Microfluidic-based G-quadruplex Ligand Displacement Assay for Alkaloid Anticancer Drug Screening

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Abstract Some natural heterocyclic alkaloids containing planar group show potential to complex with specific promoter region of proto-oncogene for stabilizing the G-quadruplex (G4) structure which nowadays promises to be a target in anticancer drug design. However, in view of the polymorphic characteristics and structural complexity of heterocyclic alkaloids, it is desirable to develop a high-throughput and low-consumption approach for anticancer drug screening. In this paper, an intensive study on alkaloid ligand/G4DNA interaction has been conducted, demonstrating that the end-stacking interaction is the favorable binding mode between the oncogene-related Pu22G4DNA and the heterocyclic alkaloid ligand. Based on structural feasibility and energy minimization, a ligand displacement assay for screening alkaloid ligand in stabilizing the oncogene target G4 has been developed, which also helps to facilitate the assessment of drug specificity. Coupled with microfluidic-based DNAzyme-catalytic chemiluminescence detection, the approach showed the advantages of high sensitivity, high throughput with low sample and reagent consumptions.

Keyword: drug screening, alkaloid, G-quadruplex, ligand displacement assay, chemiluminescence

Abbreviation:

G4: G-quadruplex; CD: circular dichroism; CL: chemiluminescence; MD: molecular dynamics; SAU: sanguinarine; BER: berberine; CHE: chelerythrine; MAT: matrine; LIG: ligustrazine; PMT: photomultiplier tube; PME: Particle mesh Ewald method; GAFF: general AMBER force field; VMD: visual molecular dynamics; PDB: Protein Data Bank.

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1. Introduction

Cancer has become the most common disease. After unremitting efforts from several generations of medical and pharmaceutical workers, chemotherapy has been used for controlling the growth of cancer and received certain curative efficacy, but the side effects of chemotherapeutic drugs limit their application. Therefore, exploring natural compounds extracted from medical plants for targeted anticancer therapy becomes an important tendency. Several features of natural compounds make them very attractive, such as the considerable variability in their chemical structure, their wide availability from natural sources, the low cost of their extraction processes, and most importantly, their general low toxicity[1-2].

DNA is the first defined target for anticancer drug development. Although dsDNA has long been considered as the focus structure, it is now well-established that DNA sequences involving repetitive G-tracts in genomic DNA can fold into higher-order intramolecular G4 structure; and stabilizing G4 in promoter region of the proto oncogene is deemed to be a promising new strategy in developing anticancer drug. However, protooncogenes in promoter sequences, including *c-KIT*, *c-MYC*, *k-RAS*, *B-RAF*, *BCL-2* and *RET*, have shown to form various G4 arrangements [3-4]. Therefore, in view of the very diversity of G4, it is not feasible to screen (or design) small molecule against all the candidate gene promoters. The practical approach is supposed to be focus on those promoter G4 targets possessing unique structural feature and to screen specific molecules solely for them [5].

Among the G4 forming motifs in the human genome, G-quadruplex in the promoter region of proto-oncogene *c-MYC* has been widely studied. Specifically, *NHE III₁* (or called *Pu27*), the upstream of the *P1* promoter of *c-MYC* overexpressed in 60–80% of all cancers, can fold into G4 structure to control about 85-90% of the transcription of the gene [6-7]. So, a potential strategy to inhibit of the gene expression, involving selectively stabilizing the G4 structure by small molecular ligands, can suppress the transcriptional activation, down-regulate expression, inhibit cell proliferation [4]. Therefore, as a promising approach for anticancer therapy, screening ligand to complex and stabilize the G-quadruplex of protooncogene has received extra attention for the development of anticancer drug.

