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The discovery of new and more potent chloropyramine (C4) analogues for the potential treatment of invasive breast cancer

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Graphical abstract

Highlights

- 17 New chloropyramine analogues were designed and synthesised.
- All new compounds were evaluated for their activity in three different breast cancer cell lines.
- For the most active compound **5c**, *in vitro* pharmacokinetic studies were performed.

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ABSTRACT

Breast cancer (BC) is the second most common cancer worldwide, accounting for 25% of all female cancers. Although the survival rate has increased significantly in the past few decades, patients who develop secondary site metastasis as well as those diagnosed with triple negative breast cancer (TNBC) still represent a real unmet medical challenge. Previous studies have shown that chloropyramine (**C4**) inhibits FAK-VEGFR3 signaling. More recently, **C4** is reported to have SASH1 inducing properties. However, **C4** exerts its antitumour and antiangiogenic effects at high micromolar concentrations (>100 μ M) that would not be compatible with further drug development against invasive breast cancer driven by FAK signaling. In this study, molecular modeling guided structural modifications have been introduced to the chloropyramine **C4** scaffold to improve its activity in breast cancer cell lines. Seventeen compounds were designed and synthesised and their anti-proliferative activity was evaluated against three human breast cancer lines (MDA-MB-231, BT474 and T47D). Compound **5c** was identified to display an average activity of $IC_{50} = 23.5-31.3 \mu$ M, which represents a significant improvement of **C4** activity in the same assay model. Molecular modelling and pharmacokinetic studies provided more promising insights into the mechanistic features of this new series.

1. INTRODUCTION

Current therapeutic strategies for breast cancer (BC) are mainly directed towards primary tumour site histopathology *i.e.* expression of estrogen (ER), progesterone (PR) and human epidermal growth factor 2 (HER2) receptors. Due to improved therapeutic strategies, the survival rate for ER-positive breast cancer in particular has increased significantly in the past few decades. However, the subgroup of aggressive and invasive breast cancers, including triple-negative disease (around 15% of cases), is characterised by rapid relapse following chemotherapy and progression to metastatic disease, with a poor overall prognosis.¹

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase and scaffold protein, which is localised to focal adhesions, and forms points of contacts between cells and the extracellular matrix. FAK is highly expressed in cancer, acts as a multifunctional mediator of cell signaling, and its involvement in cancer cell proliferation, invasion and metastasis is well documented.²⁻⁷ It is overexpressed in many types of cancer such as thyroid, brain, liver, prostate, breast, colon, head and neck. Moreover, this overexpression is associated with an invasive phenotype in such tumours.⁷ Numerous approaches are being developed to inhibit FAK catalytic function (mainly targeting the Y397 tyrosine phosphorylation site), which have successfully led to the development of clinical stage FAK kinase inhibitors such as defactinib, currently in Phase I/II trials in ovarian, non-small cell lung,

mesothelioma and pancreatic cancer (Verastem, Inc.)⁸ Alternatively, destabilisation of the FAK scaffolding function is believed to be an advantageous and potentially more specific strategy to develop FAK targeting therapeutics rather than focusing on the kinase function only.⁷⁻¹¹ *In silico* screening of the National Cancer Institute's small molecule library for compounds targeting the FAK–VEGFR3 interactions led to the identification of the clinically approved antihistamine drug chloropyramine (**C4**) which belongs to the first generation of H1-antihistamines.^{12,13} Further *in vitro* and *in vivo* studies have shown that treatment with **C4** showed reduction in tumour burden and neovascularization in mouse models of breast cancer.¹² This activity has been demonstrated, both alone and synergistically with other chemotherapeutic agents (e.g. doxorubicin and gemcitabine), sensitising breast cancer cells to chemotherapy.^{12,13} Moreover, **C4** is under development by CureFAKtor Pharmaceuticals LLC, and received orphan drug designation from the FDA in combination with gemcitabine for the treatment of pancreatic cancer.¹⁴ More recently, Burgess *et al* have independently used *in silico* connectivity mapping and *in vitro* modelling to demonstrate that chloropyramine **C4**, increased SASH1 protein levels in breast cancer cells.¹⁵ Expression of the SASH1 protein is reduced in a range of human cancers and has been implicated in apoptotic cancer cell death. Although **C4** is an attractive candidate, it exerts its antitumour and antiangiogenic effects at high micromolar concentrations (>100 μ M).¹³ In order to get more insights into the structure activity relationships (SAR) and improve the antitumour potency of **C4**, we introduced new chemical motifs into the scaffold of **C4**. These modifications were tested in a preliminary *in silico* study that suggested an improved interaction with the FAK-FAT domain. The newly designed compounds were synthesised and screened *in vitro* against three human breast cancer cell lines (MDA-MB-231, BT474 and T47D). Our findings show that compound **5c**, which displayed the best anti-proliferative potency amongst all tested analogues, is a novel compound that warrants further investigation as a candidate for breast cancer therapy.

