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Original article

Synthesis and antitumour activity of new dendritic polyamines–(imide–DNA-intercalator) conjugates: potent Lck inhibitors

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Abstract

A series of dendritic polyamines–(imide–DNA-intercalators) conjugates with different connectivity in their basic chain were synthesised and evaluated as antitumour compounds. Although their antiproliferative activity against HT-29 was not significant, conjugates **13** and **16** showed a promising profile as inhibitors of Lck.

Keywords: Antitumour; Dendritic polyamines; Intercalators; Imides; Lck

1. Introduction

Two potential targets for drug design in cancer are DNA and tyrosine-kinases. Therefore, the design of new intercalators and the discovery of specific inhibitors of protein tyrosine-kinases are two important approaches in the search of new chemotherapeutic agents.

DNA intercalating agents are among the most common anticancer drugs used in the clinical therapy of human tumours. These agents are characterised by the presence of flat chromophores bearing electron deficient polyconjugated areas bounded to polar groups [1]. Their antitumour activities are derived from DNA distortion and altered nuclear protein interaction as a consequence of reversible complex formation [2].

In our laboratory we have discovered the antitumour potential of a series of mono and bisintercalating agents bearing naphthalimide chromophores [3,4].

Two compounds from this class (Fig. 1), Amonafide and Elinafide, have been selected for phase II clinical trials [5,6].

Nowadays, there is an increasing interest in tethering polyamines to known cancer drugs and biological active agents in order to improve their activity and specificity [7,8]. Some authors have reported the combination of polyamines and DNA intercalators but they have employed linear [9,10] or macrocyclic polyamines [11], and their results suggest that the appended polyamine is a value-added fragment.

On the other hand, dendrimers are hyperbranched polymers that emanate from a central core, have a defined number of generations and functional end groups, and are synthesised stepwise by a repetitive reaction sequence. Branching is dependent on building

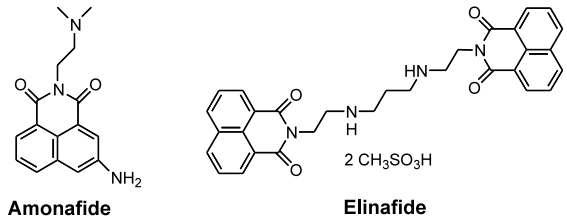

Fig. 1. Mono and bisintercalating agents.

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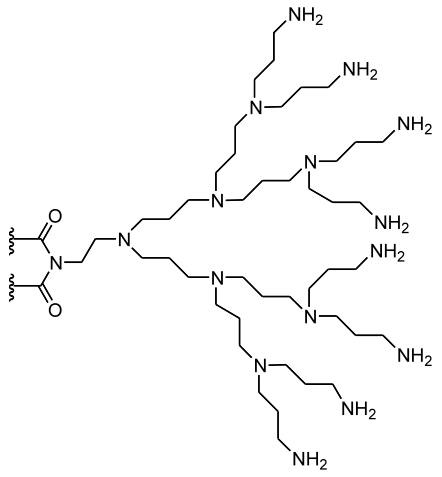

Fig. 2. PPI dendritic–(imide–DNA-intercalator) conjugates.

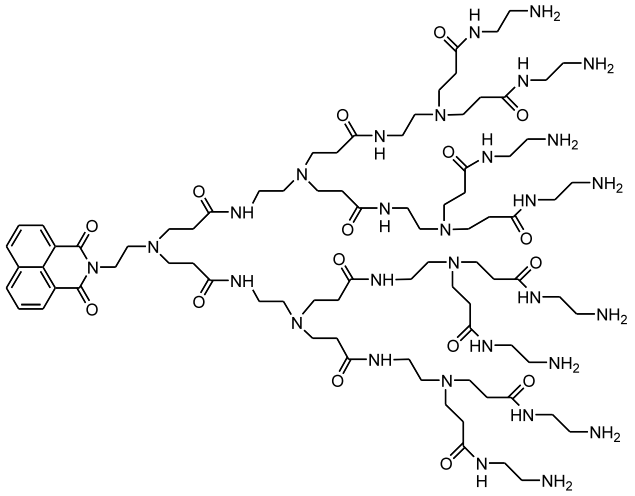

Fig. 3. PAMAM dendritic–(imide–DNA-intercalator) conjugates.

block valence (this includes the core). Thus, a core possessing one reactive moiety, such as a primary amine, is divalent and will accommodate two monomers, assuming a neutral trisubstituted amine product. Branching therefore proceeds in a 1–2 manner. PAMAM dendrimers and PPI dendrimers branch this way and both have amino terminal groups. PPI (polypropylenimine dendrimers) have *N*-branching centers and *N*-connectivity centers, whereas PAMAM (polyamidoamine dendrimers) have *N*-branching centers as well as amide connectivity. Although dendrimers have been employed in genic therapy [12,13] and they have been described as able to form a complex with DNA, their modification to join an intercalating chromophore as antitumour compounds has never been described. Thus, the synthesis and the pharmacological profile of such compounds could be of great interest.

In this work we first tried to improve the therapeutic properties of known imides by conjugating the DNA-intercalator chromophore to a dendritic polyamine as a way to increase the binding ability to DNA and, therefore the potency of the intercalator. Thus, the increase of potential hydrogen bonds and favourable electrostatic interactions between the protonated amino groups of the dendrimeric chains and the negatively charged sugar phosphate backbone should largely increase the affinity of these polymeric structures for DNA.

In this regard, Cohen and coworkers found that the conjugation of a drug to a polyammonium cation increases the DNA binding affinity and that the polyamine conjugate was more potent inhibiting tumour growth than the unconjugated drug [14,15].

The design of our conjugates is predicated upon the well-known affinity of polyamines for DNA and the established binding modes of naphthalimides [16]. DNA intercalation models predict that a multitude of anionic site charges are available for binding additional basic functions beyond those present in the intercalator framework [17]. Since polyamines exist as polycations in vivo, the conjugates which have an appended polycationic tail bind to these available sites, thereby increasing the binding affinity and potency of the intercalator. Moreover, studies have indicated that polyammonium cations (PACs) have a very high DNA affinity but are loosely bound and can 'read' DNA rapidly. These properties make PACs and related polycations ideal for drug delivery when the drug needs to reach specific sites in DNA [18]. Therefore, the combination of these agents (dendritic polyamines and DNA intercalators) is expected to provide new DNA targetering vectors.

This approach was initially supported by molecular modeling studies, in which it has been determined that these compounds are likely to bind a DNA octamer of a model d[GCACGTCG]$_2$ sequence through the central 5′-CpG-3′ step, via either the major or the minor groove [19].

Our first approach was to synthesise conjugates of dendritic polyamines with chains of amine ramification and amine connectivity polypropylenimine dendrimers (PPI-dendrimers) [20] (Fig. 2). In a second approach, we have modified the connectivity of the dendritic chain with an amide group, which possesses the ability to form additional hydrogen bonds to generate PAMAM (polyamidoamine) dendritic–(imide–DNA-intercalator) conjugates (Fig. 3). In this case the distance between nitrogen atoms in the PAMAM chain was as in Elinafide, optimum according to our experience.

On the other hand, other attractive targets for antitumoural agents are tyrosine-kinases and the signaling pathways in which they participate.

