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Development of a biotin–streptavidin-enhanced enzyme-linked immunosorbent assay (BA-ELISA) for high-throughput screening of KRAS^{G12C} inhibitors

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ABSTRACT

KRAS, the most frequently mutated oncogene in human cancers, was considered "undruggable" until the identification of small molecules that bind irreversibly to the mutant reactive cysteine at residue 12. Despite the encouraging anticancer activity of KRAS^{G12C} inhibitors in clinical trials, identification of more potent drugs is expected to achieve the maximal clinical benefit, which is hindered by the low sensitivity or throughput of current biochemical approaches. To overcome these limitations, a biotin–streptavidin-enhanced enzyme-linked immunosorbent assay (BA-ELISA) based on the competitive interaction of biotin-labeled probe and the test compound with KRAS^{G12C} was developed. Compared with reported assays, less protein was used in BA-ELISA, which significantly improves the resolution of inhibitor potency, thus contributing to the identification of highly potent inhibitors. Furthermore, BA-ELISA can also be expanded to determine the cellular potency of the inhibitors using KRAS^{G12C} mutant living cells. Using three previously disclosed compounds, ARS-1620, AMG 510, and MRTX849, we demonstrated that BA-ELISA is a highly sensitive, specific, and robust method for high-throughput screening of KRAS^{G12C} inhibitors.

Introduction

The small GTPase KRAS functions as a molecular switch, cycling between inactive GDP-bound and active GTP-bound states [1]. This transition is highly facilitated by guanine nucleotide exchange factors (GEFs; e.g., SOS1) and GTPase activating proteins (GAPs) [2]. Upon activation, KRAS interacts with downstream effectors such as Raf, PI3K, and Ral to trigger signal transduction pathways [3]. KRAS is mutated in approximately 25% of all human cancers, of which mutations in glycine 12 (G12) account for about 80% [4,5]. The mutant KRAS prevents intrinsic and/or GAPs mediated GTP hydrolysis and therefore becomes constitutively activated, promoting tumor cell growth and survival [2,6,7]. Despite the widespread prevalence of KRAS mutations in cancers, identification of potent inhibitors has been challenging. However, in recent years, breakthroughs have been made in targeting KRAS^{G12C} with covalent inhibitors, which bind to a previously undetected allosteric switch-II pocket in the GDP-bound KRAS^{G12C} directly and trap the protein in its inactive state, with high selectivity for wild-type KRAS [8,9]. Correspondingly, AMG 510 and MRTX849, two KRAS^{G12C} irreversible inhibitors, exhibited encouraging anti-tumor activity as well as favorable safety and tolerability in clinical trials [10,11]. Till now, the tremendous progress has inspired a new wave of development of small molecule KRAS^{G12C} inhibitors.

Currently, there are two major biochemical approaches to determine the activity of KRAS^{G12C} inhibitors. The first is using liquid chromatography-tandem mass spectrometry (LC/MS-MS) to quantitatively determine the covalent adduct formation at cysteine 12 of KRAS [8]. However, high technical requirements and time-consuming procedures impede its application in high-throughput screening. The other is to monitor the inhibitory activity of the compounds on nucleotide exchange. Alpha technology is applied to determine the interaction between GTP-bound active KRAS^{G12C} and the downstream effector c-Raf in the *in vitro* SOS1-catalysed nucleotide exchange system [12]. Besides, the current commercial nucleotide exchange assay kit manufactured by BPS Bioscience is designed to monitor the GDP binding states of KRAS^{G12C} by using fluorescently labeled GDP. The large amount of protein needed in the second approach restricts it from discriminating highly potent inhibitors. Furthermore, these systems can only indicate

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the binding activity of the compounds indirectly. Taken together, neither of the above two technologies is suitable for screening of highly potent $KRAS^{G12C}$ inhibitors in high-throughput format. Recently, a cell-based high-throughput NanoBRET screening platform was developed to identify modulators of the ras/raf interaction [13]. In theory, this system can also be used to indirectly indicate the activity of $KRAS^{G12C}$ inhibitors, although it has not yet. However, the cell-based screening excludes less permeable compounds and generates high rate of artifacts, hinders structure-activity relationships analysis and rational compound design. Thus, a highly sensitive, easy-to-operate, and high-throughput method to evaluate the potency of $KRAS^{G12C}$ inhibitors directly is urgently needed.

Here, we developed a BA-ELISA to high-throughput screen for inhibitors of $KRAS^{G12C}$ based on their binding affinity. To set up BA-ELISA, we synthesized a biotinylated derivative of a known covalent inhibitor as the probe, competing with the test compound for binding to the reactive cysteine of $KRAS^{G12C}$. Probe-bound $KRAS^{G12C}$ was captured by streptavidin-coated plate, followed by detection with anti-KRAS antibody and chemiluminescence. The signal intensity is inversely proportional to the test compound-$KRAS^{G12C}$ binding ability. Further, in living cells harboring $KRAS^{G12C}$ mutation, BA-ELISA can also be used to evaluate the inhibitory activity of the compounds in a cellular context. To verify both the applicability and stability of the system, we validated the assay with three reported inhibitors, ARS-1620, AMG 510, and MRTX849, demonstrating that BA-ELISA is a highly sensitive, robust, and specific high-throughput assay to screen for inhibitors of $KRAS^{G12C}$.