2. METHODS AND MATERIALS

2.1 Lead optimisation and design of novel chloropyramine (**C4**) analogues

Based on the previous promising findings with chloropyramine (**C4**),^{12,13} we sought to improve upon the chemical structure of **C4** to understand better its structure activity relationship (SAR) and to improve its anti-proliferative potency (**C4** IC₅₀ >100 μ M). To investigate the impact of various substituents of **C4** on the antitumour efficacy, a series of novel derivatives were designed and prepared, as detailed in **Figure 1**, and **Schemes 1** and **2**. Structural modifications involved replacing the pyridine moiety with other aromatic systems containing a carboxylate moiety to enhance the interaction with Lys 1032 within the FAK-FAT domain, introducing an extended carbon atom moiety

(region A, **Figure 1**) or introducing a carbonyl group into *N,N*-dimethylamino ethyl side chain (region C, **Figure 1**). The *p*-chlorobenzyl group (region B, **Figure 1**) was retained unmodified in most compounds since it is involved in key interactions via a π - π stacking with the aromatic side chain of Tyr 925. The salicylate aromatic moiety replacing the pyridine ring was reported to mimic the phosphorylated tyrosine (pTyr).^{16,17} Molecular modeling studies of the newly designed compounds showed improved binding interaction modes within the FAK-FAT pocket compared to the parent **C4** compound due to a better orientation effect on the side chain (region C, **Figure 1**). All the prepared compounds were evaluated against three breast cancer lines *i.e.* MDA-MB-231 (TNBC), BT474 (ER⁺, HER⁺) and T47D (ER⁺, HER⁻).

2.2 Chemical synthesis

The first series of compounds containing an extended carbon unit in the **C4** skeleton (**5a-d**, region A modification) were synthesised as shown in **Scheme 1**. The first step involved reductive amination of various substituted aromatic aldehydes (**1a-d**) with *N,N*-dimethyl-ethylenediamine **2** which provided intermediates (**3a-d**) in good yields.¹⁸ It is worth mentioning that the reaction conditions of this step were optimized in terms of solvent and temperature choice for the different aldehydes used (see *experimental section*). These intermediates were alkylated with *p*-chlorobenzyl chloride **4** to give the target analogues (**5a-d**) in good to moderate yield (57-69 %). Interestingly, in the case of 4-((2-(dimethylamino)ethylamino)methyl) benzonitrile **3a**, a quaternary ammonium side product **6** was obtained alongside the expected product **5a** (in 1:3 ratio, respectively). Unexpectedly, in the case of 2-cyanobenzaldehyde **7**, the cyclised isoindolenone derivative **8** was isolated instead of the desired Schiff's base, **Scheme 2 (A)**. A plausible mechanism is suggested where the initial step is the nucleophilic addition of the amine **2** to the formyl group of **7** affording the adduct **7a**, which subsequently cyclises to **7b** by intramolecular nucleophilic addition of the hydroxyl group to the cyano group of **7a**, followed by conversion to the isoindolenone derivative **8**, **Scheme 2 (B)**.¹⁹

