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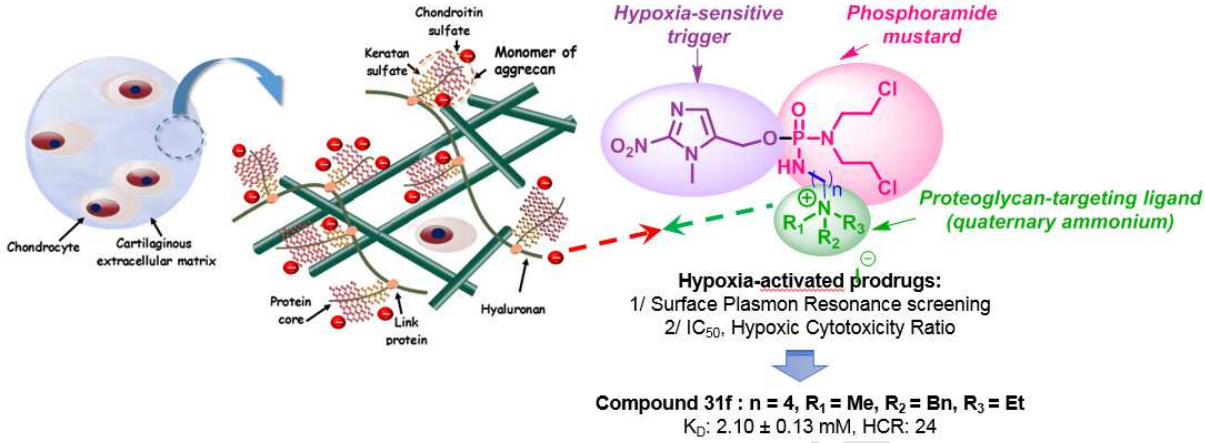
Graphical abstract

Hypoxia-activated prodrug with a quaternary ammonium function as proteoglycan-targeting ligand for a better management of chondrosarcoma: synthesis, Surface Plasmon Resonance screening, *in vitro* hypoxic selectivity and reductive activation.



Structure-activity relationship study of Hypoxia-Activated Prodrugs for Proteoglycan-targeted chemotherapy in chondrosarcoma

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Abstract

Due to an abundant chondrogenic, poorly vascularized and particularly hypoxic extracellular matrix, chondrosarcoma, a malignant cartilaginous tumour, is chemo- and radio-resistant. Surgical resection with wide margins remains the mainstay of treatment. To address the lack of therapy, our strategy aims to increase anticancer drugs targeting and delivery in the tumour, by leveraging specific chondrosarcoma hallmarks: an extensive cartilaginous extracellular matrix, namely the high negative fixed charge density and severe chronic hypoxia. A dual targeted therapy for chondrosarcoma was investigated by conjugation of a hypoxia-activated prodrug (HAP) to quaternary ammonium (QA) functions which exhibit a high affinity for polyanionic sites of proteoglycans (PGs), the major components of the chondrosarcoma extracellular matrix. Based on preclinical results, an imidazole prodrug, **ICF05016**, was identified and provided the basis for a lead optimization study. A series of 27 QA-phosphoramide mustard conjugates, differing by the type of QA function and the length of the alkyl linker, was yielded by a common multi-step sequence involving phosphorylation of a key 2-nitroimidazole alcohol. Then, a screening was realized by surface plasmon resonance technology to assess biomolecular interactions between QA derivatives and aggrecan, the most abundant PG in chondrosarcoma. Results revealed that affinity depends more on the type of QA function, than on the linker length. Moreover, the presence of a benzyle group enhanced affinity to aggrecan. Twelve compounds were shortlisted and evaluated for antiproliferative activity (*i.e.*, growth inhibiting concentration 50), under normoxic and hypoxic conditions using the human extraskeletal myeloid chondrosarcoma cell line (HEMC-SS). For all prodrugs, hypoxic selectivity was maintained and even increased, compared with the lead**.** From this study, compound **31f** emerged as the most effective PG-targeted HAPs with a dissociation constant of 2.10 µM in the SPR experiment, a hypoxia cytotoxicity ratio of 24 and an efficient reductive cleavage under chemical and enzymatic conditions.