Materials and methods

Protein expression and purification

His-tagged $KRAS^{G12C/WT/G12D}$ (full length) and SOS1 (amino acids 564-1049) were expressed in *E.coli* and High Five Sf9 insect cells, respectively. The cell pellets were resuspended in lysis buffer H (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1mM dithiothreitol, 5% glycerol) supplemented with protease inhibitor (Merck, Kenilworth, NJ, USA) and phosphatase inhibitor cocktails (Bimake, Houston, TX, USA). Cell lysates were prepared using ultrasonic cell disruptors (Branson, Danbury, CT, USA). Insoluble material was removed by centrifugation. The supernatant was loaded onto a HisTrap column (GE Healthcare, Wauwatosa, Wisconsin, USA) pre-equilibrated with lysis buffer H, followed by washing with lysis buffer H until $A_{280\ nm}$ reached baseline. The protein was eluted with lysis buffer H supplemented with 300 mM imidazole. Fractions containing the desired protein were desalted and concentrated with Amicon Ultra-4 Centrifugal Filter Devices (Merck, Kenilworth, NJ, USA).

High Five Sf9 cells expressing GST-c-Raf (amino acids 51-131) were lysed with lysis buffer G (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, 20% glycerol) and the supernatant was purified with GSTrap column (GE Healthcare, Wauwatosa, Wisconsin, USA). The protein was eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1mM dithiothreitol, 20% glycerol, 20 mM glutathione) and concentrated as described above.

Cell culture and plasmid transfection

A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). CHO, NCI-H358, HT-29, and HEK-293 were purchased from the Cell Resource Center, Peking Union Medical College, headquarters of National Infrastructure of Cell Line Resource (NSTI, Beijing, China). MIA PaCa-2 was obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2. NCI-H358 cells were cultured in RPMI 1640 medium (Biological Industries, Cromwell, CT, USA). MIA PaCa-2 and HEK-293 cells were cultured in DMEM medium (Biological Industries, Cromwell, CT, USA). A549 cells were cultured in F12K medium (Thermo Fisher Scientific, Waltham, MA, USA). CHO and HT-29 cells were cultured in DMEM/F12K medium (Thermo Fisher Scientific, Waltham, MA, USA). All mediums were supplemented with 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

In order to express biotin-labeled KRAS, a customized plasmid CS-T0178-M52 (SinoBiological, Beijing, China) was generated using Gateway cloning technology and we renamed it to pcDNA3-AVI-KRAS-IRES-BirA for greater clarity. LR recombinase catalyzed the recombination between the entry clone GC-T0178 containing KRAS ORF and the destination vector pReceiver-M52a containing FLAG-AVI tags and IRES-BirA cassette to generate an expression clone CS-T0178-M52. The plasmids were transfected into CHO cells using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). To exogenously express different KRAS mutants in HEK-293 cells, pcDNA3-FLAG-$KRAS^{G2C/WT/G12D}$ was transfected with polyethylenimine (Polyscience, Warrington, PA, USA).

Chemical compounds

ARS-1620, AMG 510, and MRTX84 were purchased from Selleckchem (Houston, TX, USA). The biotinylated probe was synthesized and modified as described previously [14,15]. The synthetic scheme and detailed synthesis process is provided in Supplemental Material.

The procedure of biochemical and cellular BA-ELISA

Reactions were performed in a 20 μL volume by pre-incubation recombinant $KRAS^{G12C}$ (final concentration 1nM) with the 10-fold serially diluted test compound in reaction buffer (50 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 0.05 % Tween 20) at room temperature for 1 hr, then adding 20 μL biotinylated probe (final concentration 100 nM). After incubation for another 1hr, wash buffer (TBST with 0.1% bovine serum albumin) was added to a final volume of 100 μL. The mixture was transferred to a streptavidin-coated 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at room temperature for 1 hr. The plate was washed three times with wash buffer. 100 μL anti-RAS primary antibody (Abcam, Cambridge, UK, catalogue number ab108602) diluted at 1:2000 in wash buffer was incubated with the plate for another 1hr. Following three washes with wash buffer, 100 μL wash buffer diluted (1:5000) horseradish peroxidase (HRP) conjugated secondary antibody (Cwbio, Beijing, China) was added to the plate and incubated at room temperature for 1 hr. After an additional washing step, 100 μL substrate solution TMB (Cwbio, Beijing, China) was added. The enzymatic reaction was stopped by adding 50 μL 0.5 M H_2SO_4 after sufficient color development. The absorbance at 450 nm was measured with Flexstation 3 (San Jose, CA, USA).

