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PRELIMINARY STRUCTURE–ACTIVITY RELATIONSHIP STUDY OF HEPTAMETHINE INDOCYANINE DYES FOR TUMOR-TARGETED IMAGING

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Our research has identified a couple of near-infrared (NIR) heptamethine indocyanine dyes exhibiting preferential tumor accumulation property for *in vivo* imaging. On the basis of our foregoing work, we describe here a preliminary structure−activity relationship (SAR) study of 11 related heptamethine indocyanine dyes and several essential requirements of these structures for *in vivo* tumor-targeted imaging.

Keywords: Heptamethine indocyanine dyes; NIR imaging; tumor targeting; SAR study.

1. Introduction

The application of near-infrared (NIR) imaging holds great promise for *in vivo* tumor imaging due to low tissue autofluorescence and high tissue penetration in the NIR wavelength (700−900 nm).[1−3] Heptamethine indocyanine dyes are representative NIR fluorescent contrast agents that have been applied extensively in biotechnology and medicine research, for example, immunoassays, DNA sequencing, labeling for proteins, and detection of some biomarkers of diseases.[4−6] Among them, indocyanine green (ICG) has been approved for clinical use as a non-targeting agent for decades.[7,8]

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Our recent studies have identified a couple of heptamethine cyanine dyes with improved near-infrared fluorescence emission profile and particularly active tumor targeting property without the necessity of chemical conjugation.[9–11] These dyes are lipophilic cations at emission wavelength around 780 nm and can preferentially accumulate in a broad spectrum of tumor cells to reach a significant signal contrast for *in vivo* tumor imaging. These dyes can reach a contrast index (CI) value up to 20 in a tumor compared with its surrounding tissue, while more than 2.5 times was considered as substantial accumulation in other published work.[12,13] However, the tumor-targeted mechanism of these dyes remains unknown. In order to investigate the essential structure requirements for tumor-targeted imaging, we conduct here a preliminary SAR study of 11 analogs of heptamethine indocyanine dyes obtained from commercial available sources.

2. Materials and Methods

2.1. *Chemicals and instruments*

As shown in Table 1, except the compound **5** synthesized by our laboratory,[9] all the heptamethine indocyanine dyes reported in this work were purchased from American Dye Source Incorporation (ADSL, Quebec, Canada), Sigma-Aldrich Corporation (SAC, Shanghai, China), or Spectrum Information Ltd (SIL, NJ, USA), respectively. These dyes were dissolved in dimethylsulfoxide (DMSO) diluted with appropriate vehicles and stored at −20°C before use. NIR fluorescence imaging was taken by using a Kodak *in vivo* FX Professional Imaging System (New Haven, CT, USA).

2.2. *Cell culture and animal tumor model*

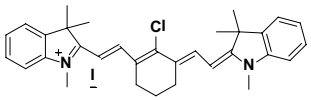
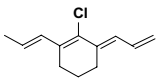
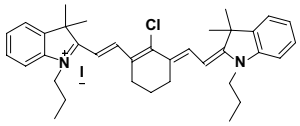
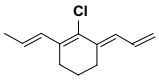
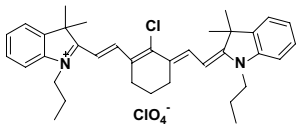
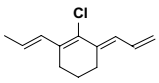
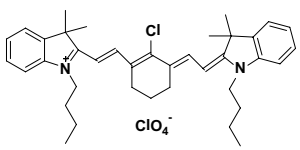
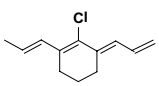
Human breast cancer (MCF-7) tumor cell lines used in this study were purchased from ATCC (Manassas, VA, USA) and cultured in ATCC recommended media, with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2.

To evaluate the tumor targeting ability of these dyes, MCF-7 tumor xenografts were established as we have reported previously.[9] Briefly, three- to four-week-old athymic nude mice were purchased from the laboratory animal center of the Third Military Medical University. Mice were injected subcutaneously in the right flank with 2×10^6 MCF-7 cultured cells suspended in 200 ul PBS. Animal protocols were in accordance with the "Animal Care and Use Committee Guidelines of the Third Military Medical University".