1. Introduction

Chondrosarcomas represent a heterogeneous group of rare and slowly-growing types of bone sarcomas sharing common features such as their mesenchymal origin and the presence of a cartilaginous extracellular matrix. Among all primary malignant bone sarcomas, chondrosarcoma is the second most common after osteosarcoma, but the first in adults.[1] To date, the therapeutic tools available for the management of the disease are very few.[2,3] Surgical resection with wide margins in surrounding non-tumour tissue is still considered the mainstay of treatement and the only recommended therapy for non-metastatic chondrosarcoma, all grades and subtypes included. Well-conducted surgery has been reported to correlate with better control of the disease, *i.e.* lower frequency of local recurrence, and improved overall survival. Nevertheless, wide margin excision is not always possible (in some anatomic locations) or could entail a significant morbidity for the patient.[2,4–6] Also, the current clinical challenge is to prevent recurrences and to offer new treatment options for patients with inoperable primary, recurrent disease, and/or metastases. As regards to radiotherapy and conventional chemotherapy, chondrosarcoma is known as a resistant lesion[7–9] due to tumour's specific hallmarks. Like hyaline cartilage, tumour tissue is marked by a dense and extensive extracellular matrix with poor blood and lymph vascularity, on which a low percentage of dividing cells is embedded. The extracellular matrix creates a physical semi-permeable barrier, which prevents cytotoxic agents reaching their target, *i.e* tumour chondrocytes, while reduced blood circulation creates severe chronic hypoxia.[10] The latter is also well known to be associated with radio- and chemo-resistance in cancer.[11–13]

Given the lack of significant improvement in terms of patient survival over the past three decades, new therapeutic targets, such as isocitrate dehydrogenase, m-TOR and Hedgehog pathway inhibitors have been investigated and are currently used in rational drug design for the development of new targeted therapies[14]. The challenges of translating these drugs into clinical use unfortunately failed and no substance has been approved so far[15,16].

In clinic, commonly used chemotherapies currently target either the highly proliferative nature of cancer cells, or specific receptors that are upregulated in cancer but often result in side effects leading to potential toxicity, and/or causing treatment failure in resistant

populations. A new promising option for improving cancer therapies appeared, based on targeting the tumour microenvironnement[17,18]. For chondrosarcoma, two physical and chemical hallmarks of the tumour could also be considered as therapeutic opportunities rather than barriers, namely hypoxia and the hyaline extracellular matrix, more precisely, its high proteoglycan (PG) content.

For more than five decades, hypoxia is well recognized as a key feature in cancer progression[11]. Hypoxia leads to low oxygen concentration areas and then to the emergence of tumour cell subpopulations with highly aggressive phenotypes, including increased drug-resistance and amplified anti-apoptotic and metastatic potential. The differences between cells in well-oxygenated normal tissue and hypoxic neoplastic cells were put to use in the development of bioreductive prodrugs activated preferentially in hypoxic regions to specifically release active cytotoxic species.[19–23] This approach emerged more than three decades ago, with tirapazamine being the first hypoxia-activated prodrug (HAP) to undergo clinical trial. However, while multiple different bioreductive prodrugs demonstrating antitumour cytotoxicity against hypoxic cells, like AQ4N, apaziquone, SN30000, evofosfamide (TH-302), PR-104, etc. have been described in literature, none are so far available in the clinical setting. HAPs have in common chemical moities used as a “trigger” or “oxygen concentration sensor” ensuring activation of the prodrug through a bioreduction process *via* one or two electrons cellular oxidoreductases. Five different chemical groups, i.e. nitroaromatics, aromatic *N*-oxides, aliphatic *N*-oxides, quinones and transition metals, are reported in literature as triggers[19–21]. After the reduction step, the link between the trigger and the anti-cancer entity is cleaved and converts the non-toxic prodrug into a toxic molecule achieving hypoxia-selective killing associated with a bystander effect. By means of HAP, the therapeutic window could be extended and higher doses could be administered, providing therefore greater therapeutic potential.