The second series of compounds (**13a-m**), featuring an additional carbonyl group in the *N,N*-dimethylamino ethyl side chain moiety of **C4**, were prepared according to **Scheme 3 (A)**. The first step involved a reductive amination reaction²⁰ between the corresponding aniline derivative (**9a-l**) and *p*-halobenzaldehyde (**10a** or **10b**) to give the key intermediates (**11a-l**). The final compounds were obtained by reacting *N,N*-dimethylaminoacetyl chloride hydrochloride **12** with intermediates (**11a-l**) in tetrahydrofuran at room temperature. The reductive amination of 5-amino-2-hydroxybenzoic acid with *p*-chlorobenzaldehyde (**10a**) was not feasible due to the instability of the intermediate product (**11m**). For this reason, we managed to get the corresponding target product **13m** via the hydrolysis of **13c** using NaOH solution in a very good yield (92 %), **Scheme 3 (A)**. Interestingly, double reductive

amination occurred in the case of compound **9m** to give **14** which was hydrolysed to **15** using NaOH. The structures of all the synthesised compounds were confirmed using analytical and spectroscopic data (NMR, mass spectrometry), which were in full accordance with their depicted structure. All final compounds were purified and tested as free bases.

3. RESULTS AND DISCUSSION

3.1 Cell Viability Assay

The anti-proliferative activity of all the synthesised compounds **5a-d**, **6**, **8**, **13a-m**, **14** and **15** was evaluated as free bases against three human breast cancer cell lines; MDA-MB-231 (TNBC), BT474 (ER⁺, HER⁺) and T47D (ER⁺, HER⁻). Chloropyramine **C4** was used as a control drug. The corresponding cell lines were seeded the day before incubation, then incubated for 72 h with the different concentrations of compound tested. CellTiter Glo reagent (25 μ L) was added to the cell plate. The luminescence was recorded after 10 mins on a PerkinElmer Envision instrument. All the twenty-one compounds were tested at 10 concentrations in half log increments up to 100 μ M in triplicate. The results indicated that three compounds (**5c**, **6** and **15**) have better anti-proliferative activity than chloropyramine **C4**, which did not show any anti-proliferative activity up to 100 μ M concentration across the three BC cell lines. The most active compound **5c** showed micromolar activity across the three BC cell lines; (MDA-MB-231, IC₅₀ = 23.5 μ M), (BT474, IC₅₀ = 73.2 μ M) and (T47D, IC₅₀ = 31.3 μ M), **Figure 2A**. Interestingly, the quaternary ammonium compound **6** and the double reductive amination product **15**, displayed better anti-proliferative activity than **C4**, **Figure 2B** and **C**, respectively. All the other tested analogues showed no observed improvement in the antiproliferative activity over the parent compound **C4** (all compounds were tested at maximum concentration of 100 μ M).

3.2 Molecular modelling

In silico docking was performed on all the designed analogues using the downloaded crystal structure of FAK (PDB ID: 1K05).²¹ The chemical structures of the compounds were constructed, rendered and minimised with the MMFF94x force field in MOE. Docking simulations were performed using Glide SP in Maestro (Glide, version 9.5, Schrödinger, LLC, New York, NY. <http://www.schrodinger.com>). Based on the surface representation of the FAK-FAT domain, there appears to be an improved interaction over the parent **C4** upon the introduction of an extended carbon linker, compounds (**5a-d**), or introduction of a carbonyl group as in compounds (**13a-m**) into the general chloropyramine scaffold. **Figure 3A** shows the predicted docking mode of the most active compound **5c** compared to that of the parent compound **C4**. The computational study showed an improved