Tyrosine-specific protein kinases are composed of two subfamilies: (1) the receptor tyrosine-kinases, which are integral membrane proteins; and (2) their nonreceptor cytoplasmatic counterparts. The former, upon coordination of specific extracellular ligands, forms

aggregates and subsequently suffers phosporylation of key tyrosine residues. Cytoplasmatic signaling proteins, including nonreceptor tyrosine-kinases, coordinate to these phosphotyrosine (pTyr) residues through Src homology 2 (SH2) domains [21]. This binding event triggers the activation of specific intracellular signaling pathways, ultimately leading to a cellular response in reaction to the extracellular stimulus. For example, antigen presentation to T cells results in the clustering of T-cell receptors (TcR) and subsequent TcR tyrosine phosphorilation by Src family member LcK (lymphoid T-cell tyrosine-kinase) [22]. These TcR pTyr moieties serve as high-affinity binding sites for the SH_2 domains of cytoplasmatic signaling proteins, which generate complexes that are required for ensuing downstream events such as an increase in cytoplasmatic Ca^{2+} levels and the ultimate production of interleukin-2. In short, SH_2 domains play a critical role in organizing coherent signal transducing complexes that are essential for the appropriate cellular response to extracellular stimuli. Constituitively, active signal transduction pathways have been identified in a variety of disease states. Ligands that are able to disrupt these inappropriately hyperstimulated pathways, by inhibition of Lck, by blocking SH_2 domain-dependent interactions, may ultimately find utility as therapeutic agents. Lawrence and coworker [23] have reported that diamines with a hydrophobic substituent and substituents that could form a hydrogen bond with residues in the SH_2 domain might promote Lck SH_2 affinity.

Another goal of this work was to know the pharmacological profile of these conjugates in other different and important targets like Lck. In our laboratory we are interested in testing some of these conjugates (which have amine groups, hydrophobic substituent and amide groups) for the inhibition of Lck, because this biological system was available in the laboratory and this enzyme is of great interest in the treatment of autoimmune diseases and T-cell based leukemias and lymphomas.

2. Chemistry

To carry out the synthesis of conjugates with chains of amine branching and amine connectivity, we first built the dendritic chain by the divergent procedure described by Vögtle and coworkers [24] and Wörner et al. [25], starting from *N*-*tert*-butoxycarbonyl derivative, **2**, which was synthesised from ethylenediamine (**1**) with di-*tert*-butylcarbonate in dichloromethane. The addition of acrylonitrile to **2** in acetic glacial acid gave us dicyano **3**. The hydrogenation of **3**, with Ni-Raney in a solution of 1.4 M NaOH provided compound **4**. This sequence of reactions was carried out successively to obtain compounds **5**–**7**, in good yields. Although first bis-cyanoethylations were accomplished in acetic glacial acid, compound **7** was obtained in acid free methanol. These conditions required a longer reaction time, but compound **7** did not need further purification. Cleavage of the *tert*-butoxycarbonyl group was carried out at room temperature with trifluoroacetic acid in dichloromethane–anisole (1:1), providing compound **8**, with the free amine group (Fig. 4).

Imide derivatives were prepared by nucleophilic addition of the dendritic amine **8** to the appropriate 1,8-naphthalic anhydride-3-substituted to provide compounds **9**–**11**. We also synthesised the imide derivative of 3,4-diphenyl maleic anhydride **12**. It is noteworthy that this system exhibits relevant cytostatic activity [26,27]. Finally, reduction of the end cyano groups of compounds **9**–**12** under the described conditions, led us to the target molecules **13**–**16** (Fig. 5).

The synthesis of dendritic polyamines–(DNA-intercalators) conjugates with PAMAM chains was carried out employing a divergent procedure already described by Tomalia [28], but using a core with an intercalating chromophore. In this way, synthesis started from *N*-(2-aminoethyl)-1,8-naphthalimide (**17**), which was synthesised from 1,8-naphthalic anhydride and ethylenediamine. A two-step process was developed: first exhaustive Michael addition of methyl acrylate to the amine core **17**, followed by exhaustive amidation of the resulting esters **18**, **20** and **22** with excess of ethylenediamine to give us the conjugates **19**, **21** and **23**, in good overall yields (Fig. 6).

3. Pharmacology

In vitro cytotoxicity of members of our synthetic series was established using the HT-29 (human colon carcinoma cell line) and a standard (MMT) assay [29]. The Lck inhibitory activity of some conjugates was determinated by an enzyme-linked immunosorbent assay (ELISA) using the universal tyrosine-kinase substrate Poly(Glu, Tyr) 4:1 at 5 μM ATP [30].

4. Results and discussion

In vitro cytotoxity against human colon carcinoma cell line HT-29 and in vitro inhibitory assay Lck was carried out (Table 1). The values obtained for both types of (polyamine dendritic–DNA intercalator) conjugates against HT-29 were not remarkable, probably due to poor permeabilities. However, compounds **13**, **16** and **21** showed significant inhibitory activity against Lck and the best values were observed when the dendritic chain had amine ramification and amine connectivity (PPI chain), and eight amine primary groups (**13** and **16**). We observed that compounds with similar or

no significant values of HT-29 exhibited the best results for inhibition of Lck. It must be noted that a slight increase in cytotoxic activity did not produce a better inhibitory activity. We must also consider that HT-29 and Lck are different biological systems and there is no correlation between the values obtained. HT-29 is an in

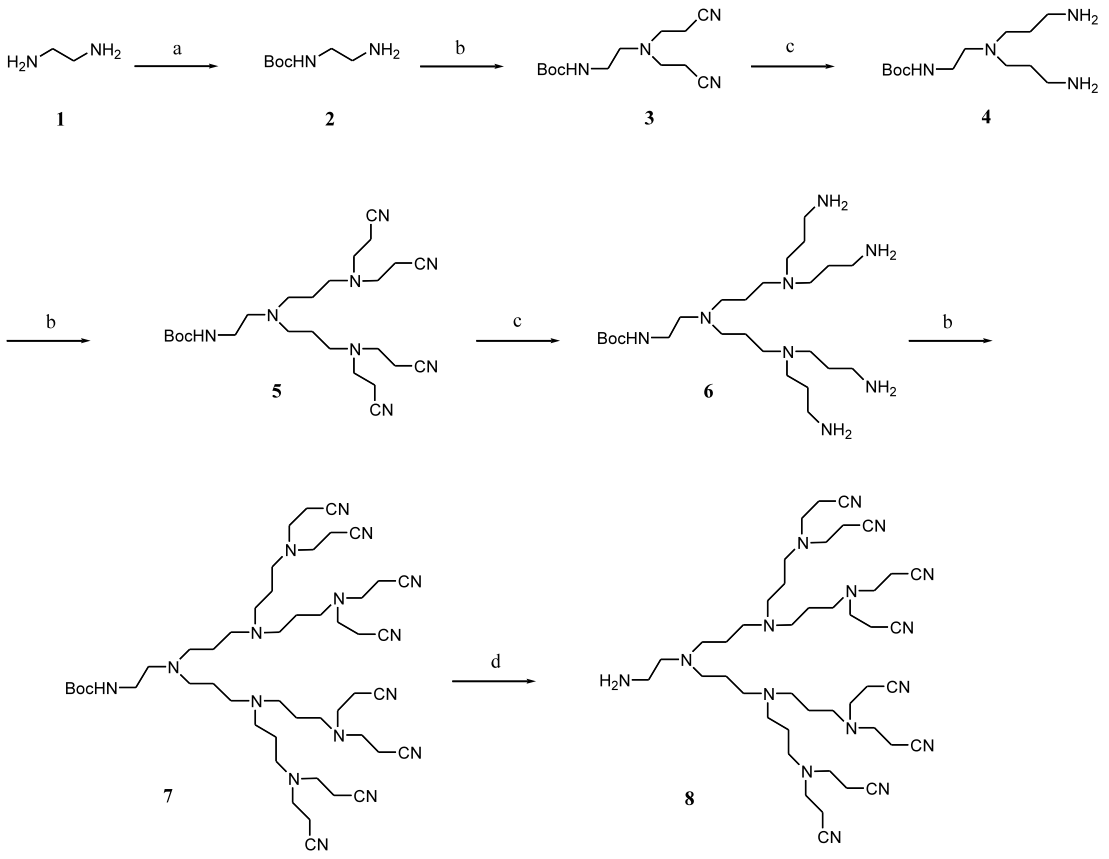

Fig. 4. (a) $(Boc)_2O$, CH_2Cl_2, 0 °C r.t., 16 h, 50%. (b) Acrylonitrile, AcOH or MeOH; **3**: 24 h, reflux, 96%; **5**: 24 h, reflux, 90%; **7**: 1 h, 10 °C, 4 days, 60 °C, 82%. (c) H_2, Ni-Raney, 1.4 M NaOH in 95% EtOH; **4**: 16 h, 80%; **6**: 36 h, 70%. (d) TFA, CH_2Cl_2–anisol, 1:1, 20 min, 80%.