Cell-based BA-ELISA was performed in NCI-H358 cells. The cells were seeded at 5×10^5 cells per well of 6-well plate and allowed to adhere for 24 hrs. The next day, the medium was replaced with 2 mL fresh medium containing 10-fold serially diluted compounds. Following 1 hr of incubation, 1000 nM probe was added to the cells and incubated for another 1hr. Then the cells were lysed with 40 μL cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). The lysates were diluted with wash buffer to 100 μL and incubated with a streptavidin-coated clear 96-well plate for 1 hr at room temperature. The following ELISA was performed as biochemical BA-ELISA.

Antibodies selection

KRAS was labeled with biotin using *in vivo* protein biotinylation technology [16]. CHO cells transfected with vector or pcDNA3-AVI-KRAS-IRES-BirA were lysed with cell lysis buffer, and the lysates were incubated with a streptavidin-coated clear 96-well plate for 1 hr at room

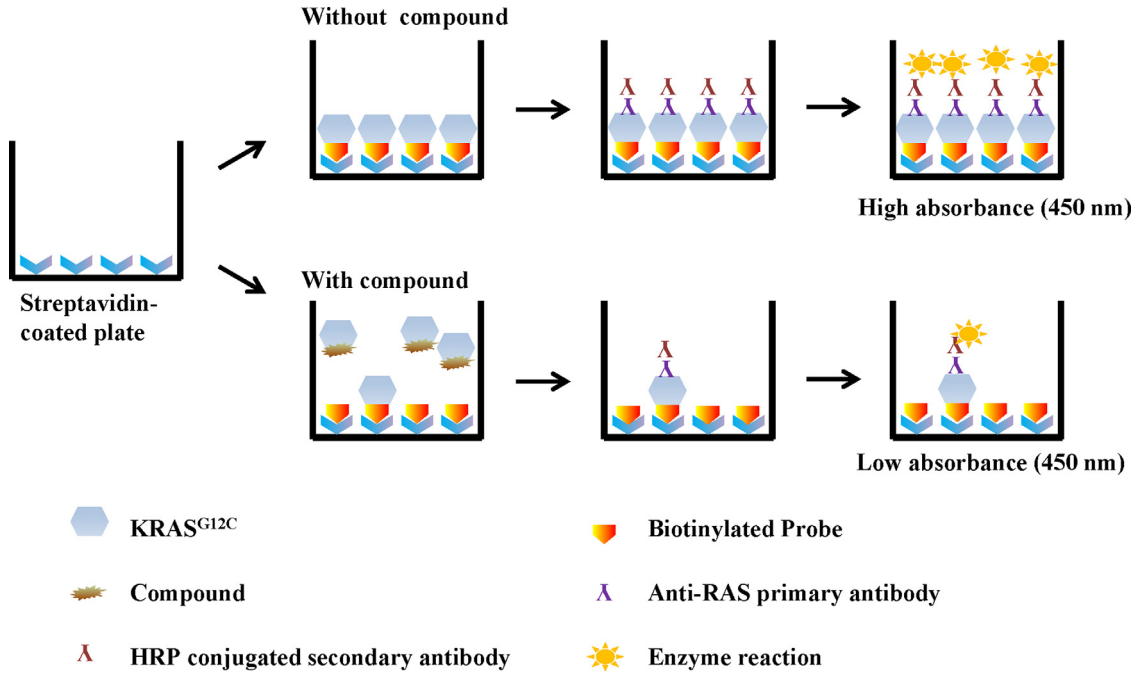

Fig. 1. Principle of the BA-ELISA for screening $KRAS^{G12C}$ inhibitors.