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interaction between the *N,N*-dimethylamino ethyl side chain and Asp 1036 of **5c**. Compound **5c** sits in a relatively polar cavity surrounded by residues Ser 910, Thr 929, Lys 1032, and Asp 1036. Key interactions of **5c** include two H-bonds between the *N,N*-dimethylamino ethyl group and both the side chain carboxylate group of residue Asp 1036 and the backbone carbonyl group of Pro 911. In addition, hydrophobic interactions were observed between the *p*-chlorobenzyl group moiety and the surrounding hydrophobic pocket formed of residues Tyr 925, Val 928, Val 932, and Leu 1035 (**Figure 3A**). The relatively higher activity of compound **5c** appears to be a result of the better directing effect of the *N,N*-dimethylamino side chain conferred by the extra carbon moiety. On the other hand, according to our model, the introduction of the carbonyl group into the *N,N*-dimethylamino ethyl moiety of **C4** (e.g. **13a**) showed an improved effect on orienting the *N,N*-dimethylamino ethyl group into the polar pocket around Asp 1036 as well as directing the other aromatic group to the hydrophobic pocket, **Figure 3B**. However, none of the carbonyl containing compounds (**13a-m**) were able to demonstrate any better anti-proliferative activity than **C4**, probably because of the reduced basicity of the *N,N*-dimethylamino moiety upon the introduction of the carbonyl group. Generally, our study of the two modified series of compounds suggests that more substantial structural modifications of **C4** are needed to significantly improve the anti-proliferative activity in BC cell lines.

3.3 *In vitro* aqueous solubility and microsomal metabolic stability

Subsequently, *in vitro* pharmacokinetic studies of the most active compound **5c** were performed. The *in vitro* aqueous solubility was investigated using five concentrations of **5c** i.e (1, 3, 10, 30 and 100 μ M), which were incubated at 37 °C for 2 hr. The estimated precipitation range (lower and upper bound) and a mid-range value were calculated for **5c**. Nicardipine and pyrene are included as control compounds. The results show that compound **5c** has a very good aqueous solubility with estimated precipitation range 100 μ M (lower bound) and >100 μ M (upper bound), **Table 1**. This relatively high aqueous solubility could prove to be a possible solution to the sedation side effect of chloropyramine because of its ability to cross the blood brain barrier (BBB).¹⁵ The calculated logP values (**C4** = 3.99, **5c** = 4.42) are similar but tPSA values (**C4** = 18.84, **5c** = 32.78) suggest that **5c** is less likely to cross the BBB.

Furthermore, the metabolic stability of **5c** was tested in human liver microsomes. Compound **5c** was incubated for 45 min with pooled liver microsomes and the intrinsic clearance (CL_{int}) and half-life (t_{1/2}) values were calculated based on 5 time points. The values of intrinsic clearance (CL_{int}) as well as half-life values of **5c** compared to control compounds, dextromethorphan and verapamil, are shown in **Table 2**. Compound **5c** has microsomal half-life (t_{1/2}) of 59.4 minutes, which exceeds that of the

registered drug controls dextromethorphan ($t_{1/2}$ = 48.2 mins) and verapamil ($t_{1/2}$ = 8.0 mins), and represents a good starting point for optimisation towards a daily administrable product.

compound	Estimated Precipitation Range (μM)		
	Lower bound	Upper bound	Calculated Mid-range
5c	100	>100	>100
nicardipine	10	30	20
pyrene	3	10	6.5

Table 1. *In vitro* aqueous solubility of **5c** versus control compounds (nicardipine and pyrene) in buffered aqueous solution

	CL_{int} ($\mu\text{L}/\text{min}/\text{mg}$ protein)	$t_{1/2}$ (min)
5c	23.3	59.4
dextromethorphan	28.8	48.2
verapamil	173.0	8.0

Table 2. *In vitro* metabolic stability of **5c** versus control compounds (dextromethorphan and verapamil) in human liver microsomes; CL_{int} : theoretical unrestricted maximum clearance of unbound drug by an eliminating organ, in absence of blood or plasma protein binding limitations, $t_{1/2}$ is the half-life.

4. Conclusion

In this study, two series of novel chloropyramine **C4** analogues were designed and synthesised. The anti-proliferative activity of compounds (**5a-d**) and (**13a-m**) was evaluated as free bases *in vitro* across three human breast cancer cell lines; MDA-MB-231 (TNBC), BT474 (ER^+ , HER^+) and T47D (ER^+ , HER^-). The results identified compound **5c** as having the most significant anti-proliferative activity, displaying micromolar activity across the three breast cancer cell lines; (MDA-MB-231, IC_{50} = 23.5 μM), (BT474, IC_{50} = 73.2 μM) and (T47D, IC_{50} = 31.3 μM). This activity represents a significant improvement to the chloropyramine **C4**, which did not display any anti-proliferative activity up to 100 μM . Further pharmacokinetic study showed that **5c** performed well in both the aqueous solubility and microsomal metabolic stability analysis. Considering the above findings, it could be

concluded that **5c** represents a promising basis for further development of breast cancer therapeutics targeting the FAK scaffolding domain.