2.3. In vivo *and* ex vivo *optical imaging*

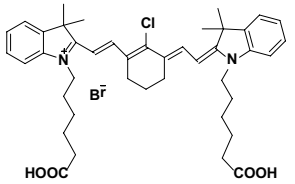
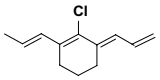
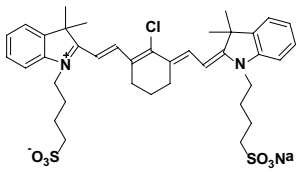
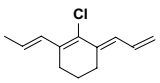
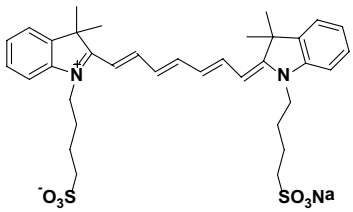
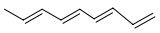
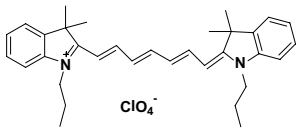
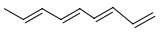
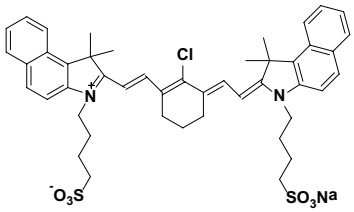
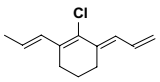
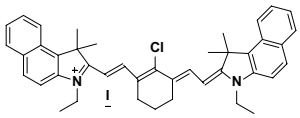
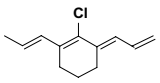
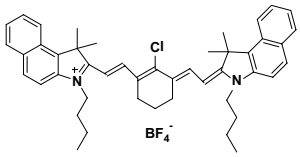
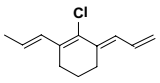
Mice-bearing human MCF-7 tumor xenografts were injected intravenously with different dyes at a dose of 0.2 mg/kg. Whole body optical imaging was taken

Table 1. Evaluation of various chemical structures of heptamethine indocyanine dyes on tumor-targeted bioactivity.

Comp. No.	Structures	R_1	R_2	R_3	R_4	X	Bio-[a]
1		CH_3		H	H	I	−[b]
2		n-Pr		H	H	I	+[c]
3		n-Pr		H	H	ClO_4	+
4		n-Bu		H	H	ClO_4	+

(*Continued*)

Table 1. (*Continued*)

Comp. No.	Structures	R_1	R_2	R_3	R_4	X	Bio-[a]
5		n-$C_6H_{10}COOH$		H	H	Br	+
6		n-$C_4H_8SO_3^-$		H	H	N[d]	+
7		n-$C_4H_{10}SO_3^-$		H	H	N	–
8		n-Pr		H	H	ClO_4	–
9		n-$C_4H_8SO_3^-$				N	–
10		CH_2CH_3				I	–
11		n-Bu				BF_4	–

[a]The bioactivity of these dyes was determined according to our published protocols.[9] The bioactivity was positive when the contrast index (CI) more than 2.5 in the MCF-7 tumor xenograft, while the dye with a CI value less than 2.5 was regarded as negative bioactivity. The fluorescent intensity was calculated using Kodak MI software 5.0.1.

[b]"–" represents negative bioactivity ($CI < 2.5$).

[c]"+" represents positive bioactivity ($CI > 2.5$).

[d]"N" represents absence of X-ion.

12 days after dye injection by using a Kodak *in vivo* FX Professional Imaging System. It was equipped with fluorescent filter sets (excitation/emission, 770/830 nm). The field of view was 100 mm in diameter. The frequency rate for NIR excitation light was 2 mW/cm^2. To evaluate the dynamic dye accumulation and retention in tumors, the contrast index (CI) values were calculated as described in the

literature.[12,13] The CI was measured according to the formula CI = [(Ftumor-Fauto)/(Fnorm-Fauto)]. Ftumor and Fnorm are the fluorescence mean intensities of tumor and normal contralateral region, respectively. Fauto is the autofluorescence from the corresponding region measured before dye injection. The fluorescent intensity was calculated using Kodak MI software 5.0.1. Then, organs and tumors were obtained for fluorescent imaging on the day of sacrifice.

2.4. *Molecular calculation*

Quantum chemical calculation was performed with molecular mechanics (MM+) force field method and PM3 semi-empirical method by using a suite of Hyperchem 7.5 programs for a preliminary analysis of three-dimensional SAR. Character and structure parameters, such as oil-water partition coefficient (LogP), polarity, and volume, can be obtained under quantitative structure-activity relationship (QSAR) module.

