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Grafting a chromophore on AMD070 analogues for CXCR4 bioimaging: Chemical synthesis and *in vitro* assessment of the inhibition properties of the CXCR4 receptor

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ABSTRACT

Thank to their particular pharmacokinetics, the use of small organic molecules can be a very promising alternative to macromolecular targeting biomolecules (*i.e.* antibodies, peptides...) for specific imaging of tumours. Herein, the potential of two AMD070-like inhibitors as CXCR4-targeting units for specific imaging of cancer cells, and the influence of chromophore-grafting on their recognition properties was investigated.

In the quest for more specific detection of tumours and enhancement of treatment selectivity, the discovery and targeting of new cellular receptors play a pivotal role. In this context, the chemokine receptor CXCR4 has emerged as a very promising target in recent years. This 7-domain transmembrane receptor has the CXCL12 chemokine (also named SDF-1 α) as its sole natural ligand in healthy tissues. This CXCR4/CXCL12 axis is strongly involved in hematopoiesis, leukocyte migration, chemotaxis or angiogenesis.¹ But its role has also been evidenced in pathological processes. Early, CXCR4 was identified as a key co-receptor (with CD4) in HIV (Human Immunodeficiency Viruses) fixation and cell infection.² More recently, CXCR4 overexpression has been detected in over 20 types of cancers, and is often associated to tumoral progression, metastatic processes and therapeutic resistance.^{3,4} Moreover, in some tumours, such as gliomas, the magnitude of CXCR4 receptor overexpression is directly linked to the grade of tumour and thus to the patient prognosis.⁵

Hence, a lot of effort has been devoted to the identification and synthesis of potent CXCR4 inhibitors. Biomolecules, such as antibodies, antibody fragments (*ex:* nanobodies)⁶ or peptides⁷ have been studied for specific targeting of CXCR4 receptor with excellent affinities, and their interactions with the receptor are well-described.^{8,9}

In addition to the potential use of such inhibitors as novel anti-HIV/ Cancer drugs, some have also been derived as CXCR4 imaging or theranostic probes, through appendage of chromophores (Fluorescence

imaging)^{10,11} or radioactive tags (in particular for Positron Emission Tomography (PET)),^{12–15} with some of these tracers in clinical trials.

However, these biomacromolecules have several drawbacks, with rather long biodistribution times that are not suitable to all applications, in addition to demanding synthesis and storage. Therefore, small synthetic organic molecules, that usually present shorter biodistribution times, easier storage and cheaper synthesis on high scale, are considered as excellent alternatives in this field. Indeed, cyclam-based CXCR4 inhibitors have been identified in the early 1990s as potential anti-HIV treatments with excellent CXCR4 specificity and nanomolar EC₅₀ inhibition values,¹⁶ in the range of previously cited peptides.

The first highly active bis-cyclam **JM1657** (Scheme 1; IC₅₀ in 0.1–1 μ M range) was found as an impurity in some cyclam samples,¹⁶ but could not be synthesized independently. So further mono- (**AMD3465**, Scheme 1) and bis-cyclams with different spacers were prepared by De Clercq et al.^{17,18} and **AMD3100** emerged as a high affinity (IC₅₀ <10 nM range) and low cytotoxicity (>500 μ g/mL) CXCR4 inhibitor. This molecule was later approved (under the name Plerixafor, also commercialized as Mozobil®) for use in therapeutic cocktails against non-Hodgkin lymphoma and multiple myeloma.^{19–21} Finally, in an effort to develop orally bioavailable drugs against HIV, another family of small organic molecules, based on a tetrahydroquinoline-benzimidazole scaffold have been identified as suitable candidates, with **AMD070** (Scheme 1) demonstrating exceptional affinity for CXCR4 (5 nM range).^{22,23}

