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A Cascade Screening Approach for the Identification of Bcr-Abl Myristate Pocket Binders active against Wild Type and T315I Mutant

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ABSTRACT

The major clinical challenge in drug-resistant chronic myelogenous leukemia (CML) is currently represented by the Bcr-Abl T315I mutant, which is unresponsive to treatment with common first and second generation ATP-competitive tyrosine kinase inhibitors (TKIs). Allosteric inhibition of Bcr-Abl represent a new frontier in the fight against resistant leukemia and few candidates have been identified in the last few years. Among these, myristate pocket (MP) binders discovered by Novartis (e.g. GNF2/5) showed promising results, although they proved to be active against the T315I mutant only in combination with first and second generation ATP-competitive inhibitors. Here we used a cascade screening appraoch based on sequential fluorescence polarization (FP) screening, *in silico* docking/dynamics studies and kinetic-enzymatic studies to identify novel MP binders. A pyrazolo[3,4-*d*]pyrimidine derivative (**6**) has been identified as a promising allosteric inhibitor active on 32D leukemia cell lines (expressing Bcr-Abl WT and T315I) with no need of combination with any ATP-competitive inhibitor.

The discovery of proteins directly involved in the pathogenesis of many tumor types has open the way to the development of selective drugs for specific cancers.[1] One of the first examples of this successful approach is represented by the use of tyrosine kinase inhibitors (TKIs) for the treatment of chronic myeloid leukemia (CML), with imatinib mesylate (IM, Gleevec™) being the first Bcr-Abl targeting drug approved by the FDA in 2001.[2] Despite the high rate of complete cytogenic response (CCyR) from patients treated each year with IM in frontline therapy, around 4-13% of these patients can develop resistance or tolerance to this drug, leading to therapeutic failure. The most common mechanism of IM resistance is represented by point mutation in the kinase domain of Bcr-Abl and, in such cases, second generation TKIs (Dasatinib, Nilotinib, Bosutinib) can be used in second line therapy.[3]

However, the major clinical challenge is currently represented by the cross-resistant Bcr-Abl T315I mutant which accounts for 15-20% of clinically observed mutations and is frequently selected on sequential inhibitor therapy. Unfortunately, only one drug (Ponatinib, Iclusig™) has recently achieved accelerated approval for the treatment of this resistant CML form, but severe side effects have limited its clinical application.[4] As a consequence, the identification of novel Bcr-Abl inhibitors active on the T315I mutant is one of the top priorities in CML research and only one additional promising candidate (Axitinib) has been recently found by Pfizer in a drug-repurposing study (Figure 1).[21]

Despite the fact that all the Bcr-Abl inhibitors approved so far for CML treatment target the conserved ATP binding pocket and share an ATP-competitive mode of action and considering the poor results obtained in the inhibition of the Bcr-Abl T315I mutant with these inhibitors, many efforts are currently devoted to the development of allosteric inhibitors of Bcr-Abl and, especially, of its T315I mutant.[5] Targeting sites outside the ATP-binding cleft could in fact present several advantages over the

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common ATP-competitive strategies: *i)* minimize the side-effects caused by the limited selectivity of competitive inhibitors; *ii)* overcome the drug-resistance induced by ATP-competitive inhibitors; *iii)* lower doses of the allosteric drugs could be used since these compounds do not have to compete with the high intracellular concentration of ATP; *iv)* allow to finely modulate the kinase activity without affecting the basal activity in order to avoid some toxic effects which can occur due to the complete abrogation of the enzyme functions. A few different classes of allosteric inhibitors active on the T315I mutant have been discovered in the last few years (Figure 1).[5,20,21]

