Novel mitochondria-targeted, nitrogen mustard-based DNA alkylation agents with near infrared fluorescence emission

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ABSTRACT

In this report, the design of two novel nitrogen mustard-based DNA cross-linking agents with fluorophores incorporated into the structure (**TN-A** and **TN-B**) is disclosed. The results indicate that **TN-A** and **TN-B** can serve directly as both reporting and imaging agents for flow cytometry and gel electrophoresis without the necessity of another fluorescent tagging agents. **TN-A** and **TN-B** both selectively locate in the mitochondria and exhibit good antitumor activity. Notably, **TN-A** is the first DNA crosslinking agent with near infrared fluorescence emission properties.

Keywords

DNA cross-linking; Mitochondria-targeted; Nitrogen mustard; Near infrared fluorescence emission; Gel electrophoresis; Cell apoptosis analysis

1. Introduction

DNA is essential for the transmission of genetic information in any living organism. Several anticancer drugs in clinical use exert their activity by the crosslinking of DNA [1-3]. A plethora of DNA cross-linking agents have been reported to date [4-9]. As one important class of DNA bi-functional alkylation agents, the nitrogen mustards (*N*-mustards) such as cyclophosphamide, chlorambucil and melphalan are frequently used as highly efficacious antitumor drugs in the clinic [10-12]. The bis(2-chloroethyl)amine moiety common to all *N*-mustard antineoplastic agents is a highly reactive alkylating electrophile. The cytotoxic effects of mustard agents are attributed to their abilities to establish interstrand cross-linkages in the major groove of DNA, which interrupts DNA biosynthesis and causes DNA damage [13-18]. Due to their high reactivity, however, *N*-mustards can demonstrate poor selectivity in the intracellular milieu, easily reacting with other cellular components including amino acids, proteins and carbohydrates, thus resulting in many unwanted side-effects such as bone marrow toxicity and genotoxicity [13, 19]. Therefore, many structural modifications have been made to the *N*-mustard scaffold over the past decades so as to increase target affinity/specificity for DNA. Nevertheless, most studies with *N*-mustards mainly focus on their overall antitumor activity; efforts to extrapolate when and where these compounds accumulate in the cell as well as to understand their associated mechanisms-of-action and specific effects on cells typically require the use of exogenous organelle-specific fluorescent dyes. To address this issue, multifunctional

compounds that contain both a bio-reactive pharmacophore and a fluorophore are believed to be a future direction for further development.

Mitochondria are the power generators of the cell and they play important roles in numerous vital cellular processes. Accordingly, malfunctions with these organelles are crucially involved in various pathologies [20-24]. Consequently, the study of mitochondria is important to biomedical research and diagnostic applications. Recent investigations have demonstrated that mitochondria, due to their role in metabolic regulation of cell death pathways, are deeply involved in drug-induced cell death [25]. The negative membrane potential of mitochondria allows for drugs and fluorescent probes with cationic charges to localize in mitochondria. To the best of our knowledge, only one example has been reported about the localization of *N*-mustard-based drugs in the mitochondria, which presented by Neamati group by utilizing of triphenylphosphonium as targeting group [26].

The high sensitivity and temporal/spatial resolution of fluorescent molecular probes has established them as vital components to modern biomedical research. Fluorescent probes that emit in the near-infrared (NIR) region (emission wavelengths from 650 nm to 900 nm) are in especially high demand for the imaging of living systems due to their superior abilities for minimizing photo-damage to living cells, avoiding interference from bimolecular auto fluorescence, and deeper tissue penetration. NIR emission fluorescent probes, thus play increasingly important roles in biological sensing, imaging, and photodynamic therapy [27-29]. Although, several

N-mustards co-functionalized with fluorophoric domains have been reported [30-32], the specific fluorescent properties of these compounds were not explored rigorously. Moreover, there are no current examples of NIR fluorescence emitting *N*-mustard-based drugs. Herein, we present two mitochondria-targeted *N*-mustard-based DNA alkylating agents with near infrared fluorescence emission, **TN-A** and **TN-B** (Fig.1). Native localization of these compounds to the mitochondria is expected to increase their selectivity and affinity for mitochondrial DNA.

2. Materials and Methods

All reagents are purchased with no special note. All of the solvents were dried according to the standard methods. All of the solvents were spectroscopic grade in the optical spectroscopic studies.