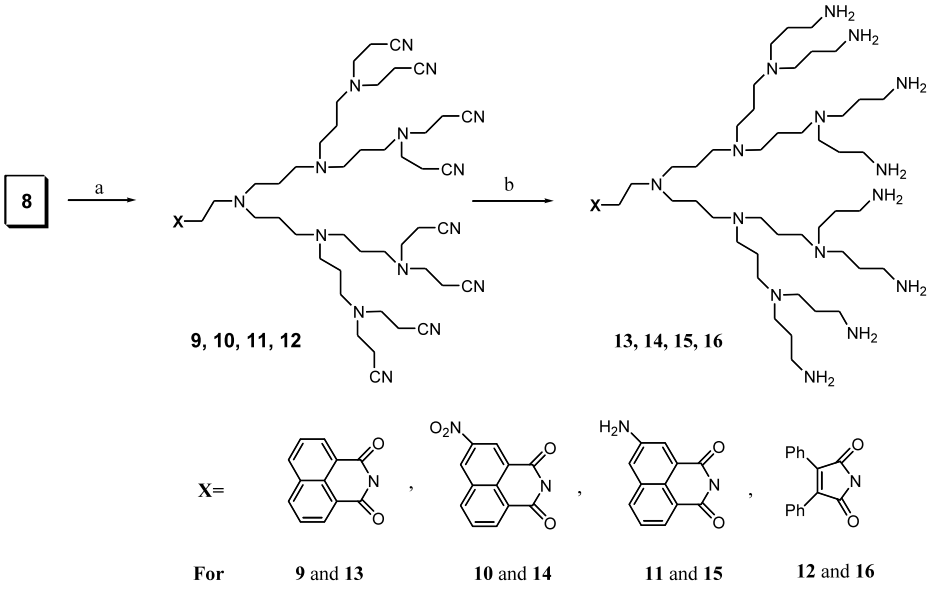

Fig. 5. (a) Aromatic anhydride, absolute EtOH; **9**: r.t., 18 h, 92%; **10**: reflux; 18 h, 83%; **11**: reflux; 48 h, 60%; **12**: reflux, 2 h, 80%. (b) H_2, Ni-Raney, 1.4 M NaOH in 95% EtOH; **13**: 48 h, 52%; **14**: 24 h, 60%; **15**: 24 h; 49%, **16**: 16 h, 77%.

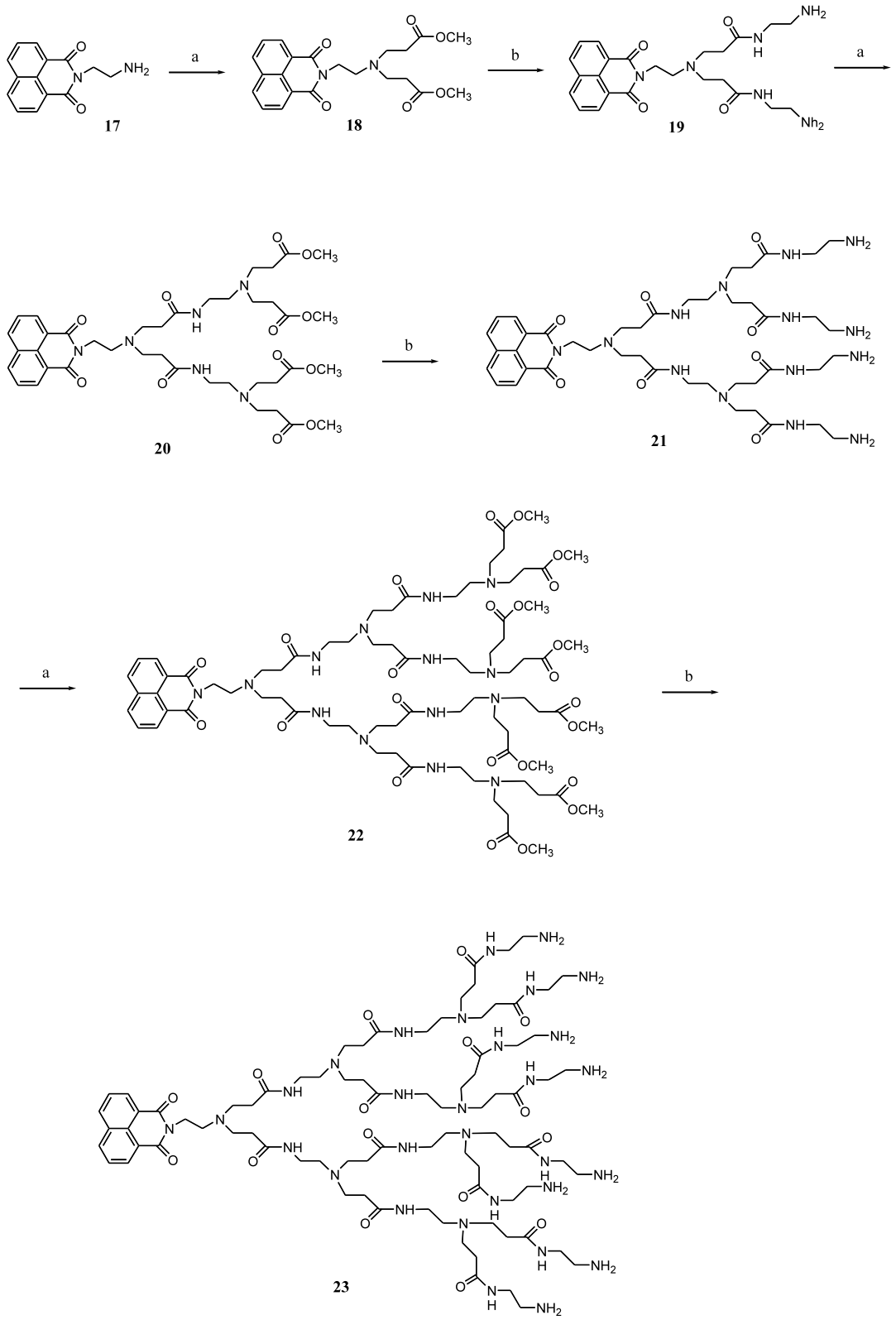

Fig. 6. (a) Methylacrilate, MeOH, reflux; **18**: 6 h, 81%; **20**: 16 h, 62%; **22**: 48 h, 67%. (b) Ethylenediamine excess, MeOH, r.t.; **19**: 58%; **21**: 60%; **23**: 40%.

Table 1
Biological activities against HT-29 and Lck

Compounds	HT-29 IC_{50} μM	Lck IC_{50} μM
8	>100	3.8
9	>100	>50
10	>100	33.8
11	>100	5.02
12	57	>50
13	44	0.39
14	– [a]	– [a]
15	– [a]	– [a]
16	100	0.10
19	>100	6.1
21	>100	1.78
23	– [a]	– [a]
Amonafide	38	– [a]
Elinafide	0.014	– [a]

[a] Not determined.

vitro assay, whereas the determination of inhibition of Lck is an ex vivo assay.

The values obtained for conjugates with PPI chains with different chromophores (**13** vs. **16**) were insignificant against HT-29 and it should be noted that a slight improvement was shown with naphthalimide chromophore. Nevertheless, these compounds showed a significant effect in the inhibition of Lck. Thus, the change of the naphthalimide chromophore for the 3,4-diphenyl maleic chromophore practically did not alter the activity against Lck and it would always be around optimal values.