In chondrosarcoma, HAP activity could be hampered by inefficient physical distribution to tumour chondrocytes due to the cartilaginous matrix barrier. Using the physical and chemical properties of the extensive cartilaginous extracellular matrix of chondrosarcoma, namely the high negative fixed charge density created by the presence of PGs, may be an attractive way of adding to these HAP therapies targeted-drug delivery. In effect, PGs are glycoproteins highly

expressed in the chondrosarcoma extracellular matrix[24], with numerous sulphate and carboxylate groups on their glycosaminoglycan moieties. This extracellular PG-targeting approach using a quaternary ammonium (QA) function was previously validated in chondrosarcoma preclinical models, with radiotracers for scintigraphic imaging[25,26] or DNA-alkylating agents for therapy.[27,28] More recently, this dual targeting therapy for chondrosarcoma was investigated and led to the identification of the first PG-targeting HAP, 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1H-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*,*N*,*N*-trimethyl-propane-1-aminium iodide, named **ICF05016**[29] (Table 1). The latter is a nitroheteroaryl-based compound designed as follows: a phosphoramidic mustard functionalized with a QA function, and a 2-nitroimidazole group tethered to the phosphorous atom to trigger fragmentation and then release the bis-alkylating mustard anion by bioreduction in hypoxic tissues. Aggrecan binding of **ICF05016** was confirmed from the dissociation constant determined by surface plasmon resonance (SPR), as well as hypoxic-cytotoxicity ratio (HCR) on the HEMC-SS cell line. In a HEMC-SS tumour-bearing mice model, treatment with **ICF05016** provides a significant inhibition of tumour growth with no associated haematological side effects, a decrease in the mitotic index and increased DNA damage predominantly found in pimonidazole-positive hypoxic areas.

Based on these results, we intend to begin a lead optimization study of **ICF05016**. Herein, we report the synthesis and characterization of a series of QA-targeted phosphoramide mustard conjugates **28-32** differing by the nature of the QA function and the length of the alkyl linker binding the QA to the mustard (Table 1). Then, we screened by SPR their direct binding ability to aggrecan. Short-listed compounds were evaluated in terms of HCR in the HEMC-SS cell line to provide more knowledge on the structure-activity relationships existing between the nature of the linker and the *in vitro* cytotoxicity of these QA-targeted HAPs. Finally, stability in both phosphate buffer and plasma, activation and phosphoramide mustard release under reductive chemical conditions, and nitroreductase-based activation were demonstrated for the most potent derivative.

2. Results and discussion

2.1. Chemistry

Target compounds **28-32** were obtained by quaternisation of the tertiary amine function of compounds **23-27** *via* a two-step reaction sequence: phosphorylation of the key hydroxymethyl 2-nitroimidazole intermediate **6** by phosphoramidic dichloride **8**, followed by the introduction of the QA alkyl chain by substitution of the remaining chloride by a series of diamines **18-22** (Scheme 1). The latter were prepared according to a Gabriel synthesis protocol starting from the appropriate alkyl halide. Briefly, *N*-bromoalkylphthalimides **9-12** with alkyl chain ranging from two to five carbons were produced in moderate to excellent yields (43-98%) by alkylation of commercial potassium phthalimide with the corresponding dibromoalcane following a slightly modified procedure of the method described by Hu et *al.*[30]. Nucleophilic substitution of derivatives **9-12** in acetonitrile with dimethyl-, diethyl-, diisopropyl- or benzylmethylamines afforded intermediates **13-17**[31,32] . Hydrazinolysis of the latters under reflux afforded directly *N*-benzyl-*N*-alkyldiamines **21a-d** and 3-(piperidin-1-yl)propanamine **22** in good to excellent yields (76 to 98%), whereas short-path distillation was required for *N,N*-dialkyldiamines **18-20** leading to moderate yields (22 to 61%). To note, elimination of the Gabriel phthalhydrazide by-product was insured by a slightly modified work up protocol compared to the litterature[31], namely a twice acidic precipitation at low temperature. Following this general three-steps procedure, the twelve diamines **18a-b**, **19a-b**, **20a-c**, **21a-d** and **22** were obtained from potassium phthalimide in overall yields ranging from 15 to 77 %.