There are mainly two categories of methods applied in investigating the interaction between G4 and ligand that are of pharmaceutical interest. The direct methods, such as nuclear magnetic resonance, mass spectrometry [8] and telomeric repeat amplification protocol assay [9], give definitive interacting evidence but usually rely on costly instruments or are time consuming. The indirect methods measure physical parameter variations, like characteristic spectral bands or melting temperature of oligonucleotides, by means of circular dichroism (CD), UV or fluorescent spectroscopy [8]. Besides those, one intriguing property of some hemin-conjugated G4s is the peroxidase-mimic DNAzyme activities toward some chromogenic or fluorescence-emitting reactions. Especially, based on such DNAzyme-catalyzed oxidation reaction of 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (*ABTS*), a colorimetric method for preliminary screening of G4 ligands was proposed [10], which was associated with the hemin/G4 ligand displacement, resulting in altered DNAzyme activity to be judged by naked eye. But the above spectrometric methods usually bore some inherent drawbacks, such as relatively low sensitivity and spectral interference; sometimes the tested G4 DNAs were artificially designed with little biological significance.

Chemiluminescence (CL) is known as a powerful analytical approach with the advantage of high sensitivity, simple instrumentation and little background interference. DNAzyme-catalyzed CL detection has already been applied in various bioassays. In the present work, a

microfluidic-based chemiluminescent ligand displacement assay is developed for screening heterocyclic alkaloid as ligand to bind the *c-MYC* oncogene *Pu22*, a DNA sequence derived from the *NHE III_l* region. Meanwhile, supplemented by molecular dynamics (MD) simulation and a group of spectrometric experiments, the ligand displacement mechanism associated with the G4/ligand interaction has also been intensively studied.

2. Experimental

2.1 Reagent and material

Common reagents or components are described in the Supplementary Material for details. **Fig S1** in the Supplementary Material illustrates the chemical structure of five alkaloids tested in the work, including *sanguinarine* (SAU), *berberine* (BER), *chelerythrine* (CHE), *matrine* (MAT) and *ligustrazine* (LIG) (Aladdin Industrial Corporation, Shanghai, China). The oligonucleotides were synthesized by Sangon Biotech Shanghai Co. Ltd. (Shanghai, China) with their names, sequences, conformations and descriptions listing in **Table S1**.

2.2 Apparatus and instrumentation

All the temperature-controlled experiments were conducted in a PCR reactor (K640, Hangzhou Jingge Scientific Instrument Co. Ltd, Hangzhou, China). The UV absorption spectrometry, including Soret band of hemin or DNAzyme, and the thermal melting experiments of DNA, was recorded by a UV-Vis spectrophotometer (Shanghai Spectrum SP-756PC, Shanghai, China) equipped with a temperature-controlled cuvette. The CD spectra of DNA oligonucleotides were measured using a circular dichroism spectrometer (MOS 450, Bio-Logic Science Instrument, Claix, France). The hemin or DNAzyme catalyzed CL intensities were investigated by a homemade μ FIA-CL system [11] as depicted in Fig 1, which consisted of a dual-channel syringe pump (PHD/2000, Harvard Apparatus, Holliston, MA, USA) for CL reagents (luminol and H_2O_2) introduction, a 25 μ L syringe pump coupled with a six-port multiposition valve (SP-SV system, KloeHN Co. Ltd., Las Vegas, MA, USA) for different DNAzyme samples solution and working buffer injection, a photomultiplier tube (PMT) (Hamamatsu Photonics China Co. Ltd., Beijing, China) for the CL signal collection and a double spiral-channel microchip for step-wise mixing of CL reagents and DNAzyme solution. In addition, the reaction and detection microchip was similar to that previously reported with 20 cm length channel and 3 μ L interior volume.

Fig1. μ FIA-CL system for DNAzyme-catalyzed CL assay

SP, syringe pump; SV, six-port multiposition valve; W, waste; PMT, photo multiplier tube; inlet 1 and 2, for introducing H_2O_2 and luminol solution, respectively; inlet 3, for introducing DNAzyme sample solution; outlet 4, for releasing waste.

