | 4.5 ± 2.1 (0.7) | 53 ± 18 (8) |
| 3   | 11 ± 3 (6.9) | 0.9 ± 0.8 (0.56) | 2.3 ± 0.4 (1.4) | 3.2 ± 0.2 (2) | 16 ± 1 (10) |
| 4a  | 6.4 ± 2.3 (5.8) | 0.6 ± 0.3 (0.54) | 3.0 ± 1.8 (2.7) | 1.0 ± 0.6 (0.91) | 3.2 ± 0.9 (2.9) |
| 4b  | 2.5 ± 0.8 (0.81) | 1.1 ± 0.1 (0.35) | 4.8 ± 2.0 (1.5) | 2.6 ± 0.5 (0.84) | 15 ± 2 (4.8) |
| 4c  | 2.6 ± 0.1 (2.0) | 0.93 ± 0.24 (0.72) | 3.8 ± 2.3 (2.3) | 3.8 ± 0.6 (2.9) | 2.6 ± 1.4 (2.0) |
| 4d  | 8.4 ± 2.3 (3.7) | 0.50 ± 0.35 (0.22) | 0.74 ± 0.13 (0.32) | 1.6 ± 0.1 (0.70) | 5.2 ± 1.6 (2.3) |
| 4e  | 3.8 ± 1.1 (1.0) | 1.7 ± 0.5 (0.46) | 2.6 ± 0.2 (0.70) | 4.8 ± 1.1 (1.3) | 22 ± 9 (5.9) |
| 4f  | 5.3 ± 0.8 (2) | 0.7 ± 0.1 (0.3) | 1.2 ± 0.6 (0.6) | 0.8 ± 0.5 (0.4) | 5.3 ± 0.4 (2) |

EC50 (nM)cd

*Cytoxic concentration resulting in 50% reduction in the level of ATP in human osteosarcoma (HOS) cells. bValues obtained from cells infected with a lentiviral vector carrying WT IN. cCells were infected with viral vectors carrying IN mutations. The table shows the EC50 values. dFold-change (FC) in EC50 relative to WT. ePreviously reported values.*

![Figure 2. DTG and 4a binding in the active site of the PFV intasome.](image)

Compounds with 6-Substituents Potently Inhibit DTG-resistant IN Mutants. We expanded the panel of HIV vectors used in the antiviral assays to include additional IN mutants that have a reduced susceptibility to DTG, including the G118R, R263K, T66L, E92Q, H51Y single mutants and the H51Y/R263K double mutant.33–16 We were particularly interested in whether 4c retained potency against these resistant mutants, because 4c had the best profile against the original panel of mutants. This compound inhibited the replication of the T66L, E92Q, and H51Y mutants as well as DTG and, encouragingly, also exhibited improved antiviral potencies relative to DTG against the G118R and R263K single mutants and the H51Y/R263K double mutant (Table 2). Importantly, none of the analogues of 4 showed appreciable cytotoxicity.

X-ray Crystal Structures of 4a and 4c Bound to the PFV Intasome. We refined a structure of the PFV intasome in complex with 4a to a resolution of 2.58 Å and compared it to the previously reported structure with DTG (PDB accession code: 3S3M).20 The 8-naphthyridine nitrogen and N-hydroxyl of 4a correspond to the 6-oxo and 7-hydroxyl heteroatoms of DTG, and the naphthyridine 2-oxo carbonyl of 4a corresponds to the ring 8-oxo carbonyl of DTG (Figure 2). As we had anticipated in our original design strategy,23 the halobenzyl carboxamido group of 4a is not involved in metal chelation. We also examined the contact residues for DTG (shown in Figure 2 and listed in the Supporting Information Table S2) and noted (as has previously been reported23) that DTG makes contact with PFV integrase Gly187 and Tyr212, which correspond to HIV-1 IN residues Gly118 and Tyr143, which are associated with RAL-resistant mutations (Table 2). In contrast, 4a does not make contact with either the Gly187 or Tyr212 residues (see Supporting Information Table S2). The 15-fold loss of potency of the GI18R virus to 4a is likely due to steric exclusion of the compound by the bulky Arg side chain. Neither DTG nor 4a directly contacts other IN residues associated with INSTI resistance. This is consistent with prior observations that for inhibitors binding to the IN active site, resistance mutations do not usually arise due to a loss of IN–drug interactions.19,20