2.1 The experimental materials and equipment

NMR spectra were measured on a Bruker AM-400. The ^1H NMR (400 MHz) chemical shifts were given in ppm relative to the internal reference TMS. The ^{13}C NMR (100 MHz) chemical shifts were given using CDCl_3 and DMSO-d_6 as the internal standard. ESI-MS and HR-MS spectral data were recorded on a Finnigan LCQDECA and a Bruker Daltonics Bio TOF mass spectrometer, respectively.

2.2 The fluorescence properties experiment

The compounds of **TN-A** and **TN-B** were respectively dissolved in DMSO, that is to say, the concentration of solution is 1 mmol/L. When measured, **TN-A** and **TN-B** were diluted into the solution you need. Such as, when measuring absorption and

emission wavelength, we bring out the original solution (1 mmol/L, 1 μ L) to dilute it to assay solution (10 μ mol/L, 1mL) by adding PBS or DMSO solution. The emission wavelength and absorption wavelength were measured by measuring instrument. Fluorescence excitation and emission spectra were obtained using Fluoro Max-4 Spectrofluoro photometer (HORIBA Jobin Yvon). UV-Vis absorption spectra were recorded on a Hitachi Pharma Spec UV-1900 UV-Visible spectrophotometer. Fluorescent quantum yields were determined by standard methods, using fluorescein (Φ =0.69 in ethanol) as a standard.

2.3 Cell lines

All cell lines used were obtained from American Type Culture Collection (ATCC). These cell lines were cultured in culture medium supplemented with 10% PBS (Phosphate Buffer Solution). All cell lines were maintained at 37 $^{\circ}$ C in a CO₂ incubator with 5% CO₂. Cell apoptosis were given in BDFACSC alibur, and data was prepared by FlowJo7.6.1.

2.4 DNA agarose gel electrophoresis

Autoradiograph of a neutral agarose gel showing DNA interstrand cross-linking by probes **TN-A** and **TN-B** with PBR 322 DNA. Drug reactions (2 h at 37 $^{\circ}$ C) were in PBS pH 7.2 buffer with 10 ng of DNA in a final volume of 20 μ L. Electrophoresis was carried out on 0.8% w/v submerged horizontal agarose gels at 80 V of 45 minutes. JUNYI Power Supply of JY300C was used for DNA agarose gel electrophoresis, data recorded in the Molecular Imager of Gel Doc TM XR+ with Image Lab TM Software

of BIO-RAD.

2.5 Confocal microscopy experiments

HeLa cells were separately cultured by using the same medium at 37 °C 20% CO₂. They were cultured 2 days. Then, the living HeLa cells were respectively incubated in a confocal dish, with either of the two compounds (2 μM) and MT Green (1 μM) in culture medium for 30 min, respectively. Finally, these cells were used for cell imaging using confocal microscopy.

2.6 Cell toxicity assay

Effects of compounds on the cell viability of cell lines were examined using a cell viability reagent, MTT. Cells were separately cultured by using the different medium at 37 °C 20% CO₂ by 1 day. The following day, drugs were added to the different cells, then, these cells and compounds were cultured 2 days. Namely, cells were seeded in a 96-well plate in the absence or presence of tested compounds for 72 h. The MTT (5mg/ml in sodium chloride) was added for the last 2 h of incubation, and the absorbance at 570 nm was measured after the dissolve in the dimethyl sulfoxide. Finally, the absorbance is measured and inhibition rate is calculated by software of Graph Pad Prism 5.01 (Graph Pad Software, USA).

3. Results and discussion

3.1 Synthesis and characterization of probe TN-A and TN-B

The role that *N*-mustards play as anticancer drugs centers on their ability to alkylate and crosslink two different biological nucleophiles [33]. Aniline-based

N-mustards usually exhibit lower reactivity, lower alkylation capacity, and lower alkalinity versus aliphatic *N*-mustards, thus allowing for better control of undesired side-effects. With this in mind, our compound design involved coupling one of two different chromophoric units (A and B) onto a 4-(bis(2-chloroethyl)amino)benzaldehyde *N*-mustard “warhead”. The two units (chromophore and warhead) were readily linked using a condensation reaction [34]; the final products **TN-A** and **TN-B** were characterized by ¹H NMR, ¹³C NMR and HRMS (See ESI). As could be expected, the chromene-based **TN-A** exhibited lower water solubility versus the less hydrophobic pyran **TN-B**.