2.3 Computational methods

2.3.1 Structure modeling

To simulate the interaction between *Pu22* G4 and ligands, molecular structure modeling was prepared first. The structure data of *Pu22* G4 DNA (*SAU*, *BER* and *CHE*) were obtained from the Protein Data Bank in the PDB entry *1XAV*, *SAU*, *BER* and *CHE*, respectively. The structure of hemin was modified from data in the Protein Data Bank (PDB entry: *HEM*). *MAT* and *LIG* were drawn manually and pre-equilibrated. The parameters of *Pu22* DNA and the ligands were all derived from the general AMBER force field (GAFF) with the AmberTools [12]. Gaussian 03 was used to calculate electrostatic potential (RESP) charge restrained by the HF 6-31G+d, as the charge method of GAFF [13]. Visual molecular dynamics (VMD) graphics software was used for trajectory inspection and molecular visualization [14].

2.3.2 System build up and molecular dynamics simulations

Periodic boundary conditions were used in our simulations with the time step set as 2 fs. Particle mesh Ewald method (PME) was applied for computing electrostatic interactions [15] and the Lorentz-Berthelot rule for calculating the Lennard-Jones potential to estimate the cross interactions among non-bonded atoms [16]. The cut-off of PME and van der Waals interaction were both set as 1.2 nm while the bond length constrained by the LINCS algorithm. The system temperature and pressure were maintained at 37°C and 1 bar, as referred to Nose-Hoover thermostat method [17-18] and isotropic Parrinello-Rahman barostat method, respectively. The isothermal compressibility was set as $4.5 \times 10^{-5} \text{ bar}^{-1}$.

After the mass center of *Pu22* G4 was set in the center of the box, one of the above mentioned ligands was added. The simple point charge (SPC) water molecular models were used to solvate *Pu22* G4 DNA, and K^+ to neutralize the system. After the energy minimization, an *NVT* ensemble (constant number of particles, volume and temperature) of 0.1 ns was to equilibrate the DNA. Then, to ensure the appropriate packing of ligand around the DNA and the restraint of *Pu22* G4 heavy atoms, a 20 ns *NPT* (constant temperature and pressure ensemble) MD simulation was conducted. After relatively reliable structures obtained, 200 ns MD simulations were conducted for each system by Gromacs 4.5.2 package. Interaction energy was calculated by the method previously reported [19] and optimized through the process of adjacent-averaging method every 3 ns to smooth out the influence of fluctuation.

2.4 Thermal melting experiments

The absorbance monitored for the G4 DNA was recorded at 295 nm. 2.5 mL of sample solution was added into a temperature-controlled quartz cuvette with 1 cm of path length, degassed and sealed with Teflon membrane to prevent solvent evaporation. The solution was kept at 90°C for 10 min and slowly cooled down to 20°C at a rate of 0.25°C/min. The absorbance was collected with the background of reference buffer subtracted.

2.5 CD measurement

CD spectra were recorded from 500 to 200 nm with a 1 cm path length quartz cuvette at 25°C. Buffer blank correction were made for all spectra.

2.6 Chemiluminescence assay protocol

The oligonucleotide test solution was prepared by diluting the DNA stock solution with Tris-HCl buffer. In a typical experiment, 1 μ L of *Pu22* DNA stock solution mixed with 59 μ L of K^+ -containing 10 mM Tris-HCl buffer (pH 7.4) was denatured at 95 $^{\circ}$ C for 10 min. After slowly cooling down to 37 $^{\circ}$ C, 10 μ L of hemin solution was added into the annealed solution and incubated at 37 $^{\circ}$ C for 30 min., Then, 20 μ L of alkaloid solution was added and incubated at 37 $^{\circ}$ C for another 30 min. The sample solution for CL assay contained 10 mM Tris, 10 mM K^+ , 1 μ M *Pu22* DNA, 5 μ M alkaloid and 0.5 μ M hemin. Finally, 0.72 M H_2O_2 and 0.5 mM luminol solutions were continuously introduced into the microchip via inlet 1 and 2, respectively, mixed in the first spiral channel of the microchip. 2 μ L of DNAzyme sample solution, preceding 23 μ L of Tris-HCl buffer (pH 7.4), was injected into the microchip via inlet 3 to catalyze luminol- H_2O_2 reaction in the second spiral channel, where the CL reaction occurred. CL signal was recorded on the peak height and the value was the mean of at least three experiments $\pm$ standard deviation.