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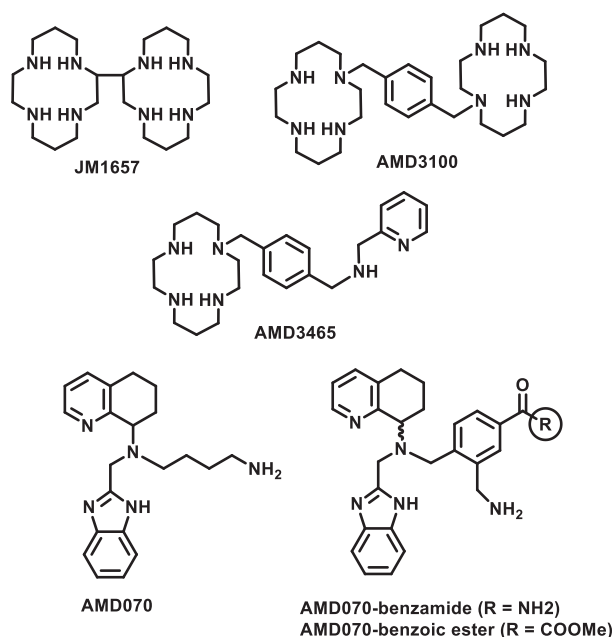
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Scheme 1. CXCR4-targeting units discussed in this work.

In this context, we sought to investigate the potential of such small organic molecules as targeting moieties for molecular imaging of the CXCR4 receptor overexpression in cancers, by tagging these compounds with the organic chromophore fluorescein.

To help us in carefully designing a suitable molecular architecture for such tracers, the interaction of these small organic molecules with CXCR4 has been evidenced at the molecular level, thank to thorough site-directed mutagenesis of the receptor and modelling experiments by several research groups.^{24–28} As cyclam-based **AMD3100** and **AMD3465** exist under multiprotonated cationic form (+4 and +3 respectively) at physiological pH, they strongly bind to anionic glutamate and aspartate residues from CXCR4 extracellular loops.^{24–26} *N*-functionalization of these structures to install the desired chromophore is thus likely to dramatically modify their acid-base properties and be detrimental to CXCR4 recognition.

Owing to its different chemical nature, the binding pattern is somewhat different for **AMD070**. Again, the +2 charge (protonation of proximal tertiary and distal primary amines) gives rise to interactions with aspartate and glutamate fragments from the receptor. But additional H-bonding and aromatic interactions of the benzimidazole core,

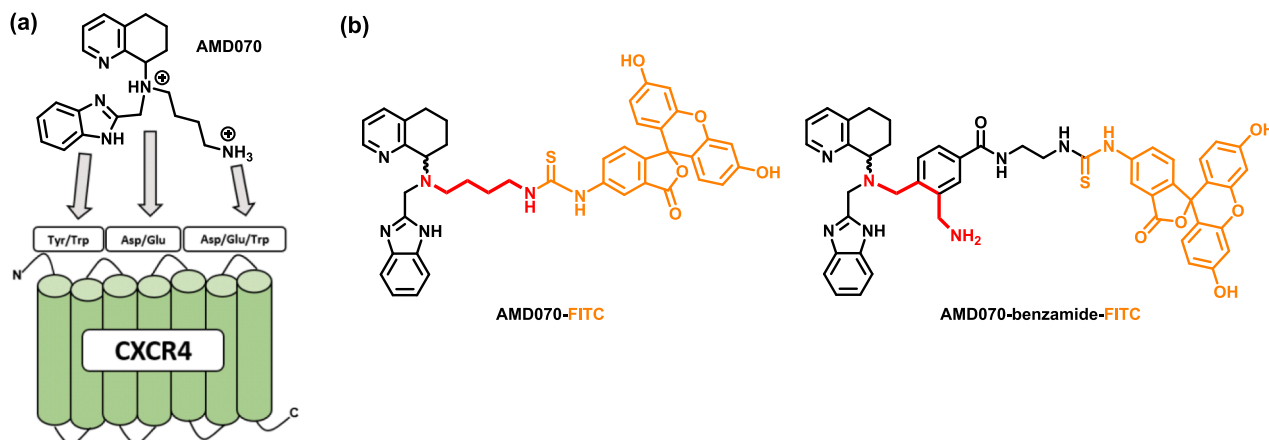
as well as cationic π -interaction of the protonated primary amine were also identified with tyrosine and tryptophan residues from CXCR4.²⁷