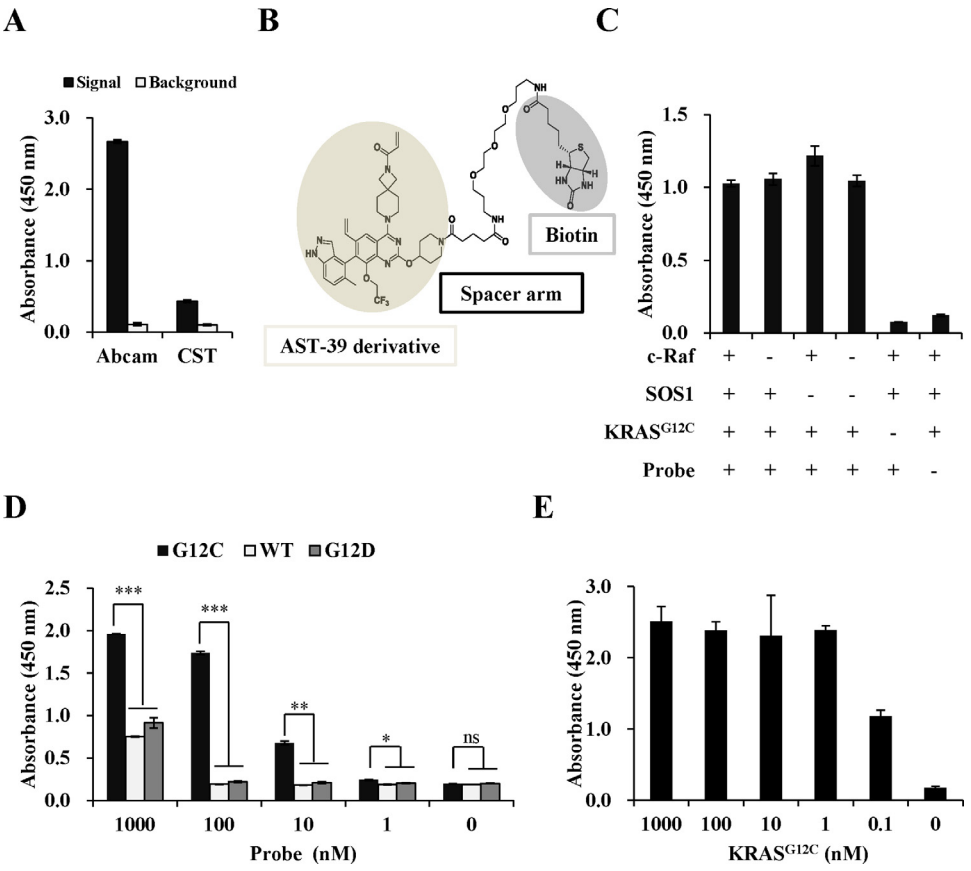

Fig. 2. Development of the BA-ELISA. (A) Comparison of the signal to background ratio of two commercial anti-RAS antibodies. The lysates of CHO cells with or without biotinylated KRAS expression were incubated with the streptavidin-coated plate and detected with antibodies as indicated. (B) The structure of the biotinylated probe. (C) Effects of c-Raf and SOS1 on the interaction between the probe and $KRAS^{G12C}$ protein. (D) Optimization of the amount of the probe and detection of the specificity of the probe. (E) Titration of the recombinant $KRAS^{G12C}$. Results were shown as the mean value ± standard deviation (S.D.). n = 3 technical replicates. Statistical analysis was performed using unpaired Student's t-tests; ns, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

temperature. The plate was washed three times with wash buffer. Two anti-RAS primary antibodies whose catalogue number is ab108602 (Abcam, Cambridge, UK) and 3965 (Cell Signaling Technology, Danvers, MA, USA), respectively, were diluted as recommended by the manufacture and incubated with the plate for another 1hr. The following ELISA was performed as described above.

BA-ELISA development and validation

Titration for probe and KRAS proteins used in biochemical BA-ELISA were performed with serially diluted probe (0-1000 nM) or recombinant $KRAS^{G12C}$, $KRAS^{WT}$, or $KRAS^{G12D}$, respectively (ranging from 0 to1000 nM). Then, the protein and probe mixture was added to a streptavidin-