Result and Discussion

3.1 The drug screening strategy

Gene promoters are the critical regulator of cellular proliferation. Deregulated promoter expression is associated with many cancers. Because certain gene promoter has been proved to be undruggable anticancer target at protein level, i.e. *c-MYC* [20], stabilizing G4 in such gene promoter for proto-oncogene transcriptional repression is supposed as a promising anticancer approach. Moreover, several promoter gene sequences, including *c-MYC*, can fold into intramolecular G4 [21] to form DNAzyme after binding with hemin, which exhibit catalytic activity towards the H_2O_2 -mediated oxidation of ABTS or luminol. Thus, if putative anticancer alkaloid had high potential to stabilize G4 via complexation interaction, the corresponding drug screening strategy would be implemented by ligand displacement assay.

In this work, the promoter gene segment *Pu22* complexed first with hemin to form G4 DNAzyme, whose activity was determined via luminol oxidation CL assay. Then, with the addition of alkaloid ligand, hemin would be substituted from the complex. Hence, the G4-stabilization effect of alkaloid could be evaluated via measuring the loss of the DNAzyme activity. The scheme is depicted in Fig2 and the mechanism details in following Section 3.2.

Fig2. Schematic illustration of the alkaloid drug screening based on G4 ligand displacement assay

For human promoter gene sequence with four or more G-tracts that expected to fold into intramolecular G4 in near-physiological solution, most studies to date are focused on intramolecular

quadruplex arrangement [5]. Among various G4 conformations, including parallel, antiparallel and mixed hybrid of parallel and antiparallel [4], the parallel-stranded propeller-type G4 is normally supposed to be the favorable structure for DNAzyme formation [22]. Here, in order to sort out appropriate promoter G4 for the proposed drug screening strategy, some typical proto-oncogene segments, including *Hum24*, *C-kit21*, *S1*, *Pu27* and *Pu22*, together with a few frequently-used artificial DNAzyme oligonucleotides *PS2.M* and *PW17* (details in Table S1 in the Supplementary Material) were tested to intercompare their peroxidase-like activities after complexed with hemin. As shown in Fig 3, the activity of the parallel-stranded propeller-type *Pu22* G4 or *Pu27* G4 DNAzyme, despite inferior to that of the artificial-designed *PS2.M* or *PW17* DNAzyme, was stronger than those of other promoter G4 DNAzymes with antiparallel or mixed hybrid of parallel/antiparallel conformations. Eventually, *Pu22* G4 was here chosen as the promoter oncogene target in virtue of its best suitability for the proposed approach.

Fig3. Intercomparison the activities of DNAzymes with various G4 conformations

Reagents: 1 μ M G4 DNA, 0.5 μ M hemin, 10 mM K⁺

3.2 Ligand displacement mechanism

In order to demonstrate the ligand displacement mechanism, the promoter G4 conformation stability in the process of hemin/alkaloid displacement was investigated. The conformational stabilities of *Pu22* G4, *Pu22* G4/hemin complex and *Pu22* G4/SAU complex were measured via CD spectroscopy. It is well known that G4 in parallel four-stranded structure shows CD spectrum with a positive band at ~264 nm and a negative band at ~240 nm; but in antiparallel structure with ~295 nm positive and ~260 nm negative bands [22]. In this work, the CD spectra of *Pu22* G4 before and after complexation either with hemin or with alkaloid SAU were all showed the similar positive 264 nm and negative 240 nm bands. Moreover, the band intensity was not sensitive to the change of ligand (SAU) concentration as well, indicating the parallel *Pu22* G4 conformation remained stable after the ligand displacement (see CD spectra in Fig S2. in the Supplementary Material).