The results for conjugates with PAMAM chains, in which the ramification grade increased (**19** vs. **21**) against HT-29, was not relevant. However, the values obtained against Lck were relevant in both cases, and it can be deduced that the increasing in the ramification grade is a favourable factor in the inhibition of Lck, but the absolute values are less than conjugates with PPI chains.

In conjugates with end cyano groups, the introduction of the chromophore in the PPI chain did not improve the results against HT-29. However, the activity against Lck was better without the chromophore. We can deduce that the presence of end cyano groups is an unfavourable factor for the inhibition of Lck and HT-29 (**9**, **12** vs. **13**, **16**), whereas one favourable factor for the inhibition of Lck is the presence of amine primary groups (**8** vs. **9**–**12**).

5. Conclusions

In conclusion, in this work we have synthesised the first examples of dendritic polyamines–(imide–DNA-intercalator) conjugates. Although dendritic polyamines have been employed in gene therapy, their modification to conjugate an intercalating chromophore as an antitumour compound has never been described. Biological assays showed no relevance for the activity of these compounds against HT-29 cell lines and values range around 44– >100 μM. However, results obtained for the inhibition of Lck seem promising and an excellent starting point for further development of this type of approximation in the search for new antitumour compounds. It appears that Lck expression is largely confined to T cells, and its over-expression may contribute to certain T-cell tumours [31,32]. Thus, selective inhibition of the Lck enzyme may be of interest in the treatment of several types of lymphomas, such as non-Hodgkin's [33] which express the Lck enzyme. These results open up a new area of future research.

6. Experimental

6.1. Chemistry

The ^{1}H- and ^{13}C-NMR spectra were recorded in a Brücker AC 300 spectrometer with Me_4Si as internal standard. IR spectra were taken on a Perkin–Elmer 1330 spectrometer. For purification of crude reaction mixtures by flash chromatography, Merck silica gel (230–400 mesh) or neutral aluminum oxide (70–230 mesh) was used as the stationary phase. Identification of products was made by analytical TLC (Merck Kiesegel 60F-254), UV light ($\lambda = 254$ nm), and 5% phosphomolydic acid solution in 95% EtOH were used to develop the plates. Aldrich celite 545 was employed as a filtrating agent. Reagents and solvents were handled by using standard syringe techniques. DMF and CH_2Cl_2 were distilled over calcium hydride. All other solvents were reagent grade. Ni-Raney (slurry in water) was purchased from Aldrich. Analyses indicated by the symbols of the element were within $\pm 0.4\%$ of theoretical values.

6.1.1. Synthesis of *N-tert-butoxycarbonylaminoethylamine* (**2**)

Di-*tert*-butyldicarbonate (9.07 g, 41.5 mmol) dissolved in $CHCl_3$ (200 mL) was added dropwise to a solution of ethylenediamine (5 g, 83.1 mmol) in 80 mL of $CHCl_3$ at 0 °C. The reaction mixture was stirred 16 h at room temperature (r.t.) and washed with water. The organic phase was dried over Na_2SO_4, evaporated to dryness in vacuo to obtain a colourless oil which was purified by silica gel chromatography ($CHCl_3$–MeOH 5:0.1). Yield: 3.3 g (50%) (colourless oil). ^{1}H-NMR ($CDCl_3$): δ 1.39 (9H, $3CH_3$), 1.90 (s, 2H, NH_2), 2.74 (t, 2H, CH_2, CH_2NH_2, $J = 6.0$ Hz), 3.12 (q, 2H, CH_2, CH_2NH, $J = 5.5$ Hz), 5.23 (bs, 1H, NH). ^{13}C-NMR ($CDCl_3$): $\delta = 156.0$, 78.9, 43.0, 41.6, 28.2. IR (neat): 3400, 2980, 1720 cm^{-1}. Anal. ($C_7H_{16}N_2O_2$) C, H, N.

*6.1.2. General procedure for synthesis of oligonitriles via cyanoethylation (**3**, **5**, **7**)*

6.1.2.1. Method A. To a solution of the amine (10 mmol) in a large excess of acrylonitrile (50 mL), glacial AcOH (20 mmol per primary amine fuction) was added and the mixture was refluxed for 24 h. The excess of acrylonitrile was evaporated in vacuo to obtain a residue, which was dissolved in $CHCl_3$ (30 mL) and added to 10 mL of concentrated aq. NH_4OH. The organic layer was separated, washed with water and dried over Na_2SO_4. Evaporation of the solvent and neutral alumine chromatography of the obtained oil yielded the pure products.

6.1.2.2. Method B. To a cold solution of the polyamine (1.0 g, 1.9 mmol) in MeOH (18 mL) were added dropwise 19 mL (288 mmol) of acrylonitrile. After stirring 1 h at 10 °C and 4 days at 60 °C, the quantitative bis(cyanoethylation) was obtained. The solvent was evaporated in vacuo to obtain an oil which was purified by low pressure distillation.

*6.1.2.3. Synthesis of N,N-bis-(2-cyanoethyl)-N′-tert-butoxycarbonyl ethylenediamine (**3**).* Following method A, from 10.0 g (64.8 mmol) of **2** was obtained 2.1 g (96%) of **3** as a colourless oil after chromatography ($CHCl_3$–MeOH 5:0.1). ^{1}H-NMR ($CDCl_3$): δ 1.39 (s, 9H, $3CH_3$), 2.46 (t, 4H, $2CH_2$, CH_2CN, $J = 6.6$ Hz), 2.64 (t, 2H, CH_2, NCH_2CH_2NH, $J = 6.0$ Hz), 2.86 (t, 4H, $2CH_2$, CH_2N, $J = 6.0$ Hz), 3.16 (q, 2H, CH_2, NCH_2CH_2NH, $J = 6.0$ Hz), 5.0 (bs, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 155.9, 118.5, 79.0, 52.8, 49.4, 38.2, 28.1, 16.9. IR (neat): 2980, 2240, 1720 cm^{-1}. Anal. ($C_{13}H_{22}N_4O_2$) C, H, N.

*6.1.2.4. Synthesis of 4-cascade–N-tert-butoxycarbonyl ethylenediamine [2]–(1-azabutylidyne)–propanitrile (**5**).* Following method A, from 3.2 g (11.7 mmol) of **4** was obtained 5.1 g (90%) of **5** as a colourless oil after chromatography ($CHCl_3$–MeOH 10:0.1). ^{1}H-NMR ($CDCl_3$): δ 1.43 (s, 9H, $3CH_3$), 1.59 (m, 4H, $2CH_2$, $CH_2CH_2CH_2$), 2.46–2.57 (m, 18H, $9CH_2$, CH_2N), 2.84 (t, 8H, $4CH_2$, CH_2CN, $J = 6.6$ Hz), 3.14 (q, 2H, CH_2, CH_2NH, $J = 6.0$ Hz), 4.99 (bs, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 155.9, 118.7, 79.0, 53.0, 51.5, 51.2, 49.5, 28.4, 24.9, 16.8. IR (neat): 2980, 2240, 1720, 1600 cm^{-1}. Anal. ($C_{25}H_{42}N_8O_2$) C, H, N.

*6.1.2.5. Synthesis of 8-cascade–N-tert-butoxycarbonylethylenediamine [2]–(1-azabutylidyne)^2–propanitrile (**7**).* Following method B, from 1.0 g (1.9 mmol) of **6** was obtained 1.5 g (82%) of **7** as a pale yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.42 (s, 9H, $3CH_3$), 1.58 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.48 (m, 42H, $21CH_2$, CH_2N), 2.83 (t, 16H, $8CH_2$, CH_2CN, $J = 6.6$ Hz), 3.13 (s, 2H, CH_2NH), 5.04 (bs, 1H, NHBOC). ^{13}C-NMR ($CDCl_3$): δ 155.9, 118.8, 78.8, 52.9, 52.0, 51.9, 51.4, 51.3, 49.4, 38.2, 29.6, 28.4, 24.8, 16.8. IR (neat): 2980, 2240, 1725 cm^{-1}. Anal. ($C_{49}H_{82}N_{16}O_2$) C, H, N.