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Supplementary data

Details of experimental procedures and spectroscopic characterisations data of the compounds **5a-d**, **6**, **8**, **13a-m**, **14** and **15** associated with this article can be found in the online version at

Figure 1. Chemical structure of chloropyramine and regions of proposed structural modification.

Scheme 1. Synthesis of compounds (**5a-d**). Reagents and conditions: a) i. **3a** and **3d**, (MeOH, rt, 4h), **3b** (DCE, reflux, 4h), **3c** (AcOH, 6h), ii. NaBH(OAc)₃, rt, 24h, b) THF, rt. 24h.

Scheme 2; A) Synthesis of isoindolene derivative **8**. Reagents and conditions; a) MeOH, rt, 4h, NaBH(OAc)₃, rt, 24h. B) proposed mechanism for the formation of the isoindolenone derivative **8**.

Scheme 3. A) Synthesis of compounds (**13a-m**). Reagents and conditions: a) MeOH, rt, 4h, NaBH(OAc)₃, 24h, b) THF, rt. 24h. **B)** Synthesis of compounds **14** and **15**. Reagents and conditions: a) DCE, reflux, 4h, NaBH(OAc)₃, r.t. 24h, b) NaOH, MeOH, reflux, 4hr.

Figure 2. Anti-proliferative activity of compounds **5c** (**A**), **6** (**B**) and **15** (**C**) against three human breast cancer cell lines; MDA-MB-231, BT474 and T47D.

Figure 3. A) Superposition of the predicted binding mode of compound **5c** (grey) versus **C4** (green) showing hydrogen bond interactions (blue dashed line) with key amino acids; Pro 911 and Asp 1036. **B)** compound **13a** (grey) versus **C4** (green); both are inside the pocket of FAK-FAT domain (golden yellow ribbon representation).

Figure 4. Mass spectrum of compound **5c**. (end of *Supporting Information*).

Figure 5. ^1H NMR spectrum of compound **5c**. (end of *Supporting Information*).

Figure 6. ^{13}C NMR spectrum of compound **5c**. (end of *Supporting Information*).