The (1-methyl-2-nitro-*1H*-imidazol-5-yl)methanol (**6**) is the key intermediate for synthesis of our QA-targeted HAPs. The 1-methyl-2-nitroimidazole group was chosen as common trigger for the designed molecules. Among bioreductive nitroaryl-triggers used in HAPs, like the 4-nitrobenzyl group, nitro-imidazole-, nitrofuran- and nitrothiophene-based groups, the 1-methyl-2-nitroimidazole group has been used the most widely, generally showing the best tendency to undergo bioreduction and, consecutively selective cleavage at low oxygen concentrations. Hence, a 1-methyl-2-nitroimidazole group was embedded in the well-investigated evofosfamide, a HAP of bromo-isophosphoramide mustard reaching phase III

clinical trials against soft tissue sarcoma[33] and pancreatic cancer (MAESTRO study)[34,35]. Evofosfamide appeared for the first time in a series of nitroaromatics including nitrothiophenes and nitrofuranes, developed by Duan *et al.* in 2008[36] and emerged as the most potent HAP of the series.

The synthesis of (1-methyl-2-nitro-*1H*-imidazol-5-yl)methanol (**6**) was built on the work published by Cavalleri *et al*.[37] and patented by Matteucci *et al.*[38], starting from *N*-methylglycine (**1**) and improved later by O'Connor *et al.*[39,40]. Synthesis of 1-methyl-2-nitroimidazole derivatives appears as a challenging and fastidious task, as recently reviewed. O'Connor *et al.* published in 2016[40] a careful and detailed description of the experimental protocol with emphasis on critical steps, precautions to be taken and the optimal reaction times for each step. More precisely, it turned out that the synthesis of the intermediate amino ester **4** represents the most critical step, associated with the lowest yields (ranging from 10-25% according to the Matteuci *et al.* patent to 50 % in the O'Connor protocol).

According to the procedure outlined in Scheme 1, *N*-methylglycine was first converted to the corresponding ethyl ester hydrochloride salt, which in turn was *N*-formylated by ethyl formate in the presence of potassium carbonate. Then, a one-pot three-steps procedure was applied to yield the 2-aminoimidazole **4**: *(i) C*-formylation of the amino ester **3** at the α-carbon by treatment with sodium hydride and ethylformate, *(ii)* removal of the unwanted *N*-formyl group under acidic conditions at reflux, and *(iii)* subsequent Marckwald cyclisation with cyanamide under reflux at pH 3 as suggested by O'Connor *et al.*[39] to minimise ester hydrolysis. This sequence finally provided the desired 2-aminoimidazole **4** in good yields (53-61 % compared to the previous 50 % yield in the O'Connor protocol[39,40]). Then, diazotization of the amino group in the presence of excess sodium nitrite afforded nitroester **5** in a 75% yield. Finally, reduction of the ester function with $LiBH_4$ formed *in situ via* the metathesis reaction between $NaBH_4$ and LiBr, allowed to obtain compound **6** in 68% yield. $LiBH_4$ was reported as a selective reducing agent for esters, with reducing power greatly enhanced by utilizing ether-type solvents containing methanol[41]. In our procedure, a mixture of $LiBH_4$-THF-MeOH was used with a strict control of the reaction medium temperature (below 10°C) in order to avoid the reduction of the nitro function.