Thus, we selected the **AMD070** framework as the targeting moiety of novel imaging tracers. To assess in particular the possibility to graft a reporter moiety on the endchain primary amine and the effect of such grafting on the target recognition properties of the compounds, two conjugated compounds were designed: (i) direct chromophore grafting on the primary amine, via the reaction with fluorescein-isothiocyanate to form a thiourea link (**AMD070-FITC**, Scheme 2); (ii) the **AMD070-benzamide-FITC** analogue (Scheme 2), as the corresponding **AMD070-benzoic ester** or **AMD070-benzamide** (Scheme 1) have excellent IC₅₀ against CXCR4 (100 and 10 nM range respectively),²⁹ an intact primary amine is kept through grafting on the benzamide function, and the same chain length (4 carbon atoms) between the proximal and distal amino groups is obtained, which has been previously identified as a key factor for optimized inhibition of CXCR4.²²

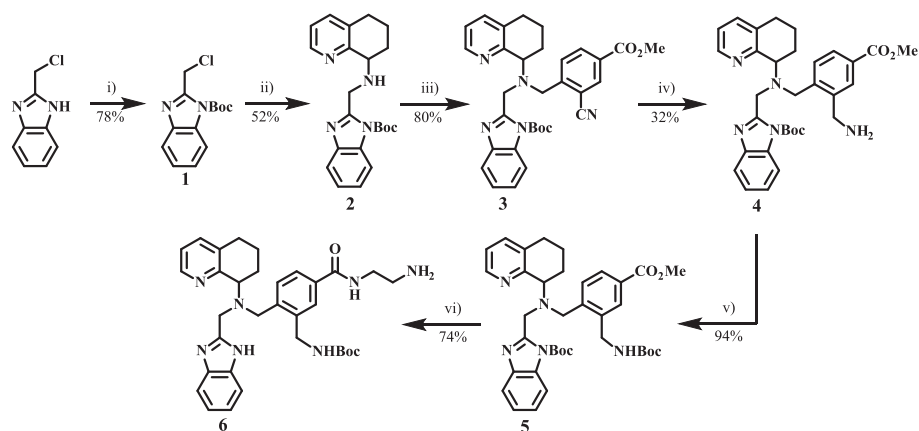
Herein, we present our synthetic work on the preparation of these two novel **AMD070** derivatives tagged with a fluorescein chromophore, and the first cellular *in vitro* experiments to investigate the effect of such grafting on their CXCR4 inhibition properties, providing a proof-of-concept of this new specific targeting strategy.

AMD070 was successfully prepared as a racemic mixture in 3 steps at the gram scale from existing building blocks (See Supporting Information).³⁰ The preparation of the benzamide analogue (Scheme 3) had, however, to be adapted from existing protocols³¹ to reach suitable scale and protect the reactive distal primary amine ahead of the subsequent coupling reaction with fluorescein-isothiocyanate.

The secondary amine of the commercial 2-chloromethylbenzimidazole was first protected by a Boc unit to afford **1**, that could then selectively monoalkylate tetrahydroquinoline-8-amine leading to **2** with 52 % isolated yield. Subsequent nucleophilic substitution on 4-bromo-methyl-3-cyanobenzoic acid, under its methyl ester protected form, afforded **3**. Raney nickel was then used under 5 bars of hydrogen in an autoclave to reduce the cyanide functional group into the corresponding primary amine of **4**. The somehow modest yield (32 %) of this step comes from the difficult purification of this compound, that has to be carefully isolated from dissolved Ni salts resulting from Raney nickel decomposition, and purified through neutral alumina column chromatography. At this stage, the primary amine was protected by a Boc group to afford **5**, in which the methyl ester moiety could react with ethylenediamine to provide, in a single step, compound **6** bearing the desired benzamido spacer and the terminal amine function available for coupling with fluorescein-isothiocyanate (FITC-NCS). Noteworthy, during this reaction step, the benzimidazole-appended Boc *N*-protecting group was surprisingly cleaved. However, this is not detrimental for further coupling reaction as the reactivity of this secondary amine is markedly different from the end-chain primary amine that is set to react



Scheme 2. (a) Schematic representation of **AMD070** molecular interaction with CXCR4 and (b) synthetic conjugates synthesized in this work.