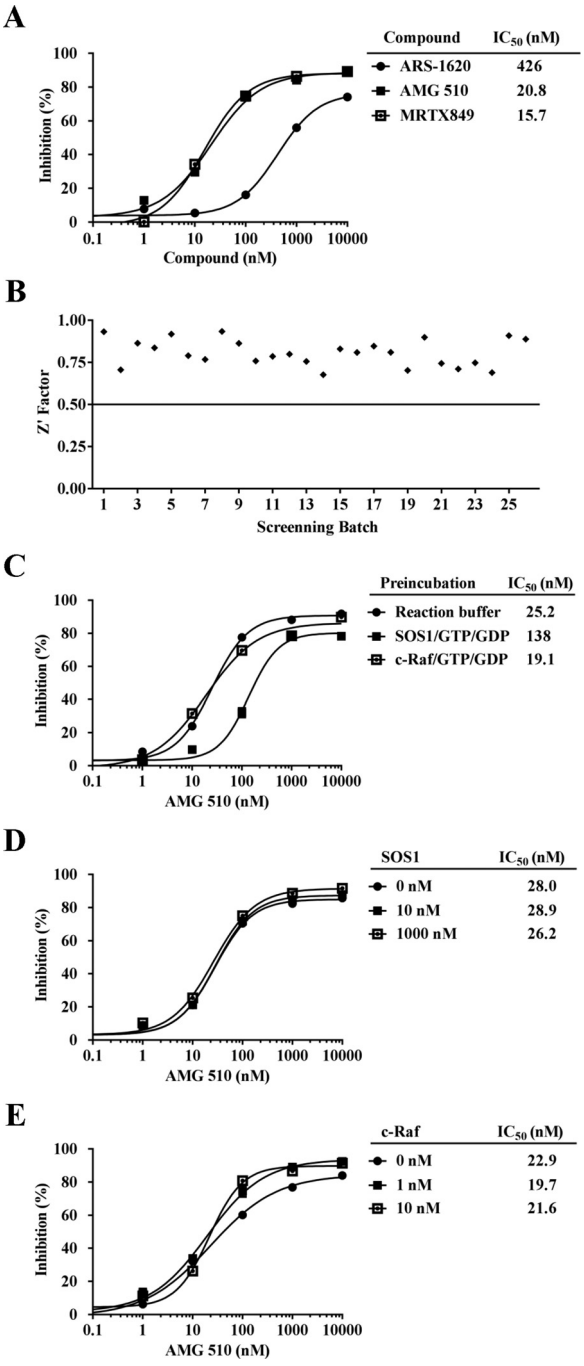

Fig. 3. Validation of the BA-ELISA. (A) Determination of half-maximal inhibitory concentrations of ARS-1620, AMG 510, and MRTX849 using BA-ELISA. (B) Z' factors of 26 separate runs of screening. 10 μM AMG 510 was used as the positive control, and 0.1% DMSO was used as the negative control. (C) $KRAS^{G12C}$ was pre-incubated with reaction buffer, SOS1/GTP/GDP, or c-Raf/GTP/GDP as indicated before performing BA-ELISA. (D and E) $KRAS^{G12C}$ was pre-incubated with varying concentrations of SOS1 (D) or c-Raf (E) alone before performing BA-ELISA. In all cases, a no compound (0% inhibition) control was included and used as the reference to calculate percent inhibition values. The data were fitted to four-parameter logistic nonlinear regression. (Mean, n = 2 technical replicates).

coated 96-well plate. The following ELISA procedures were performed as described above.

To confirm the effect of inhibitor was attenuated by increasing nucleotide exchange, $KRAS^{G12C}$ was pre-incubated with SOS1/GTP/GDP, c-Raf/GTP/GDP, SOS1 alone, c-Raf alone, or reaction buffer as indicated for 0.5 hr before incubation with AMG 510, and then BA-ELISA was performed as mentioned above.

Optimization in probe concentration and cell seeding number in cellular BA-ELISA was performed with varying probe (0-1000 nM) or NCI-H358 cells (0-7.5 × 10^5 cells per well in 6-well plate). After incubation with the probe for 1 hr, the lysates were added to the streptavidin-coated clear 96-well plate. The following ELISA procedures were performed as described above.

The specificity of cellular BA-ELISA was evaluated with NCI-H358, MIA PaCa-2, HT29, A549 cells expressing KRAS mutants endogenously and HEK-293 cells overexpressing $KRAS^{WT}$, $KRAS^{G12C}$, or $KRAS^{G12D}$, respectively. The cells were pre-treated with DMSO or 10 μM AMG 510 for 1 hr, followed by incubating with 1 μM probe for another 1 hr. After lysis with cell lysis buffer, the lysates were added to the streptavidin-coated clear 96-well plate. The following ELISA procedures were performed as described above.

Data analysis

The percentage of inhibition was calculated using the formula: Inhibition (%) = (1-Compound OD value/Control OD value) × 100, where the OD values without compound (control) are considered as 0% inhibition. Analysis and IC_{50} values were derived using GraphPad Prism 7.0. Z' factor was calculated according to the formula: Z'=1-3 (s_p+s_n) / $|m_p-m_n|$ [17]. s_s and s_c are defined as the standard deviation of the positive and negative control signal, respectively. Similarly, m_p and m_n are defined as the mean of the positive and negative control signal, respectively. 10 μM AMG510 was used as the positive control, and 0.1% DMSO was used as the negative control.

Results

Principle of the BA-ELISA

To achieve high sensitivity and specificity, we developed a competitive BA-ELISA system. As shown in Fig. 1, the test compound competes with biotin-labeled probe, a derivative of $KRAS^{G12C}$ inhibitor reported previously, for binding to $KRAS^{G12C}$ protein. In general, the process begins with a sequential incubation of the test compound and biotin-labeled probe with recombinant $KRAS^{G12C}$. Probe-bound $KRAS^{G12C}$ is then captured by the streptavidin-coated plate, while the test compound-bound $KRAS^{G12C}$ will be washed away. After incubation with anti-Ras primary antibody and HRP conjugated secondary antibody, the potency of test compound is exactly measured through subsequent HRP-mediated chemiluminescence, which is inversely proportional to the observed absorbance.

Development of the BA-ELISA

One of the key points in ELISA is using a highly specific and sensitive detection antibody. During assay development, antibody selection was performed with two commercially available anti-RAS antibodies purchased from Abcam (CAT No. ab108602) and Cell Signaling Technology (CAT No. 3965), respectively. Based on the results of ELISA, Abcam's antibody was used in the following studies due to its high signal-to-background ratio (Fig. 2A).