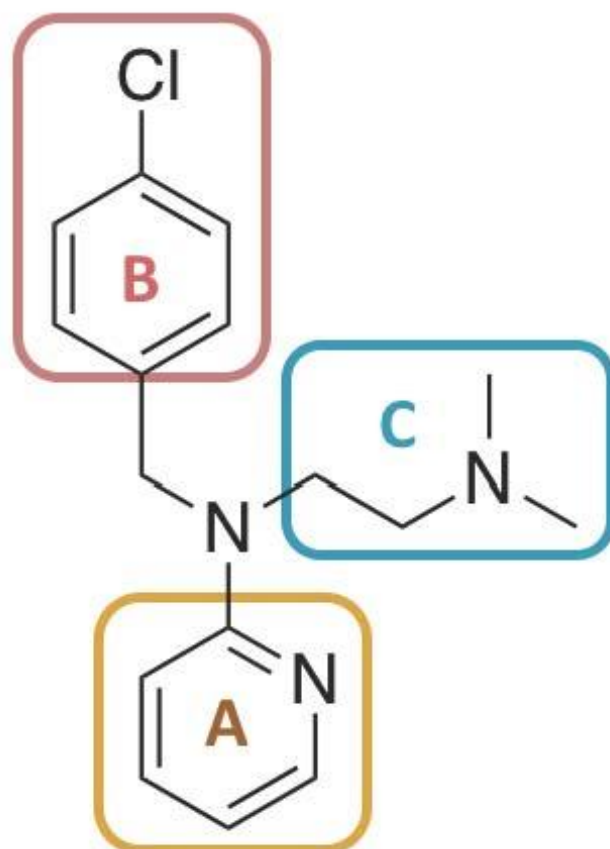
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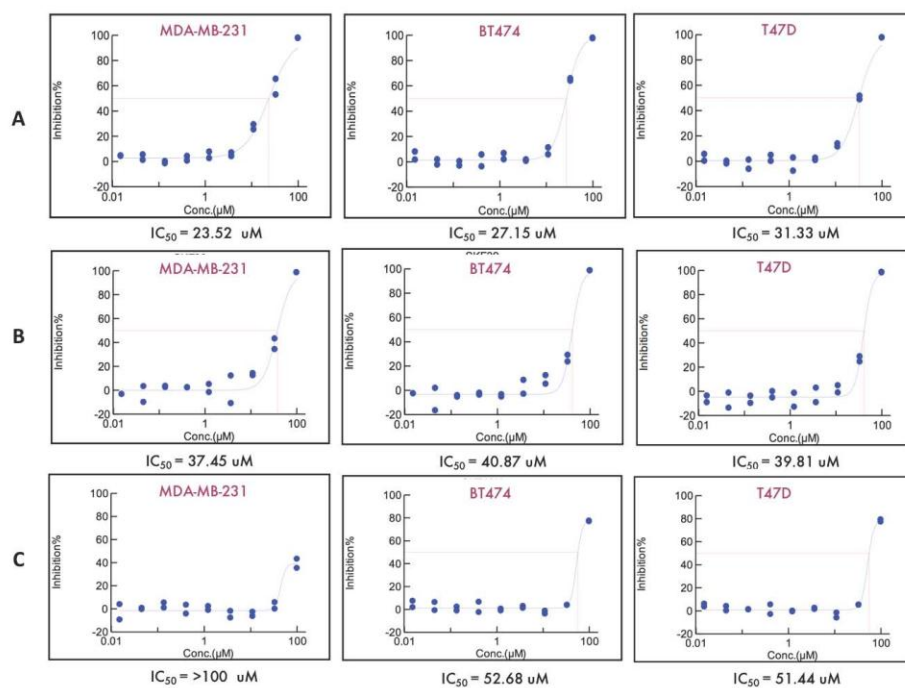
The authors declare that there are no financial/commercial conflicts of interest.