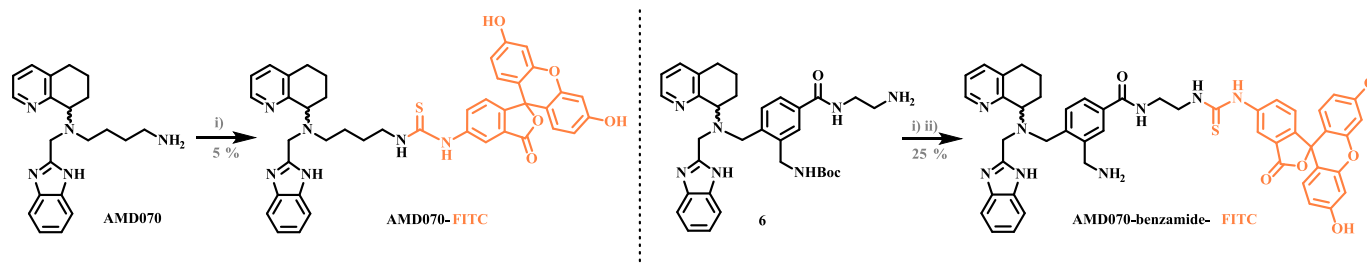
Scheme 3. Synthesis of benzamido-functionalized AMD070 derivative **6**. Reaction conditions: i) *di-tert*-butyl dicarbonate (Boc₂O, 1.9 equiv.), NEt₃ (exc.), DMAP (0.1 equiv.), dichloromethane, 0 °C, 4 h; ii) 5,6,7,8-tetrahydroquinolin-8-amine dihydrochloride (1.0 eq.), DIPEA (3.8 equiv.), KI (0.15 equiv.), CH₃CN, 30 °C, 72 h; iii) 4-bromomethyl-3-cyanobenzoic acid methyl ester (1.0 equiv.), DIPEA (1.5 eq.), KI (0.06 equiv.), CH₃CN, 60 °C, 16 h; iv) Raney Ni, H₂ (5 bars), NH₃■MeOH (7 N), 25 °C, 18 h; v) Boc₂O (1.5 equiv.), THF, reflux, 18 h; vi) ethylenediamine (exc.), RT, 5d.

with the isothiocyanate group from FITC-NCS. This coupling reaction (Scheme 4) was then conducted with the same conditions for both AMD070 and compound **6**, i.e. at room temperature in dry *N,N*-Dimethylformamide (DMF) for 3 days, in presence of an excess of triethylamine and a substoichiometric amount of commercial FITC-NCS (0.66 equiv.), as the separation of the desired products from unreacted FITC-NCS proved difficult by reversed-phase HPLC. After isolation, an additional acidic hydrolysis step was conducted in the case of AMD070-benzamide-FITC to free the distal primary amine from its Boc protecting group. Both final products were isolated with low yields, but very high purity (96 and 98 % respectively by HPLC-MS) and sufficient quantity (ca. 1–5 mg per coupling reaction) to carry out *in vitro* cellular experiments. These two new compounds, as well as the intermediates **1–5**, were fully characterized by means of high-resolution mass spectrometry, ¹H and ¹³C NMR (See Supporting Information).