With the target compounds in hand, the corresponding absorbance and fluorescence emission spectra were investigated first. As shown in Fig.2, **TN-A** exhibited a fluorescence emission centered at 676 nm when dissolved in PBS buffer (30% DMSO as co-solvent); the maximum emission wavelength of **TN-B** under the same conditions was found to be 623 nm. The Stokes shifts for both fluorophores were close to 160 nm, which should minimize excitation interference in cell imaging efforts (Fig. S1).

3.2 Quantum yield calculation

Quantum yields were determined using rhodamine B ($\Phi_{\text{standard}}=0.69$ in EtOH) as a standard according to a published method (Chemical Communications, 2014, 50(2): 183-185). The quantum yield was calculated according to the equation:

$$\Phi_{\text{sample}} = \Phi_{\text{standard}} * (I_{\text{sample}} / I_{\text{standard}}) * (A_{\text{standard}} / A_{\text{sample}}).$$

Where Φ_{sample} and $\Phi_{standard}$ are the fluorescence quantum yield of the sample and the standard, respectively; I_{sample} and $I_{standard}$ are the integrated fluorescence intensities of the sample and the standard; A_{sample} and $A_{standard}$ are the optical densities, at the excitation wavelength, of the sample and the standard, respectively.

Quantum yield of **TN-A**: $\Phi = 0.046$. Quantum yield of **TN-B**: $\Phi = 0.38$.

3.3 Mitochondrial localization experiments

Once determining that compounds **TN-A** and **TN-B** demonstrated desirable fluorescence properties for biological imaging, we further tested the utility of the two compounds in a cellular environment [35, 36]. To determine whether **TN-A** and **TN-B** could specifically locate in the mitochondria, commercially available mitochondrial dye, Mito Tracker Green (MT Green), was employed for the following co-localization studies[37, 38]. Living HeLa cells were respectively incubated with either one of our synthetic fluorescent *N*-mustards (2 μ M) and MT Green (1 μ M) in culture medium for 30 min[39, 40]. The merged picture confirmed that the fluorescence signals were only located in the intracellular area. As shown in Fig.3, the signal of **TN-A** and **TN-B** could overlay with MT Green very well. Moreover, the Pearson's co-localization coefficient for **TN-A** and **TN-B** (described as the correlation of the intensity distribution between two channels), were calculated to be 0.98 and 0.95, respectively, suggesting that these two *N*-mustard based DNA alkylating agents localized predominantly in the mitochondria of living HeLa cells. Importantly, both of **TN-A** and **TN-B** were completely taken up by the HeLa cells, indicating the excellent cell membrane

penetration abilities of these two reactive probes.

3.4 Cell apoptosis analysis by flow cytometry (FCM)

Cells were next analyzed using fluorescence-assisted flow cytometry to study the uptake ability of our fluorescent mustards [41]. The K562 chronic leukaemia myelogenous cell line (single cell suspension) was applied to these flow experiments. Our results are summarized in Fig.4, control studies without the addition of any exogenous compound are shown in panels (a) and (c), whereas Fig.4 (b) and (d) are the results of only adding either **TN-A** and **TN-B** (10 μ M), respectively. It is gratifying that no additional dye was needed; the fluorescent mustards allowed for direct observation of the results using flow cytometry. This result emphasized how use of our fluorescent mustards could help eliminate background interference and misleading data associated with non-reactive labeled dyes.

3.5 Evaluating the *N*-mustard cross-linking ability and selectivity.

Mitochondrial DNA is localized in the mitochondrial matrix; it is possible that our *N*-mustard bis-alkylating agents can react with this DNA and induce cellular apoptosis. Previous reports indicate that *N*-mustard derivatives cross-link DNA via single-molecule based substitution reactions predominantly at guanine residues [42]. In order to substantiate the cross-linking abilities of our *N*-mustard based probes, **TN-A** and **TN-B** were individually incubated with DNA and analyzed by gel electrophoresis (Fig.5). Without **TN-A** or **TN-B**, the band of free oligonucleotide was clearly visible, while in the presence of **TN-A** or **TN-B**, the band of oligonucleotide appears to be

locked in the sample-loading holes. It should be pointed out that, without the help of DNA staining reagents, we could directly see the cross-linked DNA on the holes, due to the enclosed fluorophore in the alkylation agents. Both the flow cytometry and gel electrophoresis experiments illustrate that **TN-A** and **TN-B** can reduce the number of operational processes, such as the addition of extra dyes or stains, which would make the analysis more efficient and economical.