Tertiary amine derivatives **23-27** were synthesized *via* a one-pot reaction according to a modified procedure of Hernick *et al.*[42] Synthesis began with the phosphorylation of the 2-nitroimidazole key intermediate **6** at -78 °C by sequential treatment with lithium bis(trimethylsilyl)amide and the electrophile phosphorylating agent **8** (prepared according to a literature procedure[43], on a 10 g scale from commercially available *N*,*N*-bis(2-chloroethyl)amine hydrochloride salt (**7**)). Then, addition of the appropriate diamine **18-22** ensured the formation of the corresponding phosphoramide **23**-**27**. For accurate management of this one-pot procedure, the progress of the reaction was monitored using ^{31}P NMR, initiated at addition of the *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride (**8**). When nearly complete disappearance of dichloride **8** (used in slight excess) concurrent with the formation of the phosphoramidic monochloride intermediate was observed, the appropriate amine was added to obtain the desired compounds **23-27**. For example, ^{31}P NMR monitoring for compound **24d** is depicted in Figure 1: (1) at T_0, two signals at 15.59 and 15.17 ppm appeared, relative to dichloride **8** and monochloride intermediate formed respectively; (2) after 15 min, the signal at 15.59 ppm had almost disappeared and 2-(*N*,*N*-diethylamino)ethylamine was added; (3) only 10 min later, the intermediate was consumed giving rise to compound **24d** with a signal at 17.24 ppm and an impurity at 18.97 ppm. It should be noted that this phosphorylation step was unpredictable and not always reliable, requiring a ^{31}P NMR monitoring to control the time of consumption of dichloride **8**, ranging from 5 to 80 min in different experiments. We also observed that the best results, in terms of kinetic and conversion rate, were obtained when the solution of dichlorophosphoramidate **8** in THF was pre-cooled at -78 °C and added at once in order to avoid the undesired bis-addition of alcohol **6**. We finally noted that the solubility of 2-nitroimidazole **6** in THF at -78 °C was a critical factor, partial solubility correlating with longer times and lower-yielding phosphorylation.

Quaternization of the series of tertiary amines **23-25**, **27** was achieved using alkyl iodide (methyl or ethyl iodide), leading to the corresponding ammonium derivatives **28-30**, **32.** For compounds **31a-h**, two synthesis pathways have been considered, i.e. direct methylation or ethylation of benzyl compounds **26** or benzylation of dialkyl derivatives **23** and **24**. In order to establish the best route, QA derivative **31c** was synthesized from **26c** by treatment with methyl

iodide or from **23c** by alkylation with freshly prepared benzyl iodide[44]. In the latter case, a supplementary work-up of the crude product was necessary in order to remove excess benzyle iodide, affording the desired product in a lower yield compared with the methyl iodide route (30% from **23c** *versus* 93% from **26c**). Thus, alkyl iodides (methyl or ethyl) were used for the preparation of all ammonium derivatives bearing a benzyl group within the series. It should be emphasized that the reaction took place in different ways within the series, in terms of reaction time, number of alkyl iodide equivalents required and purity status of the obtained crude product. Most QA compounds were obtained in excellent yields (mostly > 80 %), with a satisfactory purity, only after evaporation of the excess methyl or ethyl iodide. Analytical purity was evaluated by the absence of impurities revealed in the ^{1}H and ^{13}C spectra combined with ^{31}P impurities estimated at less than 5 % in global integrals. However, for five compounds (i.e. **28g**, **29g**, **29h**, **31g** and **31h**) belonging to the n = 2 or 3 series, ^{31}P spectra analyses revealed the presence of an impurity after ethylation emphasized by a signal at 24-25 ppm (in CD_3OD), and ranging from 6 to 15% for **28g**, **29g**, **29h**, **31g**, and 87% for **31h**. Purification of these QA compounds by preparative high performance liquid chromatography (C_{18} column, gradient acetonitrile in water) with subsequent lyophilisation was thus required to afford analyticaly pure compounds **28g**, **29g**, **29h** and **31g**. To better understand this side reaction, the impurity from the synthesis of compound **29h** (signal at 24.35 ppm in ^{31}P spectrum) was isolated for structure elucidation. HRMS data showed molecular ion at m/z 451.1984, which could correspond to the loss of a neutral HCl (36 amu). A comprehensive spectral analysis based on HRMS and RMN (^{1}H, ^{13}C, COSY, HSQC, ^{31}P) was in favour of the formation of the corresponding 2-oxide-diazaphospholidine obtained by intramolecular 1,5-*N*-alkylation of compound **29h**. For synthesis of compound **31h**, total conversion of **21d** required 5 days' reaction in the presence of a considerable excess of ethyl iodide (100 eq.) and led to the 2-oxide-diazaphospholidine impurity as the major product (> 80 % highlighted by ^{31}P spectrum) preventing obtention of **31h** in sufficient yield to allow biological assessment. This unwanted intramolecular 1,5-*N*-alkylation seems to be correlated with the length of the alkyl chain and/or steric hindrance of the tertiary amine substituents which hampered quaternization with ethyl iodide, requiring longer reaction time and greater excess of ethyl iodide.