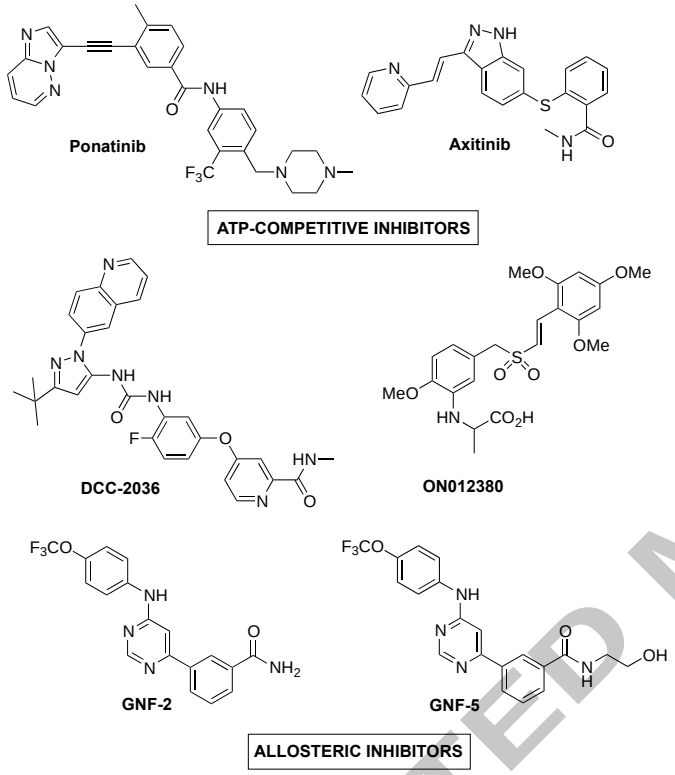

Figure 1. Representative ATP-competitive and allosteric Bcr-Abl T315I inhibitors

Deciphera Pharmaceuticals has developed T315I inhibitors (DCC-2036 and DCC-2157) that bind to structural pockets used by Abl to switch between the inactive and active conformations (switch-pocket inhibitors).[6] Onconova Therapeutics has developed a substrate competitive inhibitor (ON012380) active against 100% of IM-resistant forms of Bcr-Abl, including the T315I mutant.[7] The family of myristate pocket binders, discovered by Novartis,[8] holds a promising future. Indeed, a major issue of ATP-competitive inhibitors is represented by the easiness of mutation development due to the evolutionary pressure, that is less severe in the myristate-binding pocket (MP). For this reason MP is an ideal target for the development of allosteric drugs. The lead myristate-binder GNF-2 and its optimized derivative GNF-5 proved to be active against the T315I Bcr-Abl mutant only in combination with first and second generation ATP-competitive inhibitors (Imatinib, Dasatinib, Nilotinib), giving complete remission in a T315I Bcr-Abl mutant murine bone-marrow transplantation model.[9] Solution NMR, X-ray crystallography, mutagenesis and hydrogen exchange mass spectrometry experiments confirmed that GNF-2/5 binds to the MP of Abl and showed how this binding led to changes in the structural dynamics of both the MP and the distant ATP-binding site (both in Abl WT and T315I mutant) with functional consequences on the catalytic machinery.[9,10] All these data confirmed the existence of a cross-talk between the MP and ATP-binding site but were unable to explain why myristate-binders GNF-2/5 alone were able to inhibit only wild type Bcr-Abl and not the T315I mutant.

As a continuation of our efforts in the discovery of novel compounds active against the T315I mutant,[11-14] we became interested in the identification of compounds that could bind the allosteric site of Abl: such compounds could mimic the synergistic effect of GNF-derivatives and ATP-competitive inhibitors but could also switch from the catalytic pocket to the MP depending on the nature of the gatekeeper residue (T315 or I315). To identify compounds with the above behavior, we planned to submit our internal collection of ATP mimetics[11,15-16] to the following cascade screening approach: *1)* high-throughput fluorescence polarization (FP) screening on Abl WT (kinase domain and full-length); *2)* docking and molecular dynamics (MD) studies on the MP pocket; *3)* kinetic-enzymatic studies on Abl WT and T315I; *4)* assays on WT and T315I expressing cells. Around 200 compounds were initially analyzed for their ability to displace a GNF-2 based tracer (cmpd **1**, Figure 2) from the MP using GNF-2 as positive control and C4 N-methyl GNF-2 as negative control[17] via FP assay (experimental details are reported in the Supporting Information). To eliminate any possible inter-domain interaction effect on the displacement of the tracer **1**, FP screening (at 10 μM of tracer and test cmpds) was conducted on both the kinase domain and full-length Abl WT. Only compounds able to displace the tracer (>30% displacement at 2h) from both kinase domain and full-length Abl were considered as positive hits and submitted to further investigation (cmpds **2-6**, Figure 2). However, Lebakken and colleagues observed that a tracer binding to the ATP site of Abl could be displaced by GNF-2 as a consequence of the cross-talk between the MP and the catalytic site.[18] It is therefore reasonable to assume that a similar behavior could be observed in the displacement of the tracer **1** by ATP-competitive inhibitors, leading to the identification of false positive hits.