Next, we determined the structure of the PFV intasome crystallized with the 6-substituted compound 4c (Figure 3).
Until now, RAL was the only INSTI known to inhibit the interaction of the viral integrase (IN) Tyr143 with the viral and host DNA substrates. However, in case of RAL, the interaction depends on the position of the terminal portion of the 6-side chain of 4c, where the halobenzyl moiety is bound (Figure 4A). These interactions IN shows with the backbone amide and the phenolic side chain of Tyr212 via bridging water molecules, while the other makes close van der Waals contacts with backbone atoms of Gln186 and Gly187 in the βα-α loop (Figure 4A). Strikingly, the sulfonfylphenyl ring is bent back under the plane of the metal-chelating naphthyridinone ring system, where it makes an intramolecular π–π stacking interaction (3.3 Å separation) (Figure 4A and B). By superimposing this structure with the structures of both the uncleaved (pre-3′-P) viral DNA and the target DNA (ST) substrates (PDB codes 4E71 and 4E7K, respectively), it is apparent that the sulfonfyl group fills the envelope formed by the overlap in the positions of both the bound uncleaved viral and the bound target DNAs (Figure 4B). In addition, the position of the sulfonfylphenyl ring is similar to the position of the deoxyribose sugar of the adenine A1 in the scissile A-T dinucleotide of the uncleaved viral DNA, while other portions of the 6-side chain are similar to the position of the deoxyribose sugar of the G1 guanosine in the target DNA (Figure 4B).

The face-on-face stacking of the sulfonfylphenyl ring of 4f onto its own heterocyclic core allows it to almost completely fill the catalytic pocket. When 4f is bound, its sulfonfylphenyl ring packs against the side chain β-methylene of the metal-chelating residue D185 (≈ 3.8 Å) and against the α-methylene of the Tyr212 backbone (≈ 3.7 Å). The sulfonfylphenyl ring also nestles against the back face of the Pro214 pyrrolidine ring, a key residue that helps form the hydrophobic binding pocket where the halobenzyl moiety is bound (Figure 4A). These results demonstrate improved overlap in the bound form of 4f and portions of both target and viral DNA substrates as compared to the bound form of 4c, which was our goal in designing 4f.

INSTIs are among the most effective antiretroviral drugs for the treatment of HIV/AIDS. However, as has been true for all anti-HIV drugs, the development of resistant IN mutants has been a limitation for RAL and EVG, the first two of the
current FDA-approved members of this family. Although the most recently introduced INSTI, DTG, appears to be considerably more resilient to viral resistance, it is not infallible. Therefore, continued research is needed to obtain INSTIs that are active against all of the known resistant mutants.

In the current work, we introduced functionality at the 6-position of our previously reported INSTI 4a to take advantage of potential binding interactions afforded by the third [(R)-4-methyl-1,3-oxazinane] ring of DTG. Although the approach was initially empirical, it yielded analogues, such as 4c, that retain excellent inhibitory potencies against the major drug-resistant IN mutants. By analyzing crystal structures of the PFV intasome in complex with some of the new compounds, we realized that the 6-substituent of 4c recapitulates functionalities present in both an uncleaved viral DNA substrate in the pre-3′-P complex and the target DNA prior to ST. This represents an unusual form of "bi-substrate" mimicry, which we attempted to enhance by introducing a sulfone group at the 6-position. Among the resulting compounds is the phenylsulfone 4f, which shows potent activity against recombinant HIV IN in biochemical assays and good efficacy in assays done in cell culture against a broad panel of viral mutants carrying the major RAL and DTG IN resistance mutations. Importantly, a crystal structure of 4f within the PFV intasome shows that 4f occupies the sites where both the target and viral DNA are bound and largely fills the space that is available at the catalytic center.