*6.1.3. Synthesis of 8-cascade ethylenediamine [2]–(1-azabutylidyne)^2–propanitrile (**8**)*

Oligonitrile **7** (3.58 g, 3.86 mmol) was dissolved in 50 mL of 50% $CHCl_3$–anisol, and 25 mL of trifluoracetic acid was slowly added at 0 °C. After stirring for 25 min the solvent and the TFA were removed under vacuum. The resulting residue was dissolved in water and adjusted to pH > 8 by addition of a $NaHCO_3$ saturated solution. The resulting solution was extracted with $CHCl_3$ (3×15 mL) and the combined organic layers dried over Na_2SO_4, filtered, and concentrated in vacuo to obtain an oil which was purified by Kügelrorh distillation to yield 2.5 g (80%) of **8** as an orange oil. ^{1}H-NMR ($CDCl_3$): δ 1.57 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.40–2.58 (m, 42H, $21CH_2$, CH_2N), 2.70 (m, 2H, CH_2, CH_2NH_2), 2.81 (t, 16H, $8CH_2$, CH_2CN, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$): δ 118.7, 52.3, 51.9, 51.4, 51.3, 49.4, 39.6, 24.8, 24.3, 16.8. IR (neat): 3500, 2240 cm^{-1}. Anal. ($C_{44}H_{74}N_{16}$) C, H, N.

*6.1.4. General procedure for synthesis of primary amines via hydrogenation with Ni-Raney (**4**, **6**, **13**–**16**)*

A 1.4 M solution NaOH in 95% EtOH was added to a 500 mL hydrogenation vessel filled with the oligonitrile and Raney-Ni catalyst. The mixture was hydrogenated at 60 psi for several hours at r.t. The catalyst was filtered through celite and washed with 95% EtOH. After diluting the filtrate with H_2O, EtOH was evaporated and the residue extracted several times with CH_2Cl_2. Thereby, the NaOH concentration in the aq. phase was increased in every extraction step. The organic layers were dried with Na_2SO_4 and the solvent evaporated in vacuo yielding the polyamines as oils.

*6.1.4.1. Synthesis of N,N-bis-(3-aminopropyl)-N′-tert-butoxycarbonyl ethylenediamine (**4**).* From 4.0 g (15 mmol) of **3**, 1.5 g of Ni-Raney and 12 mL of a 1.4 M solution NaOH in 95% EtOH were hydrogenated for 16 h. In this case the filtrate was concentrated and dissolved in a small amount of water. After addition of NaOH pellets, the amine **4** begins to separate as an oil which is extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and the solvent evaporated to obtain 3.2 g (80%) of **4** as an orange oil. ^{1}H-NMR ($CDCl_3$): δ 1.22 (bs, 4H, $2NH_2$), 1.38 (s, 9H, $3CH_3$), 1.52 (m, 4H, $2CH_2$, $CH_2CH_2CH_2$), 2.41 (t, 4H, $2CH_2$, CH_2N, $J = 7.1$ Hz), 2.44 (t, 2H, CH_2, NCH_2CH_2NH, $J = 6.0$ Hz), 2.67 (t, 4H, $2CH_2$, CH_2NH_2, $J = 7.1$ Hz), 3.11 (q, 2H, CH_2, NCH_2CH_2NH, $J = 6.0$ Hz), 5.00 (bs, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 156.0, 78.8, 53.1, 51.5, 40.3, 38.2, 30.7, 28.3. IR (neat): 3500, 2980, 1720 cm^{-1}. Anal. ($C_{13}H_{30}N_4O_2$) C, H, N.

*6.1.4.2. Synthesis of 4-cascade–N-tert-butoxycarbonylethylenediamine [2]–(1-azabutylidyne)–propanamine (**6**).* From 5.2 g (10.6 mol) of **5**, 5.7 g of Ni-Raney and 16.6 mL of a 1.4 M solution NaOH in EtOH was obtained 3.7 g (70%) of **6** as an orange oil. Reaction time: 36 h. ^{1}H-NMR ($CDCl_3$): δ 1.34 (bs, 8H, $4NH_2$), 1.38 (s, 9H, $3CH_3$), 1.52 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.38 (m, 18H, $9CH_2$, CH_2N), 2.66 (t, 8H, $4CH_2$, CH_2NH_2, $J = 6.6$ Hz), 3.10 (m, 2H, CH_2, CH_2NH), 4.93 (bs, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 156.0, 78.7, 53.0, 51.8, 51.7, 51.5, 40.6, 38.2, 30.7, 28.3, 24.4. IR (neat): 3400, 2980, 1720 cm^{-1}. Anal. ($C_{25}H_{58}N_8O_2$) C, H, N.

*6.1.4.3. Synthesis of 8-cascade–N-(2-aminoethyl)-1,8-naphthalimide [2]–(1-azabutylidyne)^2–propanamine (**13**).* From 1.13 g (1.12 mmol) of **9**, 1.8 g of Ni-Raney and 25 mL of a 1.4 M solution NaOH in 95% EtOH was obtained 0.58 g (50%) of **13** as a light brown oil. Reaction time 48 h. ^{1}H-NMR (D_2O): δ 1.40–1.56 (m, 28H, $14CH_2$, $CH_2CH_2CH_2$), 2.32–2.43 (m, 42H, $21CH_2$, CH_2N), 2.70 (m, 16H, $8CH_2$, CH_2NH_2), 3.97 (bs, 2H, CH_2, CH_2NCO), 7.59 (bs, 2H, Har), 8.19 (bs, 4H, Har). ^{13}C-NMR (D_2O): δ 163.3, 128.9, 127.6, 127.5, 127.4, 57.1, 50.3, 50.1, 48.0, 38.3, 27.6, 27.0, 24.8, 20.9. IR (neat): 3500, 1700, 1610 cm^{-1}. Anal. ($C_{56}H_{110}N_{16}O_2$) C, H, N.

*6.1.4.4. Synthesis of 8-cascade–N-(2-aminoethyl)-3-nitro-1,8-naphthalimide [2]–(1-azabutylidyne)^2–propanamine (**14**).* From 1.10 g (1.04 mmol) of **10**, 1.4 g of Ni-Raney and 60 mL of a 1.4 M solution NaOH in 95% EtOH was obtained 0.68 g (60%) of **14** as a red oil. Reaction time 24 h. ^{1}H-NMR (D_2O): δ 1.10–1.40 (bs, 28H, $14CH_2$, $CH_2CH_2CH_2$), 2.29 (m, 42H, $21CH_2$, CH_2N), 2.45 (m, 16H, $8CH_2$, CH_2NH_2), 3.06 (bs, 2H, CH_2NCO), 7.21 (bs, 1H, Har), 7.47 (bs, 1H, Har), 7.52 (bs, 1H, Har), 8.16 (bs, 1H, Har), 8.81 (bs, 1H, Har). ^{13}C-NMR (D_2O): δ 165.5, 162.5, 133.9, 132.8, 132.0, 131.6, 131.4, 129.0, 54.2, 54.0, 53.9, 53.7, 53.6, 53.5, 53.0, 52.1, 36.6, 31.2, 31.0, 30.4, 24.8. IR (neat): 3400, 1700, 1610, 1590 cm^{-1}. Anal. ($C_{56}H_{109}N_{17}O_4$) C, H, N.