Since molecular dynamics are now routinely used to investigate the structure, dynamics and thermodynamics of biomolecular complexes, MD simulation of interaction between *Pu22* G4 and alkaloid or hemin was performed further for deeply exploring the *Pu22* G4 complexation with the heterocyclic molecules. As illustrated in Fig 4, the configurations of the *Pu22* G4/ligand complexes in the MD procedure indicated that either hemin or any of the five tested alkaloids (*i.e.* SAU, BER, CHE,

MAT or *LIG*) was proved complexing with *Pu22* G4 via end-stacking interaction between the heterocyclic ring of ligand and the G-quartet plane in *Pu22* G4. Moreover, the binding energies shown by thin lines in Fig5 were calculated, which contained short-range L-J potential and electrostatic potential. Then the outcomes were smoothed through the adjacent-averaging method every 3 ns to reduce the influence of fluctuation, as shown in thick lines in Fig5. The binding energies displayed the tendency of stabilizing while the systems were almost in equilibrium after 170 ns. It also demonstrated that the interaction energy for *Pu22* G4/hemin complexation reached approximately -159.2 kJ/mol, less than any of those for *Pu22* G4/*SAU*, *Pu22* G4/*BER* or *Pu22* G4/*CHE* complexations (ca. -250.0, -241.5 and -289.1 kJ/mol, respectively), but more than either of the *Pu22* G4/*MET* or *Pu22* G4/*LIG* complexation's (ca. -110.3 and -121.4 kJ/mol, respectively). These intercomparison of binding energies provided an intensive rationale for the former three isoquinoline alkaloids (*SAU*, *BER* and *CHE*), other than the latter two (*MAT* and *LIG*), had certainty in their ability to displace hemin based on the *Pu22* G4/ligand complex stability. Meanwhile, such simulation consequences were also demonstrated by the following CL ligand displacement assay, as stated in Section 3.4.

In addition, hemin, as can be seen, played a critical role in discriminating ligand complexation stability with *Pu22* G4 DNA. In fact, previous studies have already revealed that not only endogenous heme is biosynthesized and extensive existing in almost all mammal cells [23], but many cell-cycle regulator genes influencing cancer promotion and suppression, such as *c-MYC*, are also under circadian control which modulates heme biosynthesis reciprocally. Accordingly, when certain analogue competed against heme in binding to the cell-cycle regulator genes, such competitive displacement would produce potential anticancer effect on oncogene expression as well [24]. Therefore, it is reasonably believed that the mechanism of the proposed drug screening strategy based on ligand displacement assay might also be biologically feasible.

Fig4. Schematic diagrams showing the side view of the interaction between each of the five alkaloids or hemin and the *Pu22* G4 structure of *c-MYC*. The main chain of G4 is shown in ribbon while the bases in blue leaflet and the deoxyribose in green leaflet. The ligands are shown in ball-and-stick model, respectively.

Fig5. Interaction energies for *Pu22* G4/ligand complexation during 200 ns GMD simulation procedure (thin lines) and smoothed by adjacent-averaging method every 3 ns (thick lines)

3.3 Spectrometric evidences of G4/ligand complexation

The complexation of *Pu22* G4 DNA with ligand is commonly studied with the aid of molecular absorption or fluorescence spectroscopy investigation, and the UV absorbance melting curve technique [8]. Here, the putative alkaloid drug *SAU* [2] was used as the ligand for exploring *Pu22* G4/alkaloid interaction.

From the perspective of G4 DNA, owing to the π - π stacking arising from the interaction between G-quartet and chromophoric ligand, quadruplex DNA often produces remarkable bathochromic shift and hypochromic effect on the ligand [25]. In this work, pronounced changes in the UV-Vis spectrum of *SAU* solution, including a red shift (~ 10 nm) of maximum absorbance (λ_{max}) and a remarkable attenuation of absorbance ($\sim 33\%$), had been observed after the addition of *Pu22* DNA (details in Fig S3I in the Supplementary Material). And also the fluorescence spectrum of *SAU* quenched apparently with the *Pu22* DNA addition (details in Fig S3II in the Supplementary Material), indicating the interaction between *Pu22* DNA and *SAU*.

In addition, UV absorbance melting curve associated with the hypochromic effect at 295 nm is widely applied for monitoring the stability of G4 DNA or G4 DNA/ligand complex [8]. Here, we measured 295 nm absorbance of *Pu22* DNA as a function of temperature to get its thermal stability index (T_m), which at the mid-point of melting curve for 50% dissociation of G4 DNA forming oligonucleotides. As illustrated in Fig 6I, a sigmoidal curve obtained from the *Pu22* DNA thermal melting experiment demonstrated the existing of the *Pu22* G4 with its T_m at 74.8°C. And the increase of T_m value 79.5°C measured after the addition of *SAU* provided the evidence of stability raised with the *Pu22* G4/*SAU* complex formation.