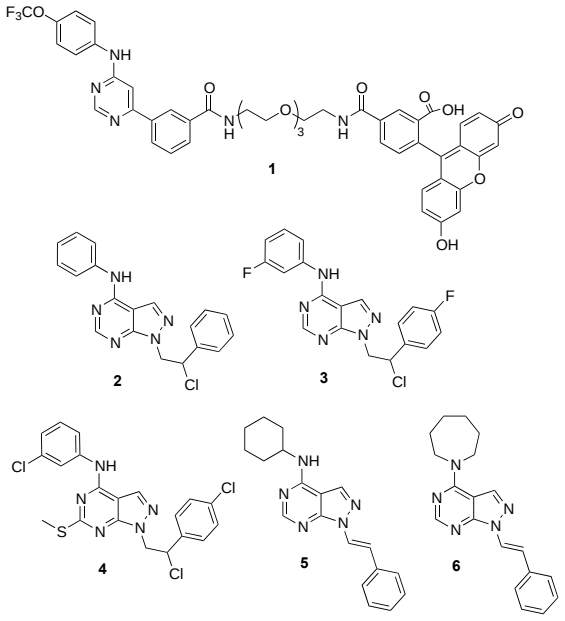

Figure 2. GNF-2 based fluorescent probe (**1**) and compounds selected from FP assay (**2-6**).