2.2. Biology

The interaction between synthesized QA-targeted prodrugs **28-32** and aggrecan was evaluated by Surface plasmon resonance (SPR) analyses. SPR technology has emerged as a powerful technique to investigate the interaction between low-molecular-weight molecules and target proteins and also as an effective tool for drug screening.[45–47] Aggrecan (≈ 250 kD), with approximately 150 glycosaminoglycan side chains of chondroitin sulphate and keratan sulphate, is the most abundant PG in chondrosarcoma.[24,48] A preliminary screening of the different prodrugs was performed by binding level analysis (Figure 2A). Compound **ICF05016** was used as positive control and its non-QA equivalent (*O*-[(1-methyl-2-nitro-1*H*-imidazol-5-yl)methyl]-*N,N*-bis(2-chloroethyl)-*N'*-propylphosphorodiamidate named **ICF05017**[29]) as negative control for aggrecan binding measurements. The hit selection by initial screening of all synthesized QA-HAP was based on at least equivalent relative binding to aggrecan (Response Units) compared with **ICF05016**.

Some structure-activity relationships appeared in terms of interaction with aggrecan: affinity seemed to be related more to the type of the QA function, rather than the length of the alkyl chain between the QA function and the nitrogen mustard, since the twelve short-listed compounds were of various chain lengths (n = 2, 3, 4, 5). Concerning QA substituents, the first emerging conclusion was that the presence of a benzyl group enhanced the affinity to aggrecan. In fact, all synthesized compounds bearing a benzyl moiety showed a higher binding affinity than the lead compound (almost two-fold difference in relative binding, except for compounds **31f** and **31c**) with the best affinity obtained for analogue **31a**, characterized by a pentyl chain and a benzyl-dimethyl QA function. Improved binding affinity to aggrecan was also observed with derivatives with ethyl substituents on the QA function. None isopropyl derivatives were short-listed, suggesting that steric hindrance perhaps hampers the inter-molecular recognition process between the isopropyl QA function and aggrecan.

Based on this selection criterion, twelve hits with Response Units over 24 were further evaluated to determine their dissociation constants (K_D) in a concentration-dependent secondary experiment (Figure 2B). This analysis yielded K_D values ranging from 2.10 ± 0.13 to 5.03 ± 0.21 mM (Table 1) for all selected prodrugs, which demonstrates fairly similar affinity for aggrecan as **ICF05016**.