*6.1.4.5. Synthesis of 8-cascade–N-(2-aminoethyl)-3-amine-1,8-naphthalimide [2]–(1-azabutylidyne)^2:propanamine (**15**).* From 1.0 g (0.98 mmol) of **11**, 1.44 g of Ni-Raney and 50 mL of a 1.4 M solution NaOH in 95% EtOH was obtained 0.51 g (49%) of **15** as a yellow oil. Reaction time 24 h. ^{1}H-NMR (D_2O): δ 1.25–1.33 (bs, 28H, $14CH_2$, $CH_2CH_2CH_2$), 2.15–2.30 (m, 42H, $21CH_2$, CH_2N), 2.45 (m, 16H, $8CH_2$, CH_2NH_2), 3.03 (bs, 2H, CH_2, CH_2NCO), 6.82 (bs, 1H, Har), 6.94 (bs, 1H, Har), 7.15 (bs, 1H, Har), 7.49 (bs, 1H, Har), 7.61 (bs, 1H, Har). ^{13}C-NMR (D_2O): δ 161.9, 158.7, 131.3, 130.9, 130.7, 130.0, 128.9, 126.6, 124.6, 122.7, 54.2, 53.7, 53.6, 53.5, 52.2, 41.7, 40.3, 36.3, 31.0, 30.4, 24.8. IR (neat): 3400, 1740, 1660 cm^{-1}. Anal. ($C_{56}H_{111}N_{17}O_2$) C, H, N.

*6.1.4.6. Synthesis of 8-cascade–N-(2-aminoethyl)-2,3-diphenylmaleimide [2]–(1-azabutylidyne)^2–propanamine (**16**).* From 0.46 g (0.44 mmol) of **12**, 0.70 g of Ni-Raney and 25 mL of a 1.4 M solution NaOH in 95% EtOH was obtained 0.37 g (77%) of **16** as a yellow oil. Reaction time 36 h. ^{1}H-NMR (D_2O): δ 1.26–1.39 (m, 28H, $14CH_2$, $CH_2CH_2CH_2$), 2.08–2.27 (m, 42H, $21CH_2$, CH_2N), 2.33 (t, 16H, $8CH_2$, CH_2NH_2, $J = 7.1$ Hz), 3.91 (bs, 2H, CH_2, CH_2NCO), 6.99–6.87 (m, 10H, Har). ^{13}C-NMR (D_2O): δ 170.2, 138.1, 129.2, 128.4, 127.8, 127.5, 57.1, 50.3, 50.1, 48.0, 38.3, 27.6, 27.0, 20.8. IR (neat): 3400, 1740, 1650 cm^{-1}. Anal. ($C_{60}H_{114}N_{16}O_2$) C, H, N.

*6.1.5. General procedure for synthesis of imide derivatives **9**, **10**, **11**, **12***

To a solution of the 8-cascade–ethylenediamine [2]–(1-azabutylidyne)^2–propanitrile (**8**), the corresponding anhydride was added. The mixture was stirred and the solvent removed under reduced pressure to obtain the imide derivatives after chromatography in neutral aluminum oxide using $CHCl_3$–MeOH (5:0.1) as eluent.

*6.1.5.1. Synthesis of 8-cascade–N-(2-aminoethyl-1,8-naphthalimide [2]–(1-azabutylidyne)^2–propanitrile (**9**).* From 0.89 g (1.08 mmol) of **8** and 0.21 g (1.08 mmol) of 1,8-naphthalic anhydride in absolute EtOH (50 mL) were stirred 18 h at r.t. to obtain 0.89 g (92%) of **9** as a light brown oil. ^{1}H-NMR ($CDCl_3$): δ 1.64 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.50 (t, 24H, $12CH_2$, CH_2CH_2N, $J = 6.6$ Hz), 2.59 (t, 16H, $8CH_2$, NCH_2CH_2CN, $J = 7.1$ Hz), 2.75 (t, 2H, CH_2, $NCH_2CH_2N(CO)_2$, $J = 7.1$ Hz), 2.86 (t, 16H, $8CH_2$, CH_2CN, $J = 7.1$ Hz), 4.26 (t, 2H, $NCH_2CH_2N(CO)_2$, $J = 7.1$ Hz), 7.78 (t, 2H, Har, $J =$ 7.7 Hz), 8.24 (d, 2H, Har, $J = 8.2$ Hz), 8.5 (d, 2H, Har, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$): δ 163.8, 133.8, 131.3, 130.8, 127.7, 126.7, 122.1, 118.7, 52.3, 51.5, 51.2, 50.9, 49.1, 44.1, 37.6, 24.3, 23.9, 16.5. IR (neat): 2240, 1700, 1610 cm^{-1}. Anal. ($C_{56}H_{78}N_{16}O_2$) C, H, N.

*6.1.5.2. Synthesis of 8-cascade–N-(2-aminoethyl)-3-nitro-1,8-naphthalimide [2]–(1-azabutylidyne)^2–propanitrile (**10**).* From 0.81 g (0.98 mmol) of **8** and 0.24 g (0.98 mmol) of 3-nitro-1,8-naphthalic anhydride in 50 mL of 50% absolute EtOH–CH_2Cl_2 were refluxed 18 h to obtain 0.72 g (83%) of **10** as a red oil. ^{1}H-NMR ($CDCl_3$): δ 1.65 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.48–2.65 (m, 40H, $20CH_2$, CH_2N), 2.76 (m, 2H, CH_2, $NCH_2CH_2N(CO)_2$), 2.85 (t, 16H, $8CH_2$, CH_2CN, $J =$ 6.6 Hz), 4.26 (t, 2H, CH_2, $NCH_2CH_2N(CO)_2$, $J = 7.1$ Hz), 7.95 (t, 1H, Har, $J = 7.1$ Hz), 8.43 (d, 1H, Har, $J = 8.2$ Hz), 8.76 (d, 1H, Har, $J = 7.1$ Hz), 9.14 (s, 1H, Har), 9.23 (s, 1H, Har). ^{13}C-NMR ($CDCl_3$): δ 162.8,

162.3, 145.9, 135.6, 134.2, 130.8, 129.9, 129.0, 124.3, 122.8, 118.8, 52.3, 51.6, 51.3, 51.1, 49.3, 44.2, 38.1, 24.6, 16.6. IR (neat): 2240, 1700, 1610, 1590 cm^{-1}. Anal. ($C_{56}H_{77}N_{17}O_4$) C, H, N.

*6.1.5.3. Synthesis of 8-cascade–N-(2-aminoethyl)-3-amine-1,8-naphthalimide [2]–(1-azabutylidyne)^2–propanitrile (**11**).* From 0.98 g (1.19 mmol) of **8** and 0. 25 g (1.19 mmol) of 3-amine-1,8-naphthalic anhydride in 50 mL of 50% absolute EtOH–DMF were refluxed 48 h to obtain 1.0 g (60%) of **11** as a yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.75–2.10 (m, 14H, $6CH_2 + NH_2$, $CH_2CH_2CH_2$), 2.53–2.65 (m, 42H, $21CH_2$, CH_2N), 2.84 (t, 16H, $8CH_2$, CH_2CN, $J = 6.0$ Hz), 4.24 (t, 2H, CH_2, $CH_2N(CO)_2$, $J = 7.1$ Hz), 7.31 (s, 1H, Har), 7.61 (t, 1H, Har, $J = 7.7$ Hz), 7.95 (d, 1H, Har, $J = 7.1$ Hz), 8.02 (s, 1H, Har), 8.25 (d, 1H, Har, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$): δ 162.8, 162.3, 133.5, 131.8, 127.1, 122.1, 119.1, 119.0, 118.8, 113.8, 51.5, 51.3, 50.7, 49.4, 44.5, 36.4, 31.4, 18.9, 17.0. IR (neat): 3500, 2240, 1700, 1650 cm^{-1}. Anal. ($C_{56}H_{79}N_{17}O_2$) C, H, N.