From the perspective of ligand, hemin complexation with G4 DNA may give rise to hyperchromicity and red-shift of its Soret band [11], which had also been demonstrated in this work as shown in Fig 6II (trace c vs. a). Then again, we investigated the change of the Soret band after further addition of alkaloid ligand (*SAU*) and found such hyperchromicity and red-shift effect decreased obviously (trace d vs. c), despite *SAU* increased the background absorbance (trace b vs. a). Such spectroscopic analysis result helped to

demonstrate the displacement of SAU for hemin in *Pu22* G4 complex as well.

Fig6I. UV melting curves monitoring *Pu22* G4 in the absence or presence of SAU

Fig6II. UV Soret bands of hemin, hemin with *Pu22* G4 and hemin with SAU and *Pu22* G4

Reagents: 10 μ M *Pu22* DNA, 20 μ M SAU, 10 mM K⁺, 2 μ M hemin

3.4 Alkaloid drug screening

Alkaloids are natural compounds with diverse biological activities, some of which are reported to have anticancer properties [2, 26]. Alkaloid compounds containing heterocyclic moieties often exhibit higher bioavailability on account of the improved solubility. Because previous biophysical data showed that the planar groups were essential for effective G4/ligand complexation via π - π stacking, some hetero-aromatic polycyclic compounds [4] are considered as putative ligands with potential antiproliferative activity to downregulate *c-MYC* expression in cancer cells [27]. However, in view of the polymorphic characteristics and structural complexity of alkaloids, to seek a high-throughput and low-consumption approach for efficiently screening alkaloid *Pu22* G4 stabilizer becomes a key part of this work.

Here, we proposed a microfluidic-based ligand displacement assay for screening anticancer drug. After the optimization of the CL assay system as referred to Fig S4-S7 in the Supplementary Material, five commonly used heterocyclic alkaloids SAU, BER, CHE, MAT and LIG extracted from the traditional medicine herbs were used to validate the proposed screening approach. The experimental outcomes, as illustrated in Fig 7, showed that the alkaloid ligand-related DNAzyme-catalyzed CL signals decreased obviously with the addition of SAU, BER or CHE; but instead, MAT or LIG did not induce such effect. The results suggested that the three isoquinoline alkaloids SAU, BER and CHE, other than the two negative controls MAT and LIG, could effectively displace hemin in complexation with *Pu22* G4, even their concentrations were 100 times lower (5 μ M vs. 500 μ M). That clearly agreed with their previously proven anticancer properties [2, 26].

In addition, by taking the advantage of microfluidic-based CL detection, this approach consumed just 2 μ L of sample solution for one measurement containing only ~10 fmol of alkaloid and ~1 fmol of G4 DNA. The CL assay process is totally program-controlled with a high throughput of 75 /h and a good precision of RSD value 1.8% (n=11).

Fig7.The CL assay result of screening putative alkaloid ligands

Reagents: 0.5 μ M Pu22 DNA, 5 μ M SAU, 5 μ M CHE, 5 μ M BER; 500 μ M MAT, 500 μ M LIG, 10 mM K⁺

3.5 Ligand selectivity assessment for G4 DNA over dsDNA

The affinity of the anticancer drugs for G4 DNA is essential to efficiently establish its biological functions, such as chromosomal stability or transcriptional regulation[28]. However, in view of the putative alkaloid drug having in their ability to bind either with dsDNA or G4 DNA[25], it would be valuable to know if they indeed specifically targeted G4 DNA over dsDNA. Therefore, a 17 base-pair dsDNA *ds17* (sequence details in the Supplementary Material) was chosen as the dsDNA reference control[29] to investigate the preferential selectivity of alkaloid drug for Pu22 G4 DNA.