The selected prodrugs were evaluated for antiproliferative activity (i.e., Growth Inhibiting Concentration 50: IC_{50}) respectively to **ICF05016** and evofosfamide, under normoxic and hypoxic conditions using the human extraskeletal myeloid chondrosarcoma cell line (HEMC-SS). The IC_{50} values for all tested compounds are given in Table 2 and confirmed their relevance as hypoxia-activated prodrugs. Based on the inhibition of proliferation, almost all compounds were more potent than **ICF05016**, with a higher activity observed in hypoxia compared to normoxia. Hypoxic-cytotoxic ratio (HCR, IC_{50} under normoxia *vs.* IC_{50} under hypoxia) was maintained and even increased, compared with the HCR of 7 obtained for **ICF05016**. Substitution of methyl groups by ethyl on the QA function or modification of the alkyl chain length did not lead to significant change in potency or in hypoxic selectivity of the prodrugs, as compared with **ICF05016**. However, substitution of one methyl by a benzyl group appears more effective in terms of hypoxic selectivity, as for all benzylated prodrugs (**31a-g**) selectivity was maintained and even increased of 2-fold to 3-fold for three compounds, mainly for compound **31f**, emerging as the most effective (HCR = 24). Compound **31f** exhibited selectivity for hypoxia similar to evofosfamide with a HCR of 23 in HEMC-SS cells. However, the potency of evofosfamide on HEMC-SS cells in normoxia was higher (IC_{50} = 2.9 ± 1.5 µM) compared to compound **31f** (IC_{50} = 40.7 ± 8.8 µM). This cytotoxicity could be attributed to the superoxide species, generated under normoxia and is time-dependent with longer drug exposures as previously reported[49]. This may lead to toxicity in healthy cells, which could be expected to occur to a lesser extent with compound **31f**.

2.3. Chemical Stability in PBS and plasma

A stability evaluation, carried out in phosphate buffer solution (10 mM, pH 7.4, 0.13% DMSO) on compound **31f** at 37 °C and monitored by reversed-phase HPLC, demonstrated promising results. Compound **31f** remains structurally intact under these conditions with no significant changes over a 24 h period (< 5%). Stability in rabbit plasma at 37 °C was also demonstrated by ^{31}P NMR experiments, as no cleavage or degradation over a 24 h period was observed (Figure 3B).

2.3. Reductive Chemical Activation

A ^{31}P NMR experiment was carried out on compound **31f** under model physiological conditions (pH 7.4, 37 °C) to ascertain that initial reductive activation of the phosphoramidate prodrug **31f** leds to the rapid formation of the corresponding phosphoramidate anion, hypothesized as being the active cytotoxic species. Prodrug **31f** was proved to be stable in these conditions over a period of 24 h. The phosphoramidate prodrug **31f** was chemically reduced with 3 equivalents of sodium dithionite (0.1 M cacodylate buffer, pH 7.4), a reductant system used to mimick bioreduction in hypoxic tissue. Figure 3 is an example of a ^{31}P NMR stack plot showing the results from the dithionite reduction of **31f**. As expected, the resonance corresponding to prodrug **31f** at 17.99 ppm rapidly disappears (half-life < 2 min) with the concurrent appearance of the resonance at 12.39 ppm, which corresponds to the ^{31}P signal of the substituted bis(2-chloroethyl)phosphoramidate anion. The phosphoramidate anion is a highly reactive specie and give raised to the formation of a variety of by-products arising both from subsequent solvolysis reactions (bisulfite and water substitution) and cleavages of P-N bonds[50,51]. At least 90% conversion of prodrug **31f** occured in less than 8 min at 37 °C under chemical reductive conditions, efficiently leading to the release of the bis-alkylating mustard anion under hypoxia mimic conditions (Figure 3B).