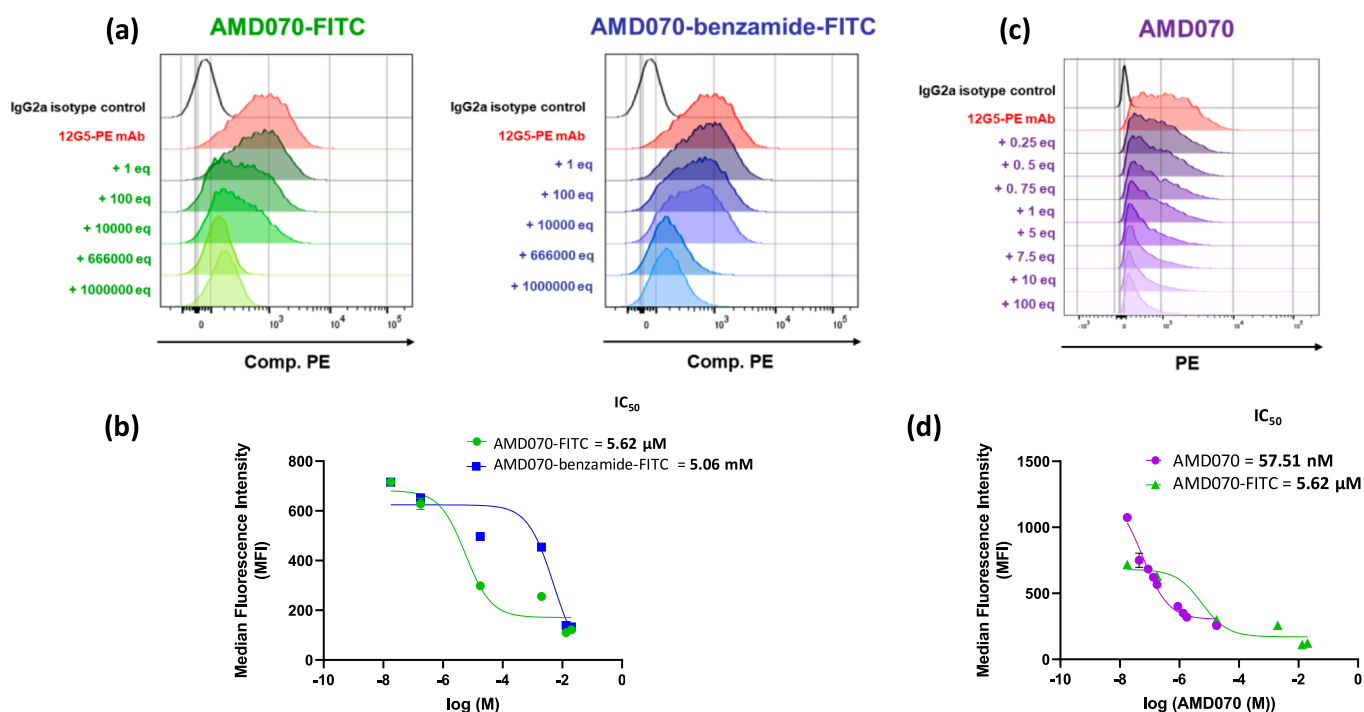
Among several cancer types overexpressing the CXCR4 receptor, our research groups have focused part of their investigations on multiple myeloma (MM) in order to develop specific probes for imaging and therapy. Therefore, the first cellular *in vitro* tests of our novel AMD070-based probes were conducted on human multiple myeloma cell line U266.B1, a common MM cell line with publicly available data,³² and were analyzed through flow cytometry experiments (Fluorescence-Activated Cell Sorting (FACS)). Their affinity for the CXCR4 target was evaluated by competition with a reference phycoerythrin (PE)-tagged human anti-CXCR4 monoclonal antibody (clone 12G5-PE, Miltenyi) that possesses nanomolar affinity for the receptor (K_d = 7.65 ± 0.45 nM), which allows comparison with non-fluorescent compounds, such as original AMD070. First, the concentration of 12G5-PE to be used in competition experiments (C = 2.66 × 10⁻⁸ M, n = 2.66 × 10⁻¹² mol) was determined from a saturation curve obtained with increasing concentrations of the antibody (See supporting Information). Then U266.B1