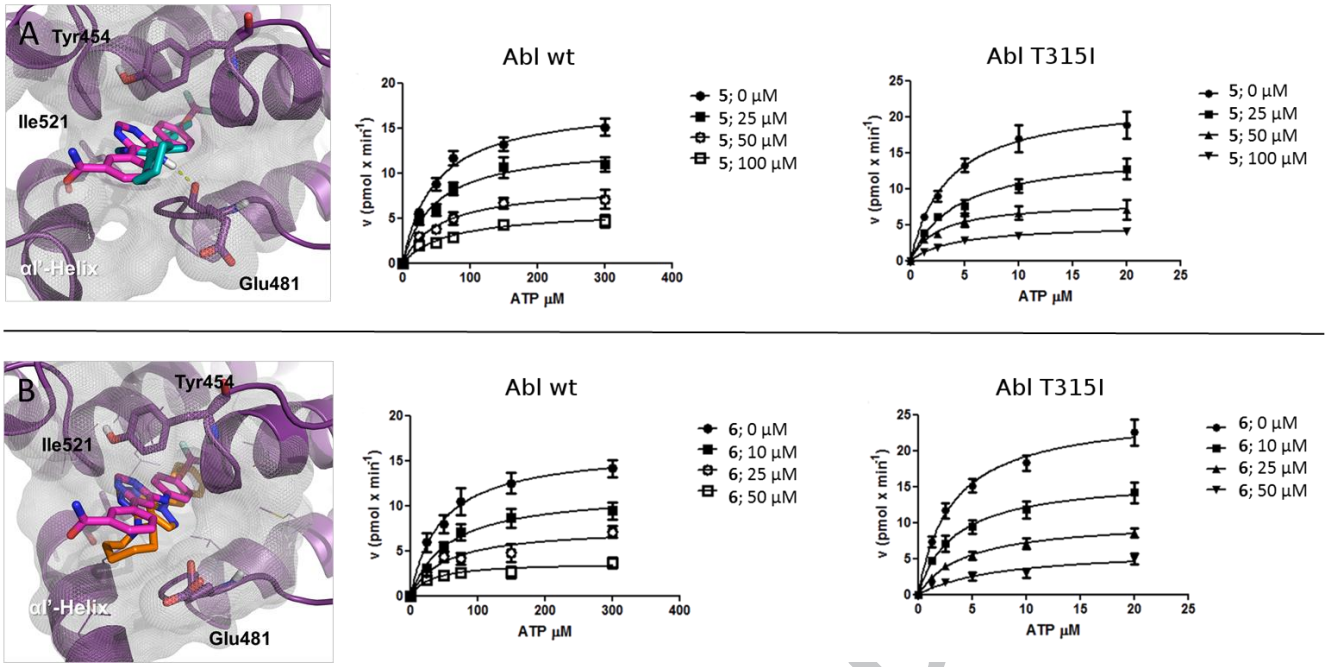

Figure 3. A) From left to right: superimposition of GNF-2 (mangenta) binding mode (3K5V crystal structure) and 5 (blue) in the MP of Abl wild type (the same binding mode was found in the MP of T315I) from the last frame of MD simulation; variation of the reaction velocity of Abl WT and Abl T315I as function of ATP concentration at different fixed concentrations of compound **5**. Error bars represent ± S.D. B) From left to right: superimposition of GNF-2 (magenta) binding mode (3K5V crystal structure) and **6** (orange) in the MP of Abl wild type (the same binding mode as in the MP of T315I) from the last frame of MD simulation; variation of the reaction velocity of Abl wt and Abl T315I as function of ATP concentration at different fixed concentrations of compound **6**. Values are the means of three independent experiments.

For this reason, additional *in silico* and *in vitro* experiments were conducted to validate the allosteric mechanism of action of the selected hits. The interaction between compounds **2**-**6** and Abl (WT and T315I mutant) was initially analyzed *in silico*: molecular docking, molecular dynamics (MD) simulations together with MM-GBSA analysis were applied according to the protocol previously described by us.[19] Docking results showed that only compounds **5** and **6** were able to fill in the MPt (Figure 3A and 3B) with a binding mode comparable to the one of GNF-2 (PDB code: 3K5V).[9] Indeed, the styryl group of **5** and **6** perfectly superimposed the p-trifluoromethoxyl group of GNF-2 oriented toward the bottom of the pocket (Figure 3).

Furthermore, hydrophobic interactions were found with residues Leu395, Val357, Val358, Ala356, Leu359, Leu360, Ala452 and Val487 while a hydrogen bond occurred between the amino group of **5** and the backbone of Glu481. Compounds **5** and **6** were then submitted to a more detailed MD and MM-GBSA analysis (a detailed description of the protocol has been provided in the Supporting Info file) to investigate their effect on the stabilization of the SH2-SH3/kinase domain interaction, which is responsible for the allosteric inhibition of Bcr-Abl (both WT and T315I).

MD simulations of 30 ns were performed and RMSD (Root Main Square Deviation) for α carbons was calculated in order to evaluate the stability of the system (Figure S2). The production stages of all simulations were used for extracting 150 frames from the dynamics. RMSFs (Root Main Square Fluctuations) for all residues were also calculated (Figure S3) for finding high fluctuation areas during the simulations. Surprisingly, compound **6** showed a stable RMSFs profile in particular considering the T315I mutant. Lower fluctuations were found for **6**, in comparison to **5,** which seemed to be less able to stabilize the assembled conformation. High values of binding free energy for both ligands in the MP of Abl WT were found (-35 kcal/mol, -40 kcal/mol for **5** and **6** respectively). Unlike GNF-2,[19] **5** and **6** maintained a high ΔG also in the MP of the T315I mutant (-35 and -39 kcal/mol respectively). These results led to speculate an allosteric mechanism of action not only against the wild type but also against the T315I mutant. The binding free energy calculated was decomposed into the contribution of each residue (Table S1). Both **5** and **6** were able to establish contacts with Tyr454 and Cys483 identified as hot spots in our previous analysis. Weak contacts (lower than 1 kcal/mol) were detected with Arg351. Furthermore, analysis of the binding free energy of interaction between SH2 and kinase domains showed a considerable ability of **6** to stabilize the compact conformation when it is bound to the T315I MP rather than to the WT MP (-59 and -50 kcal/mol respectively). By comparison, **5** was characterized by a weaker stabilization of the SH2/kinase domain interaction both on WT and T315I mutant, showing lower ΔG values calculated at the interaction interface (-42 kcal/mol and -50 kcal/mol respectively). From our analysis what emerged was an interesting and high capability of **6** to stabilize the autoinhibited conformation by allowing the formation of strong interactions at SH2/kinase interface. Hot spots residues were identified in Arg189, Tyr158, Tyr361, Glu526 and Asp523, which were the same amino acids detected in the interaction of GNF2 with the MP. Features like the π-π interactions involving Tyr158 and Tyr361 were detected with a high energy associated to these two residues. Noteworthy, hydrogen bonds between Arg189 and Asp523 and Glu526 were more stable in the case of **6** bound to MP of T315I rather than **5** (Tables S3 and S4). All these results suggested an allosteric mechanism of action for both **5** and **6** against Abl wild type and T315I mutant with a higher potency of **6** against both forms of the enzyme.