3. Results and Discussion

The heptamethine indocyanine dyes (as shown in Fig. 1) generally consist of two indole rings with N-alkylation chain (R_1), heptamethine moiety (R_2), different substitution groups (R_3/R_4) in indole rings, and often including some kind of halogen ion (X^-).[14] Herein, our structure−activity relationship study is carried out by means of altering the kinds of R moieties and X-ion (Table 1).

According to the bioactivities of compounds with various R_1 chains in Table 1, compounds **2**–**6** with N-aliphatic hydrocarbon chains (3−6 carbons) exhibited significant accumulation in tumor tissues for *in vivo* imaging, while **1** with N-methyl (one carbon) did not show the selectivity, suggesting the length and kind of carbon chain are closely related to the tumor targeting property. Less than two carbons in R_1 chain would significantly weaken the ability of selective tumor accumulation.

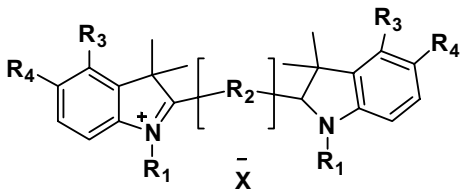


Fig. 1. General formula of heptamethine indocyanine dyes.

For evaluating the importance of chlorcyclohexene and heptamethine moiety (R_2) on its tumor targeting bioactivity, we conducted the comparison of bioactivities of some typical structures, such as **7**–**8** without chlorcyclohexene in heptamethine moiety. It was testified that **7**–**8** did not show the bioactivity of preferential accumulation in tumor cells. This indicates both cyclohexene and heptamethine moiety are the essential groups for their tumor targeting property.

Through comparison of selectivity of **9**, **10**, **11** in which structures comprise benzo ring between R_3 and R_4, we found that these compounds lost the property of tumor targeting. It indicates that the formation of benzo ring between R_3 and R_4 in indole ring is disadvantage to retain the tumor selectivity. Furthermore, we observed that **2** with form of iodum ion ($X = I^-$) and **3** with form of perchlorate ($X = ClO_4^-$) both manifested favorable tumor selectivity, suggesting that the kind of X-ion had little effect on their tumor targeting property.

Chemical structure determines the molecular properties. However, it is improvident to evaluate various organic structures all through experiment method. To shed light on the structure−activity relationship between heptamethine indocyanine dyes and tumor targeting activity, quantum chemical calculation was performed with MM+ force field method[15] and PM3 semi-empirical method[16,17] using a suite of Hyperchem 7.5 programs for a preliminary analysis. It could build a reasonable three-dimensional structure via energy minimization and structure optimization.[18] Scientists also often use hyperchem to compute geometrical, physico-chemical and electronic properties for chemical product design.[19,20] Character and structure parameters, such as oil−water partition coefficient (LogP), polarity, and volume, can be obtained under QSAR module.

The results of structure calculation (Table 2) indicate that the dyes with a certain range of LogP (around 6∼7), show tumor-targeted bioactivity. The range-limited LogP value is apprehensible, because on one hand, these dyes need to penetrate the lipophilic phospholipid layers of cytomembrane and mitochondrial membrane into the mitochondria of the tumor cells, certain lipophicity is essential. On the other hand, hydrophilicity is also indispensable to insure dyes with certain water-solubility

Table 2. LogP, polarity, volume and molecular length of **1**–**11** from molecular calculation.