*6.1.5.4. Synthesis of 8-cascade–N-(2-aminoethyl)-2,3-diphenylmaleimide [2]–(1-azabutylidyne)^2–propanitrile (**12**).* From 0.80 g (0.97 mmol) of **8** and 0.24 g (0.97 mmol) of 2,3-diphenylmaleic anhydride in 50 mL of absolute EtOH were refluxed 2 h to obtain 0.82 g (80%) of **12** as a yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.58 (m, 12H, $6CH_2$, $CH_2CH_2CH_2$), 2.42–2.54 (m, 40H, $20CH_2$, CH_2N), 2.71 (m, 2H, CH_2, $NCH_2CH_2N(CO)_2$), 2.80 (t, 16H, $8CH_2$, CH_2CN, $J = 6.6$ Hz), 3.70 (t, 2H, CH_2, $NCH_2CH_2N(CO)_2$, $J = 6.6$ Hz), 7.44–7.35 (m, 10H, Har). ^{13}C-NMR ($CDCl_3$): δ 170.7, 136.2, 129.8, 128.7, 128.6, 128.5, 118.8, 52.5, 51.8, 51.5, 51.2, 49.5, 36.4, 24.7, 16.8. IR (neat): 2240, 1700, 1640 cm^{-1}. Anal. ($C_{60}H_{82}N_{16}O_2$) C, H, N.

*6.1.6. Synthesis of N-(2-aminoethyl)-1,8-naphthalimide (**17**)*

1,8-Naphthalenedicarboxylic anhydride (2 g, 10.1 mmol) was added in portions to a solution of 1.01 mL (15.1 mmol) of ethylenediamine in 250 mL of EtOH and the mixture was heated to reflux during 6 h. The mixture was filtered under vacuum and the filtrate was evaporated under reduced pressure. The residue was recrystalised from EtOH to give **17** as a solid with 80%. ^{1}H-NMR ($CDCl_3$): δ 1.50 (bs, 2H, NH_2), 3.08, (t, 2H, CH_2, CH_2NH_2, $J = 6.6$ Hz), 4.29 (t, 2H,CH_2, $CH_2N(CO)_2$), $J = 6.6$ Hz), 7.75 (t, 2H, Har, $J = 7.7$ Hz), 8.21 (d, 2H, Har, $J = 8.2$ Hz), 8.59 (d, 2H, Har, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$):δ 164.9, 134.4, 131.9, 131.7, 128.6, 127.3, 122.9, 43.5, 40.9. IR (neat): 3345, 1700, 1660 cm^{-1}. Anal. ($C_{14}H_{12}N_2O_2$) C, H, N.

*6.1.7. General procedure for synthesis of polyesters **18**, **20**, **22***

Methyl acrylate (75 equiv. per primary amine function) was added to a solution of conjugate with amine terminal groups and the mixture was heated to reflux. MeOH and excess of methylacrylate were eliminated to yield the polyester as an oil after chromatography in silica gel using $CHCl_3$–MeOH 10:0.1.

*6.1.7.1. N-[2-(N,N′-bis(2-metoxycarbonylethyl)amine)-ethyl]-1,8-naphthalimide (**18**).* From 987 mg (4.11 mmol) of **17** and 27 mL (308 mmol) of methyl acrylate in 40 mL of MeOH was obtained **18** as a colourless oil with 81%. Reaction time: 6 h. ^{1}H-NMR ($CDCl_3$): δ 2.48 (t, 4H, $2CH_2$, $NCH_2CH_2CO_2CH_3$, $J = 7.1$ Hz), 2.81 (t, 2H, CH_2, NCH_2CH_2NCO, $J = 7.1$ Hz), 2.90 (t, 4H, $2CH_2$, $NCH_2CH_2CO_2CH_3$, $J = 7.1$ Hz), 3.52, (s, 6H, $2CH_3$), 4.26 (t, 2H, CH_2, NCH_2CH_2NCO, $J = 7.1$ Hz), 7.74 (t, 2H, Har, $J = 8.2$ Hz), 8.20 (d, 2H, Har, $J = 8.2$ Hz), 8.56 (d, 2H, Har, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$): δ 172.9, 164.1, 133.8, 131.5, 131.2, 128.1, 126.9, 122.6, 122.6, 51.3, 50.9, 49.6, 38.0, 32.8. IR (neat): 1730, 1700, 1650 cm^{-1}. Anal. ($C_{22}H_{24}N_2O_6$) C, H, N.

*6.1.7.2. 4-cascade–N-(2-aminoethyl)-1,8-naphthalimide [2]–1, 4-diaza-5-oxoheptylidine–methylpropanoate (**20**).* From 19 (458 mg, 0.98 mmol) in 10 mL of MeOH and 13.2 mL (146.7 mmol) of methyl acrylate **20** was obtained as a yellow oil with 62%. Reaction time: 16 h. ^{1}H-NMR ($CDCl_3$): δ 2.45 (t, 12H, $6CH_2$, $J = 6.6$Hz), 2.54 (t, 4H,$2CH_2$, $J = 6.0$ Hz), 2.77 (t, 8H, $4CH_2$, $J = 6.6$ Hz), 2.83 (t, 2H, CH_2, NCH_2CH_2NCO, $J = 7.7$ Hz), 2.96 (t, 4H, $2CH_2$, $J = 6.6$ Hz), 3.27 (c, 4H, $2CH_2$, $CH_2NHCOCH_2$, $J = 6.0$ Hz), 3.65 (s, 12H, 4MeO), 4.28 (t, 2H, CH_2, NCH_2CH_2NCO, $J = 7.14$ Hz), 7.12 (t, 2H, 2NH), 7.76 (t, 2H, Har, $J = 7.1$ Hz), 8.23 (d, 2H, Har, $J = 7.7$ Hz), 8.57 (d, 2H, Har, $J = 6.6$ Hz). ^{13}C-NMR ($CDCl_3$): δ 172.7, 171.9, 163.7, 133.8, 131.3, 130.8, 127.7, 126.7, 122.2, 52.7, 51.3, 50.1, 50.0, 48.9, 37.7, 36.8, 33.6, 32.4. IR (neat): 3300, 1730, 1650, 1630 cm^{-1}. Anal. ($C_{40}H_{56}N_6O_{12}$) C, H, N.

*6.1.7.3. 8-cascade: N-(2-aminoethyl)-1,8-naphthalimide [2]–(1,4 - diaza - 5 - oxoheptylidine)^2–methylpropanoate (**22**).* From **21** (255 mg, 0.27 mmol) in 20 mL of MeOH and 7.2 mL (81 mmol) of methyl acrylate **22** was obtained as a yellow oil with 67%. Reaction time: 48 h. ^{1}H-NMR ($CDCl_3$): δ 2.38–2.44 (m, 32H, $16CH_2$), 2.49–2.54 (m, 8H, $4CH_2$), 2.56 (bs, 2H, CH_2), 2.74 (t, 16H, $8CH_2$, $J = 6.6$ Hz), 2.79–2.85 (m, 8H, $4CH_2$), 2.93 (t, 4H, $2CH_2$, $J = 6.6$ Hz), 3.29 (c, 12H, 6 CH_2, $J = 5.5$ Hz), 3.66 (s, 24H, 3OMe), 4.28 (t, 2H, NCH_2CH_2NCO, $J = 7.1$ Hz), 7.07 (m, 4H, NHCO), 7.69 (bs, 2H, NHCO), 7.77 (t, 2H, Har, $J = 7.1$ Hz), 8.23 (d, 2H, Har, $J = 8.2$ Hz), 8.58 (d, 2H, Har, $J = 7.7$ Hz). ^{13}C-

NMR ($CDCl_3$): δ 173.0, 172.3, 172.2, 164.0, 134.0, 131.6, 128.1, 127.0, 122.5, 52.8, 52.5, 51.6, 50.2, 49.9, 49.2, 37.1, 33.7, 32.6, 29.6. IR (neat): 3300, 1700, 1680 cm^{-1}. Anal. ($C_{76}H_{120}N_{14}O_{24}$) C, H, N.