3.6 The cell toxicity test of probe TN-A and TN-B.

It has been reported that compared with normal cells, cancer cells are more sensitive to nitrogen mustards [26, 33]. Finally, to test the cell toxicity of our two DNA alkylation agents, standard MTT assays were performed by changing the concentration (10 μ M) of **TN-A** and **TN-B** in different cell lines. As shown in Fig.6, generally, **TN-B** exhibited a better inhibition ratio than **TN-A** at the same concentration. Compared with other cell lines, **TN-B** exhibited the highest inhibition activity in the K562 cell line, suggesting a potential role as anti-cancer agents.

4. Conclusion

In conclusion, we developed two related multifunctional cross-linking reagents (**TN-A** and **TN-B**) that can selectively locate in the mitochondria of living cells. In addition, **TN-A** is the first *N*-mustard based probe with NIR emission. The fluorescent properties of these compounds allow for direct analysis, without the help of extra dyes or stains, in flow cytometry and gel electrophoresis experiments. Last but not least, we provide a strategy for the coupling of a traditional drug pharmacophore with a NIR

fluorescent probes. We anticipate this kind of design concept could include more fluorophores and inspire the production of more efficient DNA alkylation agents.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at <http://www.journals.elsevier.com/talanta>.

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Figure captions

Figure 1. Nitrogen mustard based DNA alkylation agents TN-A and TN-B.

Figure 2. The absorption and emission spectrum of TN-A and TN-B in aqueous PBS/DMSO (10 μ M). (a) The absorption spectrum of probe **TN-A** and **TN-B** was measured in the concentration of 10 μ M. **TN-A** exhibits a fluorescence absorption centred at 510 nm, the maximum absorption wavelength of **TN-B** was found at 464 nm. (b) The emission spectra of **TN-A** and **TN-B** was measured in the concentration of 10 μ M. **TN-A** exhibits a fluorescence emission centred at 676 nm, the maximum emission wavelength of **TN-B** was found at 623 nm.

Figure 3. HeLa cells were stained with TN-A and TN-B (2 μ M) and MT Green (1

μM) for 30 min. (a, f):**TN-A, TN-B**; (b, g): MT Green; (c, h):merged images of (a) and (b), or (f) and (g), (d, i): bright field of (a) or (f); (e, j):co-localization images across HeLa cells. Ex@488 nm for the red channel (570–750 nm), and Ex@488 nm for MT Green (470-520 nm).

Figure 4. Flow cytometry with only TN-A and TN-B. (a) and (c): control, only cells and medium, without probes; (b) and (d): in addition to the cells and culture media, adding **TN-A** (10 μM) and **TN-B** (10 μM), respectively.

Figure 5. DNA agarose gel electrophoresis. Channel 1, 2, 3 and 4 with Gold view (DNA fluorescent dye); Channel 5, 6, 7 and 8 without Gold view. Channel 1: DNA marker; Channel 2: PBR 322; Channel 3: **TN-A** and DNA; Channel 4: **TN-B** and DNA; Channel 5: **TN-B** and DNA; Channel 6: **TN-A** and DNA; Channel 7: **TN-A**; Channel 8: **TN-B**.

Figure 6. MTT assay for different cell lines. The inhibition rate of **TN-A** and **TN-B** for different cells was assayed in the same drug concentrations (10 μM), respectively. All the cell lines were used to MTT assay including BGC-823 (human gastric cancer cell line), CT-26 (colon cancer cell line), HepG2 (human hepatica cell line), BCPAP (human thyroid cancer cell line), eca-109 (human oesophageal cancer cell line), A549 (lung cancer cell line), Jurkat (acute T-cell leukaemia cell line), HeLa (cervical cancer cell line) and K562 (single cell suspension).

Figure1

TN-A

TN-B

Figure2

Figure3

Figure4

Figure5

Figure6

Tiger with wings: Combine nitrogen mustard-based DNA alkylation agent with near infrared emission fluorescence probe into one.