The molecular interactions of 4c and, to a greater extent, 4f exemplify a more general concept of "substrate envelope" originally used to explain why some HIV protease inhibitors are able to retain efficacy against multiple resistance mutations. The rationale underlying this principle is that any mutant form of an enzyme that retains good enzymatic activity must be able to bind its cognate substrate(s). Thus, an inhibitor that binds to an enzyme in a fashion such that the inhibitor remains entirely

Figure 4. PFV Intasome crystal structure complexed with 4f. (A) Crystal structure oriented to show the complexed Mg^{2+} ions (blue spheres) and the semitransparent surface of 4f (orange surface with carbons in cream), 3′ P DNA (purple surface), and protein (gray surface) with key protein residues shown (carbons in light gray). Important binding interactions are indicated. (B) Bound 4f with superimposed pre-3′-processed DNA (carbons in purple; PDB code: 4E71[^3]) and target DNA (carbons in black; PDB code: 4E7K[^3]). Regions where the side chain of 4f overlaps with DNA are indicated by black dashed ovals.
within the envelope defined by the structure of the normal substrate(s) will be able to bind efficiently to both WT and (enzymatically active) mutant forms of the enzyme. The fact that our 6-substituted analogues have structural components that bind in ways that are similar to the target and viral DNAs points the way to developing new and more broadly effective INSTIs.25

Shape complementarity also plays an important role in protein–ligand recognition.34 While simultaneously remaining with an envelope defined by the cognate substrates, the 6-substituted analogues, 4c and 4f, occupy more of the catalytic site volume (as defined by the structure of the protein and the 3′-processed DNA) than the unsubstituted parent 4a. Thus, the compounds have good shape complementarity without protruding beyond the substrate envelope. This has resulted in potent inhibitors that retain high efficacy against the entire panel of resistant mutant forms of IN.

Our findings provide a unifying theme for understanding why there are common structural features in some of the most successful second generation INSTIs. For example, although it is known that the terminal (R)-1-methyl-1,3-oxazinane ring of DTG is important for its potency against resistant mutant forms of IN, this portion of the molecule was developed empirically.30 In the PFV cocystal structures of DTG (PDB code: 3S3M),20 the position where the third ring is bound overlaps with portions of a bound MK-2048 molecule (PDB code: 3OYB).19 MK-2048 is another second generation tricyclic INSTI that retains good inhibitory potency against RAL-resistant mutants. For both DTG and MK-2048, the overlapping regions project toward the β4-α2 loop in the respective PFV intasome cocystal structures (see Figure 2). Examination of structures of the PFV intasome with other second generation INSTIs bound, including “Compound G” (PDB code: 3OYG)19 and MK0536 (PDB code: 3OYH;19 these structures are shown in the Supporting Information, Figure S2) and its more recent analogues,31 shows that these molecules bind within a volume that is defined by the bound substrates. We previously proposed that the contacts made between the compounds and HIV-1 IN may be an important feature that helps some INSTIs retain efficacy against RAL-resistant mutants.19

Our current work shows that portions of the most successful INSTIs can be viewed as occupying regions defined by the binding of the precleaved viral DNA and target DNA, which helps to explain why the compounds are broadly effective against the known drug resistant IN mutants. The principle of maximizing shape complementarity and recapitulating the structural components (structural complementarity) of two of the substrates, the target and viral DNAs, could serve as a guiding principle for the development of new INSTIs.

**METHODS**

**Synthetic.** Detailed synthetic procedures and analytical data for the synthesis of final products 4b–4f are provided in the Supporting Information.

**Recombinant Proteins, Biochemical Assays, and Crystallography.** IN proteins were produced in *E. coli*; HIV-1 IN biochemical assays and PFV crystallography were carried out as previously described.31,32 Detailed experimental protocols are provided in the Supporting Information.

**Single-round HIV-1 Infectivity Assay.** Assays were performed using the human embryonic kidney cell culture cell line 293 acquired from American type culture collection (ATCC) and the human osteosarcoma cell line, HOS, obtained from Dr. Richard Schwartz (Michigan State University, East Lansing, MI) using methods that have been previously reported36,37 and are described in detail in the Supporting Information.

**X-ray Crystallography.** PFV intasome crystals grown as previously described38−40 were soaked in the presence of 0.5–1 mM INSTIs in cryoprotection solution prior to snap freezing in liquid nitrogen. X-ray diffraction data were collected on beamline I04 of the Diamond Light Source (Oxfordshire, UK) and processed using XDS.38 Structures were solved using rigid-body refinement of 4BDZ (protein and DNA components), and small molecules were fitted into difference maps. The structures were built in Coot,41 refined in Phenix,42 and validated using MolProbity.43 Data collection and refinement statistics are given in Supporting Information Table S1.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00948.

**Accession Codes**

Coordinates and structure factors were deposited with the Protein Data Bank under accession codes SFRM, SFRN, and SFRO.

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**Notes**

The authors declare the following competing financial interest(s): Compounds described in the paper are included in pending or filed patent applications.

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