As for the probe design, the most potent compound 39 in the patent detailing $KRAS^{G12C}$ inhibitors from Astellas Pharma Inc. (WO 2018/143315 A1) was biotinylated at piperidine via a 20 atoms spacer arm as a highly sensitive and specific probe that binds to $KRAS^{G12C}$ (Fig. 2B), with little disturbance on the pharmacophore according to

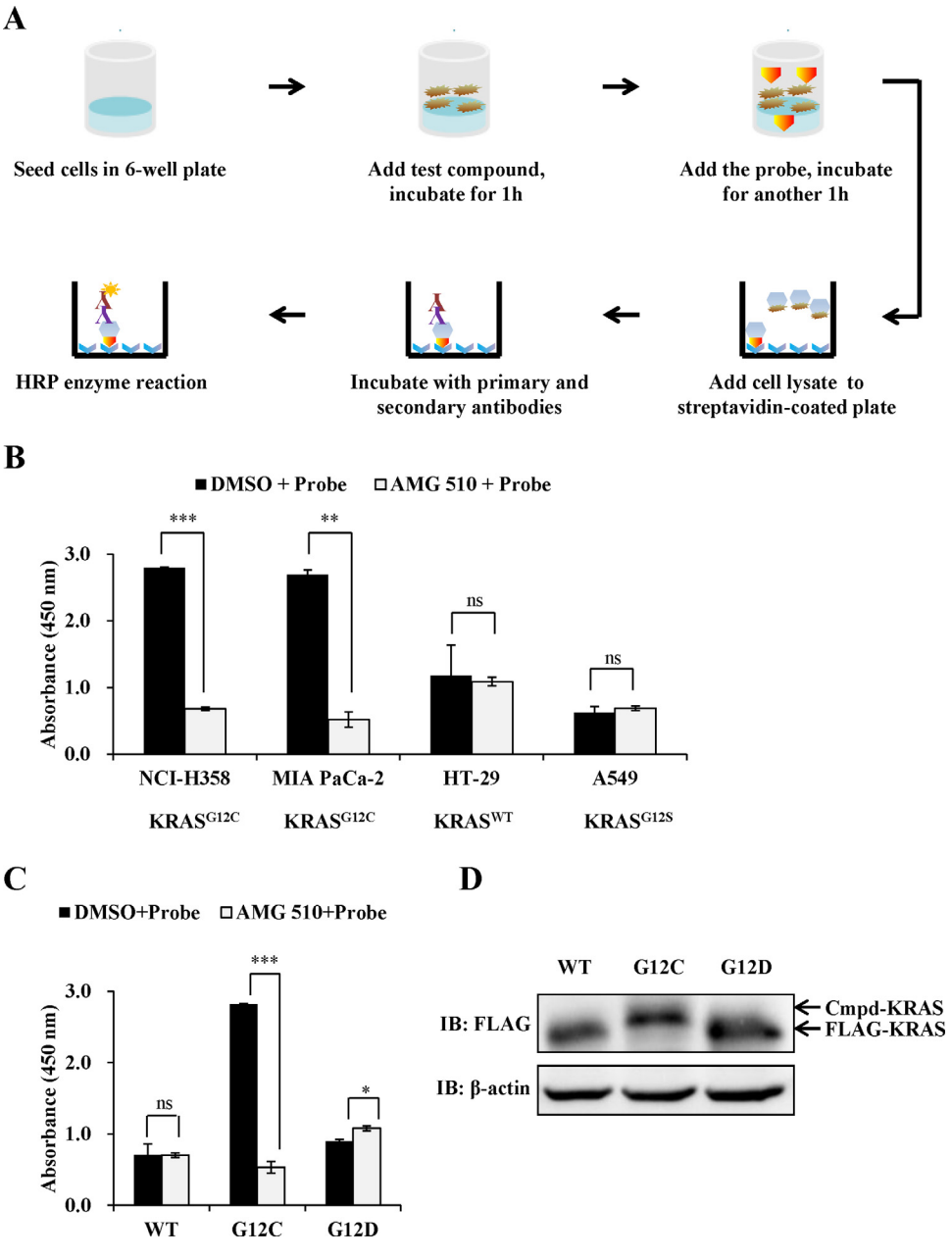

Fig. 4. The specificity of cellular BA-ELISA. (A) The workflow of cellular BA-ELISA. (B) Cell-based BA-ELISA was performed with NCI-H358 (heterozygous $KRAS^{G12C}$), MIA PaCa-2 (homozygous $KRAS^{G12C}$), HT-29 (homozygous $KRAS^{WT}$), A549 (homozygous $KRAS^{G12S}$) cells. (C) Cell-based BA-ELISA was performed with HEK-293 overexpressing $KRAS^{WT}$, $KRAS^{G12C}$, or $KRAS^{G12D}$. Results were shown as the mean value ± standard deviation (S.D.). n = 3 biological replicates. Statistical analysis was performed using unpaired Student's t-tests; ns, $P > 0.05$,*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (D) Representative western blot showing the comparable expression of exogenous FLAG-tagged KRAS in HEK-293 cells. β-Actin was used as a loading control.