In order to confirm our results, enzymatic studies were conducted to evaluate the mechanisms of action of compounds **5** and **6**. *In vitro* kinase inhibition assays were performed using recombinant enzymes, obtaining IC_{50} values of 50 μM and 20 μM against T315I by **5** and **6** respectively. We also analyzed the reaction velocity as a function of the ATP concentration, in the

presence of different fixed amounts of inhibitors **5** and **6** (Figure 3). Kinetic parameters for **5** and **6** are summarized in Table 1: as it can be observed, both compounds decreased $Vmax_{app}$ either towards Abl WT or T315I mutant but did not affect the apparent

Table 1. Kinetic parameters of **5** and **6** inhibition toward Abl WT and T315I (mean ± S.D.)

	Wild Type (WT)			
5 [μM]	0	25	50	100
Kmapp [μM]	48.18 ±5.91	47.52 ±5.73	53.81± 4.47	54.69 ±5.87
Vmaxapp [pmol/min^{-1}]	17.70 ±0.94	13.14 ±1.06	8.57 ±0.84	5.83 ±0.62
	T315I			
Kmapp [μM]	3.48 ±0.53	4.11 ±0.70	2.9 ± 0.69	4.08 ±0.29
Vmaxapp [pmol/min^{-1}]	22.97 ±1.50	14.96 ±1.34	8.06 ±0.82	4.95 ±0.15
	Wild Type (WT)			
6 [μM]	0	25	50	100
Kmapp [μM]	46.11 ±5.59	55.3 ±5.85	50.09 ±4.79	35.31 ±4.75
Vmaxapp [pmol/min^{-1}]	16.35 ±1.27	11.52 ±1.03	7.53 ±1.00	3.66 ±0.45
	T315I			
Kmapp [μM]	3.168 ±0.46	3.29 ±0.52	4.30 ±0.67	4.9 ±0.8
Vmaxapp [pmol/min^{-1}]	25.27 ±1.39	16.11 ±1.22	10.28 ±0.84	6.30 ±1.3

affinity towards ATP (Km_{app}) as expected for a non-competitive inhibitor. Overall, these results confirm the capacity of **5** and **6** to bind the MP and inhibit the kinase activity with an allosteric mechanism of action.

Finally, compounds **5** and **6** were evaluated for their cytotoxic effect on murine 32D parental myeloid cell line and both the wild type Bcr-Abl (p210) and the T315I mutant expressing cells. Compounds **5** and **6** have been tested for 48 h at increasing concentration (0.5-50 μM) and their efficacy was calculated considering the number of viable cells in respect to the control (Figure 4A). Notably, both molecules showed no cytotoxicity on parental 32D cells while compound **6** displayed a higher cytotoxic activity (1.2 μM and 13.8 μM against IM-sensitive and IM-resistant cell lines respectively) compared to **5** (8.5 μM and 25.1 μM) in the leukemic cell lines. Surprisingly, **5** and **6** showed higher activity in cells rather than on isolated enzymes, suggesting that inhibition of additional targets may be responsible for the observed cytotoxic effect. Moreover the phosphorylation levels of the Bcr-Abl substrate CrkL protein have been analyzed by western blot (Figure 4B). It was observed a reduction of the phosphorylation on CrkL on Tyr207 after treatment with our compounds **5** and **6**, confirming their efficacy in inhibiting Bcr-Abl.