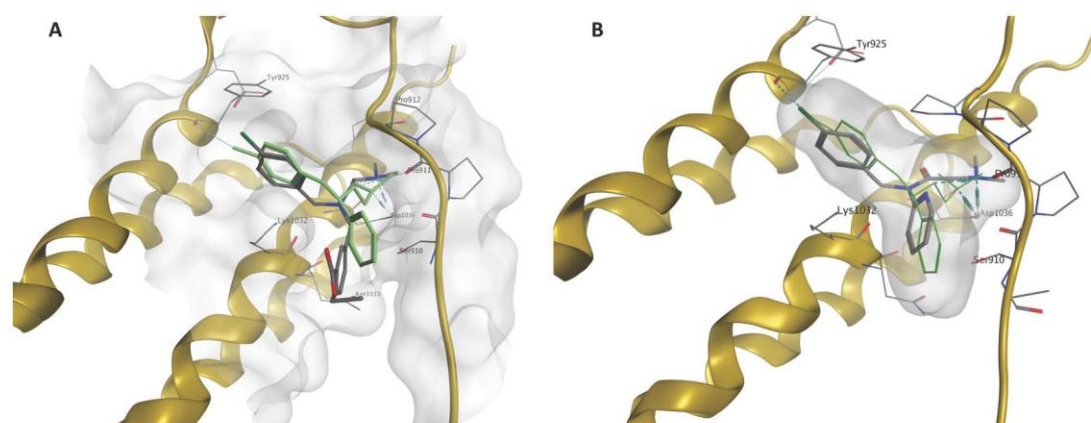
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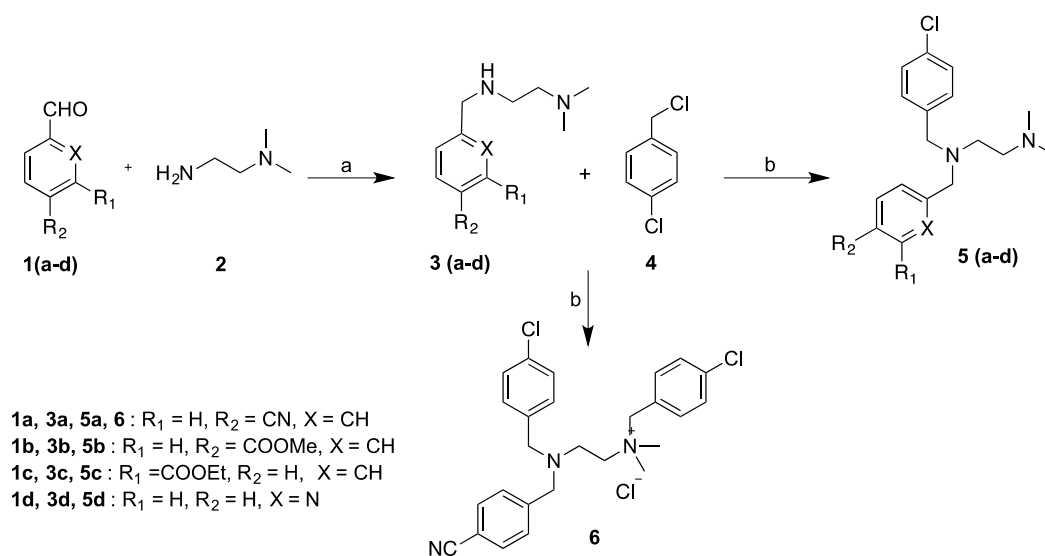
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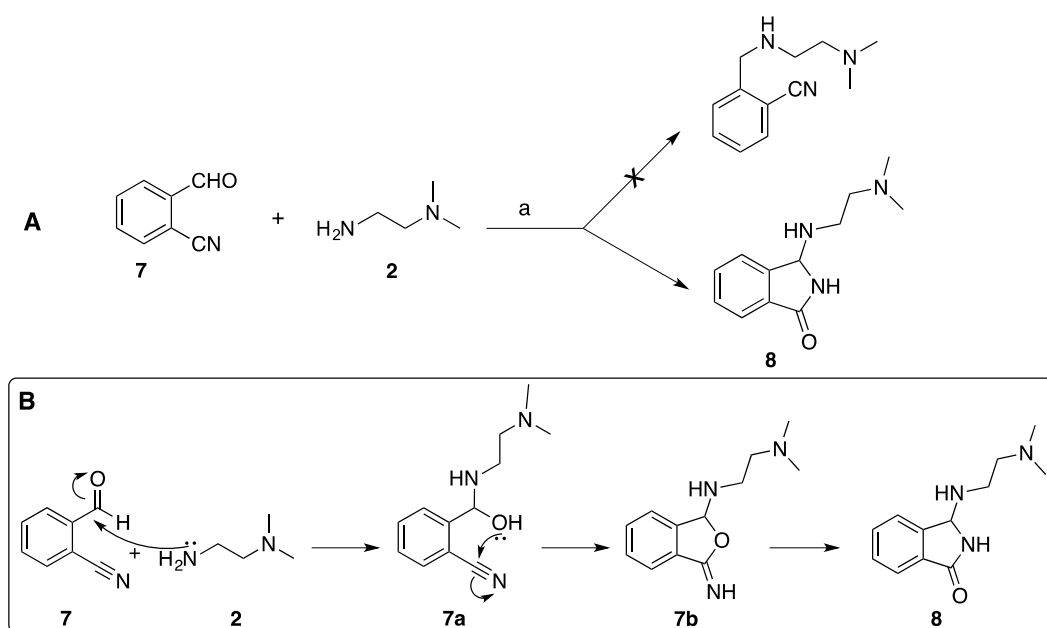