6.1.8. General procedure for amonolysis of polyesters ***19***, ***21***, ***23***

Ethylenediamine (54 equiv. per terminal ester group) was added dropwise to a solution of the polyester in MeOH and the mixture was stirred at r.t. The excess of ethylenediamine and MeOH were eliminated under reduced pressure. The crude was purified by chromatography using $CHCl_3$–MeOH 10:0.5 to obtain the polyamide as an oil.

*6.1.8.1. N-[2-(N,N-Bis(2-methoxycarbonylethyl)amine)-ethyl]-1,8 naphthalimide (**19**).* From **18** (1.38 g, 3.32 mmol) in 30 mL of MeOH and 244 mL (364.8 mmol) of ethylenediamine was obtained **19** (900 mg) with 58% as a yellow oil. Reaction time: 24 h. ^{1}H-NMR ($CDCl_3$): δ 1.75 (bs, 4H, NH_2), 2.45 (t, $2CH_2$, $J = 6.0$ Hz), 2.76–2.83 (m, 6H, $3CH_2$), 2.89 (t, 4H, $2CH_2$, $J = 6.0$ Hz), 3.25 (c, 4H, $2CH_2$, $CH_2NHCOCH_2$, $J = 6.06$ Hz), 4.26 (t, 2H, CH_2, NCH_2CH_2NCO, $J = 7.1$ Hz), 7.32 (t, 2H, 2NH), 7.77 (t, 2H, Har, $J = 7.7$ Hz), 8.23 (d, 2H, Har, $J = 7.7$ Hz), 8.59 (d, 2H, Har, $J = 7.1$ Hz). ^{13}C-NMR ($CDCl_3$): δ 172.7, 164.4, 134.4, 131.6, 131.4, 128.1, 127.0, 122.3, 51.0, 50.4, 42.1, 41.5, 38.5, 34.5. IR (neat): 3500, 1700, 1650 cm^{-1}. Anal. ($C_{24}H_{32}N_6O_4$) C, H, N.

*6.1.8.2. 4-Cascade–N-(2-aminoethyl)-1,8-naphthalimide [2]–1,4-diaza-5-oxoheptylidine–3-aza-4-oxohexanamine (**21**).* From **20** (338 mg, 0.42 mmol) in 10 mL of MeOH and 6.06 mL (90.7 mmol) of ethylenediamine was obtained **21** with 60% as a yellow oil. Reaction time: 3 days. ^{1}H-NMR (D_2O): δ 2.18–2.23 (m, 12H, $2CH_2 + 4NH_2$), 2.30 (c, 4H, $2CH_2$, $J = 6.0$ Hz), 2.50–2.54 (m, 8H, $4CH_2$), 2.63–2.67 (m, 4H, $2CH_2$), 2.89–2.82 (m, 20H, $10CH_2$), 3.11 (t, 2H, CH_2, $J = 6.0$ Hz), 3.22 (t, 8H, $4CH_2$, $J = 5.5$ Hz), 3.74 (m, 2H, CH_2), 7.35–7.42 (m, 2H, Har), 7.85–7.93 (m, 4H, Har). ^{13}C-NMR (D_2O): δ 178.0, 177.4, 167.4, 137.8, 134.1, 133.4, 129.8, 129.3, 123.0, 51.8, 51.4, 42.9, 42.2, 41.8, 41.4, 40.6, 40.5, 38.9, 35.0. IR (neat): 3300, 1700, 1650 cm^{-1}. Anal. ($C_{44}H_{72}N_{14}O_8$) C, H, N.

*6.1.8.3. 4-Cascade–N-(2-aminoethyl)-1,8-naphthalimide [2]–(1,4-diaza-5-oxoheptylidine)^2–3-aza-4-oxohexanamine (**23**).* From **22** (42 mg, 0.026 mmol) in 30 mL of MeOH and 0.75 mL (11.3 mmol) of ethylenediamine was obtained **23** (900 mg) 40% as a yellow oil. Reaction time: 4 days. ^{1}H-NMR (CD_3OH): δ 2.22 (bs, 28H, $14CH_2$), 2.42 (bs, 16H, $8CH_2$, CH_2NH_2), 2.69 (t, 28H, $14CH_2$, $J = 6.0$ Hz), 3.25 (t, 42H, $21CH_2$, $J = 6.0$ Hz), 4.01 (bs, 2H, CH_2NCO), 7.68 (bs, 2H, Har), 8.23 (bs, 2H, Har), 8.42 (bs, 2H, Har). ^{13}C-NMR (CD_3OH): δ 177.6, 176.8, 176.0, 166.4, 131.0, 126.2, 122.7, 118.8, 59.3, 43.8, 41.7, 41.5, 37.2, 31.8, 30.7. IR (neat): 3300, 1700, 1650 cm^{-1}. Anal. ($C_{84}H_{152}N_{30}O_{16}$) C, H, N.

6.2. Pharmacology

6.2.1. Materials and methods

6.2.1.1. Cytotoxicity assay in HT-29 cells. The cytotoxicity of synthesised conjugates was measured using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The human carcinoma cell line, HT-29, was obtained from the American Type Culture Collection and cultured in the recommended media. Exponentially growing cells were plated at 3000-well into 96-well plates in 150 μL complete DMEM media containing 10% fetal bovine serum (FBS). Cells were allowed to attach for 24 h before the addition of a serial (1:4) dilution of the drug in 50 μL of fresh medium. After 72 h of incubation at 37 °C (5% CO_2), MTT was added to each well and the plates incubated for 4 h. Formazan crystals formed by MTT metabolism were solubilised by the addition of 50 μL of 25% SDS (pH 2) to each well and incubated overnight. The cellular metabolism of MTT was then quantified by reading the absorbance of the solubilised product at 550 nm with a 96-well plate reader, IC_{50} values were calculated as the concentration of drug inhibiting cell growth to 50% of controls.

6.2.1.2. ELISA for determination of Lck activity. Human recombinant Lck catalytic domain was produced in a baculovirus expression system. Purified protein was used. The susbstrate Poly(Glu, Tyr) 4:1 for ELISA plate coating was diluted to 1 mg mL^{-1} in phosphate-buffered saline (PBS) containing 0.02% Na azide; 100 μL per well of this polymer solution was used to coat 96-well microtiter ELISA plates. Plates were covered and incubated overnight at 4 °C. Immediately prior to use, plates were washed four times using TBS–Tween 20 (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20). The enzyme mix was prepared immediately prior to use in a kinase buffer. The final concentrations of components in the Lck kinase assay were 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ 5 μM ATP and 1 ng of Lck enzyme. Fifty microlitres of kinase buffer containing Me_2SO was added to each well and inhibitor dilutions were carried out. Compounds were dissolved in Me_2SO, giving final concentrations of 5%. Positive and negative controls contained the appropiate Me_2SO concentration. Negative controls contained 10 μL of 0.5 M EDTA, which was added prior to the addition of the enzyme. All assays were carried out in duplicate. The reaction was started by the addition of 50 μL of kinase buffer containing diluted enzyme to each well. Plates

were mixed and incubated for 10 min at 22 °C. Reactions were terminated by washing the ELISA plates four times with TBS–Tween. Washed plates were blocked with 3% BSA–TBS–Tween (100 mL per well) for 45 min and then washed four times with TBS–Tween; 100 mL of anti-phosphotyrosine antibody was added per well (diluted to 0.1 μg in 3% BSA–TBS–Tween) and incubated for 45 min at r.t. Plates were washed four times with TBS–Tween. HRP-conjugated goat *anti*-mouse IgG was added to wells and bated for 45 min. ELISA plates were finally washed with TBS–Tween and then with TBS and developed by the addition of 100 mL of HRP substrate reagent per well. The resulting absorbance was read at 415 nm in an ELISA autoreader. IC_{50} values were defined as the drug concentration that resulted in 50% inhibition of enzyme activity.

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