the docking result (Data not shown). As expected, the probe binds to $KRAS^{G12C}$ successfully, which is independent of the presence of c-Raf or SOS1 (Fig. 2C and Suppl. Figs. S1A, S2). To balance the tradeoff between the high sensitivity and large dynamic range, the amount of the probe and $KRAS^{G12C}$ protein was optimized subsequently. The signal reached a steady plateau with 100 nM probe, which was used for further assay development. To exclude the potential of non-specificity, the system was verified with recombinant $KRAS^{WT}$ and $KRAS^{G12D}$ protein. The probe exhibits a much weaker affinity to $KRAS^{WT}$ and $KRAS^{G12D}$ as compared to $KRAS^{G12C}$, indicating that the probe binds to $KRAS^{G12C}$ selectively (Fig. 2D and Suppl. Fig. S1B). Finally, the usage of $KRAS^{G12C}$ protein was titrated, and the signal is saturated at approximately 1 nM $KRAS^{G12C}$, which was set as optimum (Fig. 2E).

Validation of the BA-ELISA

To explore the feasibility of using the BA-ELISA system for potential $KRAS^{G12C}$ inhibitors screening, a tool compound with moderate activity against $KRAS^{G12C}$, ARS-1620, and two potent $KRAS^{G12C}$ inhibitors, AMG 510 and MRTX849, were tested in a dose-response assay. As expected, all compounds significantly inhibited the interaction between $KRAS^{G12C}$ and the probe. Among them, AMG 510 and MRTX849 showed approximately 20-fold higher potency (IC_{50} = 20.8 nM and 15.7 nM, respectively) than ARS-1620 (IC_{50} = 426 nM), which is consistent with the reported activity [11,12,18] (Fig. 3A). Of note, the calculated Z' factor for BA-ELISA is 0.62, indicating the high robustness and quality of the system (Fig. 3B).

The covalent inhibitors preferentially interact with GDP-bound $KRAS^{G12C}$ and stabilize the inactive form. Indeed, co-mutations in $KRAS^{G12C}$ that promote nucleotide exchange reduced the effect of the inhibitors [9,11]. Similarly, the inhibitory activity of AMG 510 on $KRAS^{G12C}$ was attenuated when nucleotide exchange occurs through pre-incubation with SOS1/GTP/GDP (Fig. 3C). In contrast, pre-incubation with SOS1 alone (Fig. 3D) or c-Raf (Fig. 3C and E) didn't modulate the potency of AMG 510. These data confirm that BA-ELISA

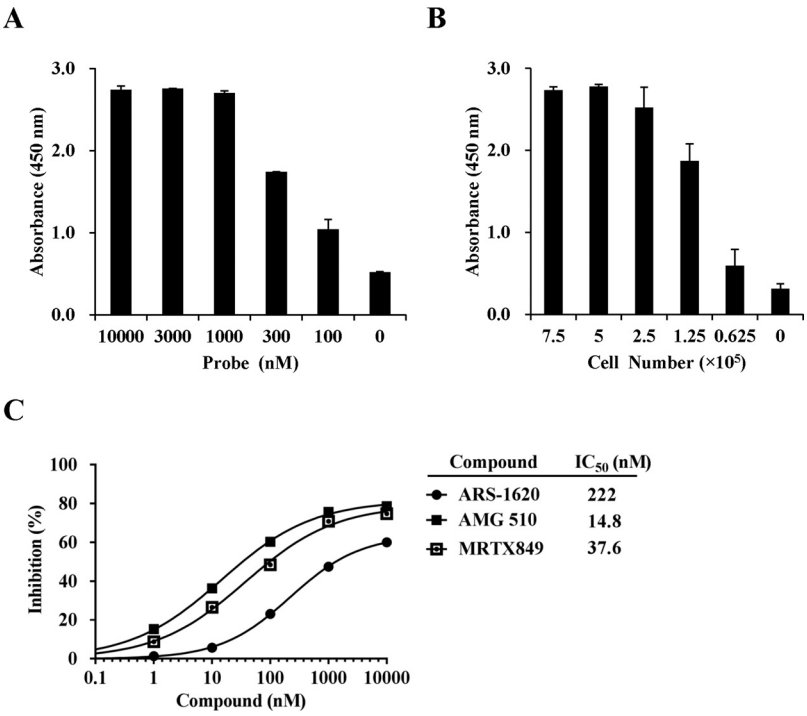

Fig. 5. Development of the cellular BA-ELISA. (A and B) Optimization of the amount of biotin-labeled probe (A) and cell number of NCI-H358 (B). Cell number refers to the seeding number at the time of plating. Results were shown as the mean value ± standard deviation (S.D.). n = 3 biological replicates. (C) The cellular potency of ARS-1620, AMG 510, and MRTX849 in NCI-H358 cells was determined by cellular BA-ELISA. (Mean, n = 2 biological replicates).

is an effective method to detect the binding activity of compounds to $KRAS^{G12C}$ *in vitro*.