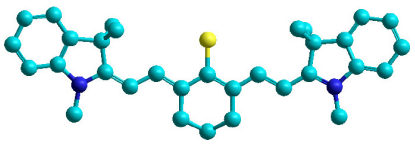
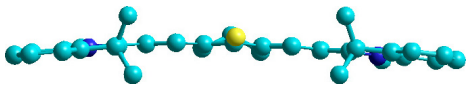
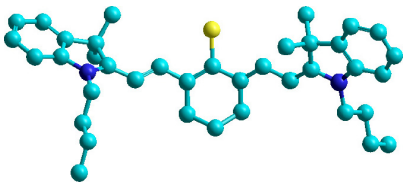
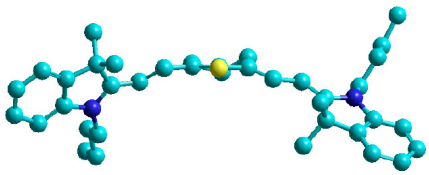
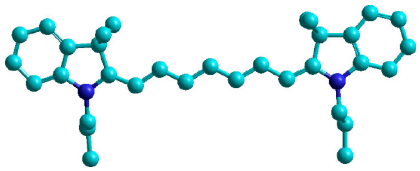
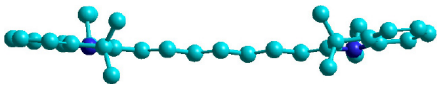
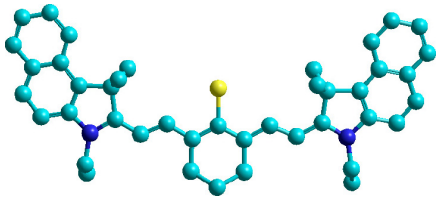
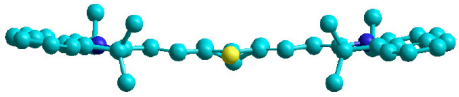
Parameters	1	2	3	4	5	6	7	8	9	10	11
LogP	4.46	6.08	6.08	6.87	5.99	7.22	7.47	6.10	7.60	5.29	7.40
Polarity	58.36	65.70	65.70	69.37	78.16	71.10	65.36	59.05	84.38	74.40	81.74
Volume	1397.35	1600.80	1600.80	1731.24	1790.94	1881.53	1824.33	1463.94	2073.30	1732.51	1930.43
Molecular Length^{G}	20.67	20.24	20.24	20.04	20.76	20.41	19.57	19.34	19.12	24.94	16.80

Note: **G**: the ground state.

and stability in blood circulation. Another possibility is that tumor cell contains binding sites that allow **2**–**6** compounds with the suitable LogP value to efficiently attach to tumor specific sites, while other negative dyes, such as **1** with a LogP below 6 (calculated as 4.66) and **9** with a LogP beyond 7 (calculated as 7.40), may have lower binding efficiency.

In addition, relevance between molecular steric configuration and tumor-tageted property is worth studying by comparison of the spatial characteristic of both tumor targeting and non-targeting heptamethine indocyanine dyes. As seen in Table 2, the molecular length seems related to the tumor-targeted bioactivity. It shows that a certain range of molecular length (between 20∼21 Å) would benefit them to exhibit tumor-targeted bioactivity, such as **2**–**6** compounds. Furthermore, configuration with certain torsion angle ranging from 147° to 168° appears to benefit the tumor-targeted property (Table 3), such as tumor-targeted compound **4** with a torsion angle of 147° (∠N-Cl-N). Conversely, **1**, **7**, and **9** compounds without tumor-targeted property show approximate coplanarity in their whole structures. The unique steric configuration concluded from these target dyes may relate to another target mechanism, which may endow them to bind

Table 3. Optimized structures of **1**, **4**, **8**, **10**.

Comp. No.	Vertical orientation[a]	Horizontal orientation
1		
4		
8		
10		

[a]In the ball-and-stick representation, carbon, nitrogen and chlorine atoms are colored in cyan, blue and yellow. Hydrogen atoms are not showed.

with certain specific receptor expressed on cell surface of tumors.

It is noteworthy that some structures of compounds are exceptional for the general rules concluded above. For example, the tumor-targeted compound **2** shows approximate coplanarity in their whole structures. Therefore, these calculated structures should be determined by X-ray crystallography analysis, and further investigation on the structure−activity relationship is required.

4. Conclusion

Our preliminary structure-activity relationship study of heptamethine indocyanine dyes for tumor-targeted near-infrared imaging reveals that cyclohexene and heptamethine moieties are the essential groups. The N-alkylation chain R_1 being an aliphatic hydrocarbon chain with an appropriate length (3−6 carbons) would benefit its tumor-specific accumulation; and the formation of benzo ring between R_3 and R_4 is a disadvantage for the specificity, while the kinds of X-ion has no significant effect on the tumor-targeting ability. Through the analysis of the simulated spatial structures, appropriate oil−water partition coefficient and torsion configuration appear to play an important role in retaining tumor-targeted bioactivity.

Acknowledgments

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