In summary, a cascade screening approach has been successfully applied to our internal collection of ATP mimetics leading to the identification of a hit compound (**6**) acting with an allosteric mechanism of action against both Abl WT and T315I mutant. A fast FP screening protocol followed by computational studies (combining docking and dynamics simulations) have been applied for the initial identification of two MP binders (**5** and **6**). Kinetic enzymatic studies confirmed the allosteric mechanism of action of both compounds not only against the WT Abl enzyme but, most importantly, against the T315I mutant. The pyrazolo[3,4-*d*]pyrimidine **6**, which showed the higher potency, represents a promising candidate able to allosterically inhibit the Abl T315I mutant with no need of combination with an ATP-competitive inhibitors (differently from GNF-2/5). Our work open the way to the discovery of a new class of MP binders which could be further optimized to provide new drug candidates to fight the T315I mutant form of drug-resistant leukemia.

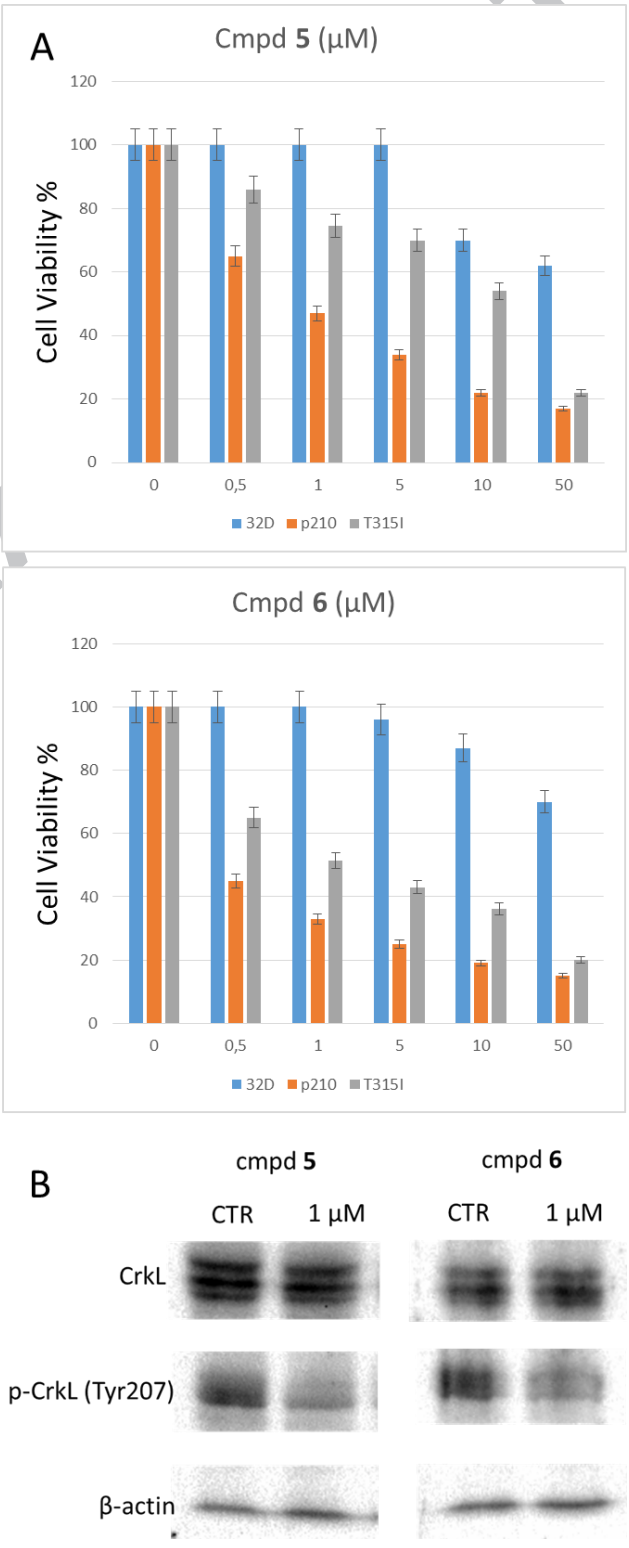

Figure 4. A) Cell death in response to treatment with compounds **5** and **6** for 48 h. Assays were conducted on murine leukemia 32D expressing Bcr-Abl (WT or T315I mutated).; B) Protein levels of CrkL and p-CrkL (Tyr207)

were analyzed by western blot in 32D-T315I mutated cells treated for 24h with 1μM compounds **5** and **6**. As loading control β-actin was utilized.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at

Graphical Abstract

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