cells (500,000 cells/well) were incubated at 4 °C for 60 min with 2.66 × 10⁻¹² mol of 12G5-PE and increasing concentrations of AMD070-FITC or AMD070-benzamide-FITC (5 data points for each, from 2.66 pmol to 300 nmol) in phosphate buffered saline (PBS) with 5 % fetal bovine serum (FBS). Analysis was subsequently performed by flow cytometry experiments (FACS) (Scheme 5) using BD FACS Canto II™ (BD Sciences). Data were then analysed using FlowJo™ software (BD Sciences). Cells were gated based on their size and their viability using 4',6-diamidino-2-phenylindole (DAPI), which is non-permeant to live cells. Then, 12G5-PE displacement by AMD070-FITC and AMD070-benzamide-FITC was assessed from mean fluorescence value of PE after applying a compensation of FITC signal in PE band-pass filter (564–606 nm) (Scheme 5, (a)). Inhibition curves, adjusted by non-linear regression to a three-parameter log(dose)-inhibition model with GraphPad Prism software (v. 8.0.0 for Windows, GraphPad Software, San Diego, California USA), allowed the determination of half-maximal inhibitory concentration (IC₅₀) for both compounds (Scheme 5, (b)).

The FACS analysis clearly demonstrates a different behaviour of the two fluoresceine-tagged compounds towards the CXCR4 receptor, with a 1000-fold difference between their calculated IC₅₀ (5.62 μM for AMD070-FITC and 5.06 mM AMD070-benzamide-FITC) demonstrating that grafting of a tag on the free primary amine of AMD070 gives superior inhibition ability than modification of the scaffold by introduction of the benzamide spacer. To assess the potential loss of target recognition of the AMD070 moiety upon grafting of the fluoresceine tag, the IC₅₀ of native AMD070 was also measured in the same experimental conditions vs. 12G5-PE (Scheme 5, (c) and (d)) and a value of 57.5 nM was obtained, demonstrating a 100-fold downgrade of the CXCR4 inhibition properties. This activity loss can be attributed to the derivatization of the primary amine that plays a role in the receptor recognition, but might also be explained by the additional steric



Scheme 4. Synthesis of fluoresceine conjugates. Reaction conditions: i) fluoresceine isothiocyanate (FITC-NCS, 0.66 equiv.), NEt₃ (1.6 equiv.), DMF, RT, 3 days; ii) hydrochloric acid (1.5 M), THF, 40 °C, 3 h.



Scheme 5. CXCR4 antibody competition by CXCR4 ligands. FACS analysis of 12G5-PE competition experiments with (a) AMD070-FITC, AMD070-benzamide-FITC and (c) AMD070. Inhibition of 12G5-PE binding to CXCR4 receptor by (b) AMD070-FITC, AMD070-benzamide-FITC and (d) AMD070. A fixed 12G5-PE concentration was deposited on U266.B1 cell and small molecules were diluted in PBS/SVF 5 % and added immediately afterwards. After 1 h incubation, cells were washed and then labelled with DAPI to assess their viability. Analysis was performed by flow cytometry on a Canto II cytometer (BD Biosciences).

hindrance and lipophilicity brought by the fluoresceine fragment that might alter the compounds ligation to the receptor.

However, the IC_{50} obtained for the new **AMD070-FITC** derivative is still encouraging for the fate of this direct grafting strategy in future work. *In vitro* cellular imaging will be conducted in the near future with this compound to assess its capabilities as a CXCR4-staining compound for biological assays. Different tags will also be evaluated to better maintain the inhibition properties of the AMD070 motif, with different chromophores, or to allow detection of the CXCR4 receptor by other imaging techniques, such as Positron Emission Tomography (PET) or single-photon Emission Tomography (SPECT).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ANR-16-IDEX-0007). This initiative takes place in the Pays de la Loire region, in Nantes Métropole.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmcl.2024.130027>.

Data availability

Data will be made available on request.

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