Development of cell-based BA-ELISA

Generally, cell-based measurement of a compound is perhaps the most critical step for activity reassessment as well as a prerequisite for *in vivo* studies. We further developed a cell-based BA-ELISA to evaluate the intracellular activity of the test compounds in $KRAS^{G12C}$ mutated cancer cells. The related workflow of cell-based BA-ELISA is shown in Fig. 4A. In brief, $KRAS^{G12C}$ mutant cells were pre-seeded in a 6-well plate for overnight attachment. After a sequential incubation with the test compound and the probe, the cells were harvested and lysed. And then the lysates were incubated with a streptavidin-coated plate, followed by KRAS detection with antibodies and HRP-mediated chemiluminescence.

To investigate the specificity of cell-based BA-ELISA, we compared the activity of AMG 510 in cells harboring endogenous $KRAS^{G12C}$ mutation (NCI-H358 and MIA PaCa-2) and cells expressing $KRAS^{WT}$ (HT-29) or $KRAS^{G12S}$ (A549). The results showed that pre-incubation with AMG 510 significantly reduced the amount of probe-bound $KRAS^{G12C}$ in NCI-H358 and MIA PaCa-2 cells. In contrast, neither AMG 510 nor the probe can bind to non-G12C mutant KRAS in HT-29 and A549 cells (Fig. 4B). In addition, in an exogenous study by ectopically overexpressing FLAG-tagged $KRAS^{WT}$, $KRAS^{G12C}$, or $KRAS^{G12D}$ in HEK-293 cells, we also found that interaction specifically occurred between $KRAS^{G12C}$ and the probe, which was inhibited by pre-incubation with AMG 510, demonstrating the specificity of cellular BA-ELISA (Fig. 4C). The $KRAS^{G12C}$-compound adducts formed by covalent bonds migrate more slowly than the corresponding free KRAS in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [11,12]. In line with it, the shifted mobility was exactly observed only in the G12C mutant (Fig. 4D).

To finally establish a cell-based screening system, we developed the cellular BA-ELISA in NCI-H358 cells. The amount of the probe and cell seeding number were optimized. As shown in Fig. 5A and B, 1000 nM probe and 5×10^5 cells per well in a 6-well plate were apposite, which were the minimal probe and cells usage to reach the signal plateau. Based on the optimized condition, the inhibitory potency of ARS-1620, AMG 510, and MRTX849 in NCI-H358 cells highly corresponds to that in the biochemical BA-ELISA (Fig. 5C).

Discussion

Despite the pivotal role of $KRAS^{G12C}$ in tumor initiation and progression, the currently available $KRAS^{G12C}$ inhibitors have relatively modest activity compared to other approved therapies targeting other classic oncogenic drivers [10,19-21]. A more potent inhibitor capable of achieving near-complete $KRAS^{G12C}$ inhibition may translate into a more effective treatment for patients with $KRAS^{G12C}$ mutations. However, compromised *in vitro* compound screening systems render the identification of potent inhibitors challenging.

The BA-ELISA described here provides a high-throughput platform for $KRAS^{G12C}$ inhibitors screening with high sensitivity as well as excellent specificity and robustness. The integration of the tight binding of biotin-labeled probe to $KRAS^{G12C}$ and the extraordinarily high affinity of biotin for streptavidin reduced the amount of $KRAS^{G12C}$ protein used in BA-ELISA greatly. Less protein typically improves the ability to discriminate potent inhibitors, since the lowest IC_{50} value measurable is equivalent to half the protein concentration used [22,23]. This is evident in the case of *in vitro* SOS1-catalysed nucleotide exchange assay and commercial nucleotide exchange assay kit, which use 20 nM and 1 μM $KRAS^{G12C}$, respectively, compared with 1 nM $KRAS^{G12C}$ used in BA-ELISA. For AMG 510, the IC_{50} values obtained in the two nucleotide exchange systems were 90 nM and 502 nM, respectively [12,24]. The potency of ARS-1620 measured in SOS1-catalyzed nucleotide exchange assay is about 1 μM [12]. While BA-ELISA improved roughly 2 to 25-fold of potency over nucleotide exchange systems, demonstrates that BA-ELISA is a highly sensitive platform to identify $KRAS^{G12C}$ inhibitors. Benefiting from the high sensitivity of BA-ELISA, improving the resolution of inhibitor potency can be achieved without compromising robustness. The calculated Z' factor of BA-ELISA is 0.62, indicating the excellent screening assay quality. Notably, the biotinylated probe binds to $KRAS^{G12C}$ selectively *in vitro* and in cells, as compared with other KRAS mutants, suggesting BA-ELISA is highly specific. Although both the quantitative LC/MS-MS and BA-ELISA reported here determine the binding activity of compound to $KRAS^{G12C}$ directly, BA-ELISA is more

amenable to high throughput screening as it omits time-consuming, laborious procedures and enables screening in 96-well plates. In summary, BA-ELISA, which is ideal for high-throughput screening of highly potent $KRAS^{G12C}$ inhibitors, will greatly facilitate the drug discovery to fulfill the unmet medical needs.

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Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slasd.2021.12.007.

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