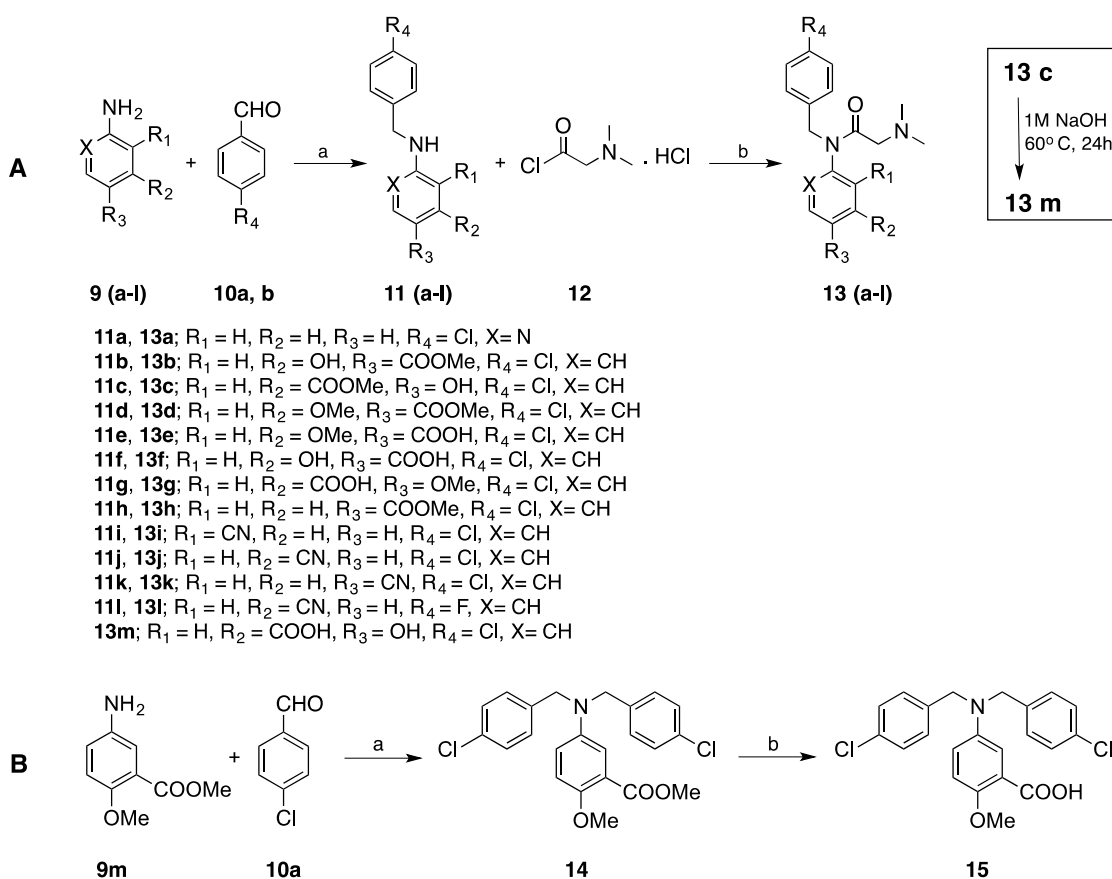




Scheme 1. Synthesis of compounds (**5a-d**). Reagents and conditions: a) i. **3a** and **3d**, (MeOH, rt, 4h), **3b** (DCE, reflux, 4h), **3c** (AcOH, 6h), ii. $NaBH(OAc)_3$, rt, 24h, b) THF, rt. 24h.



Scheme 2; A) Synthesis of isoindolene derivative **8**. Reagents and conditions; a) MeOH, rt, 4h, NaBH(OAc)₃, rt, 24h. B) proposed mechanism for the formation of the isoindolenone derivative **8**.



Scheme 3. A) Synthesis of compounds (**13a-m**). Reagents and conditions: a) MeOH, rt, 4h, NaBH(OAc)₃, 24h, b) THF, rt. 24h. **B)** Synthesis of compounds **14** and **15**. Reagents and conditions: a) DCE, reflux, 4h, NaBH(OAc)₃, r.t. 24h, b) NaOH, MeOH, reflux, 4hr.