But first, the species distribution of the *ds17* and mixed *ds17*/Pu22 DNA solutions were calculated using two thermodynamic software packages DINAMelt and NUPACK, as referred to our previous works[11, 30]. The software packages had been applied to simulate the species normalized concentrations in near-physiological (100 mM Na⁺) condition, as illustrated in Fig 8I & II. The results indicated that the heterodimer *ds17* remained fairly stable and almost no dissociated strand available to hybridize with Pu22 DNA at body temperature (37°C) condition.

Fig8I. Temperature-dependent concentration distribution and T_m from DINAMelt simulation of 10 μ M of each of the complementary strand A and B. The Au and Bu lines indicate the concentrations of the unfolded single strands, and the Af and Bf lines show the folded single strands. The AA and BB curves correspond to the two homodimers and the AB curve to the heterodimer.

Fig8II. NUPACK prediction (at 37 °C) of thermodynamic equilibrium population distribution of three strands: 10 μ M complementary strands A & B and 1 μ M Pu22 DNA strand C, suggesting both strand A and B were dominated by the formation of Watson-Crick base-pairs *ds17* DNA between the complementary bases, whereas strand C was self-assembled as Pu22 G4 structure.

Then, for those *Pu22*-hemin DNAzyme and mixed *Pu22*-hemin DNAzyme/alkaloid (*i.e.* *SAU* or *BER* or *CHE*) solutions, their peroxidase-like activities before and after mixed with *ds17* (even 10 times the amount of *Pu22*) were investigated and the CL intensity variations concerning alkaloid affinity of G4 DNA over dsDNA were intercompared, including *Pu22*-hemin DNAzyme *vs.* mixed *ds17*/*Pu22*-hemin DNAzyme, and mixed alkaloid/*Pu22*-hemin DNAzyme *vs.* mixed alkaloid/*ds17*/*Pu22*-hemin DNAzyme, respectively. Furthermore, by using duplex DNA intercalator ethidium bromide (*EB*) as positive control of the dsDNA binder, the catalytic activities of mixed *EB*/*Pu22*-hemin DNAzyme before and after the *ds17* addition (*i.e.* mixed *EB*/*Pu22*-hemin DNAzyme *vs.* mixed *EB*/*ds17*/*Pu22*-hemin DNAzyme) were also investigated. Through intercomparing those variations of the DNAzyme-catalyzed CL assay results, it is reasonably perceived that the more CL intensity enhanced with the addition of *ds17*, the more influence of *ds17* would be expected to compete against G4 DNA in binding with alkaloid ligand. As illustrated in Fig 9, the selectivity order of the alkaloids to *Pu22* G4 over *ds17* should be *BER* > *CHE* > *SAU*. Meanwhile, all these putative alkaloid drugs, as compared with typical duplex DNA binder *EB*, unsurprisingly gave better priority to *Pu22* G4. Such intercomparison information facilitated at least in one respect the assessment of drug specificity for candidate oncogene target, which should obviously be desirable in drug screening test regarding genetic therapy.

Fig9. DNAzyme activities intercomparison for assessment ligand selectivity for G4 DNA over dsDNA
Reagents: 0.5 μ M *Pu22* DNA, 5 μ M *SAU*, 5 μ M *CHE*, 5 μ M *BER*, 5 μ M *EB*, 10 mM K^+ , 100 mM Na^+ [#]

Conclusion

Recent advances in molecular biology and genetic research appeal to discover relevant promising anticancer drug screening approach. In order to screen potential alkaloid drug for stabilizing promoter oncogene-related G4 with specific parallel structural feature, a ligand displacement assay based on G4 DNAzyme catalyzed chemiluminescence detection has been developed. In the work, not only the

[#]Other than 10 mM K^+ , the presence of 100 mM Na^+ in the sample solution just induced a limited DNAzyme activity enhancement and marginally increased the overall signal intensity in CL assay.

end-stacking interaction associated with the ligand displacement mechanism has been demonstrated to be the favorable complexation mode between the G4 DNA and putative alkaloid drug, but the microfluidic-based chemiluminescence detection used in the assay has also showed its advantage of automatic, high throughput with just trace amount of sample and reagent consumption.

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