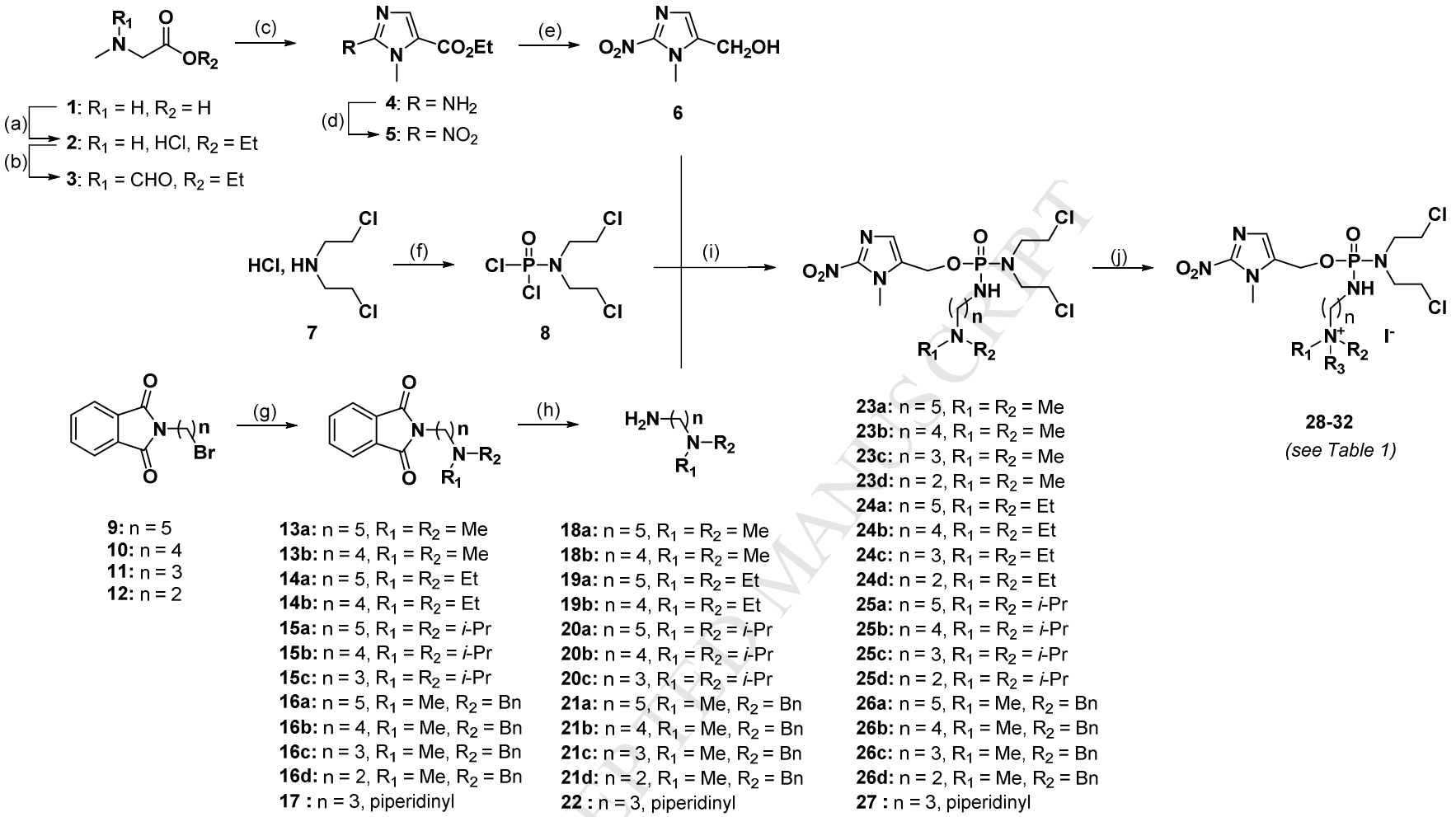
2.3. Bioreductive activation via nitroreductase

Prodrug **31f** was evaluated as substrate of an oxygen-insensitive nitroreductase from *E. coli*, to verify activation following an enzymatic reduction. This enzyme, which involves a two electrons reduction process, provides an experimental setting not affected by the environmental oxygen level. This offers the great advantage to bypass the oxygen-sensitive

prodrug radical, which is reoxidised to the original prodrug in the presence of oxygen and thus hampered the phosphoramidate anion release, as observed for nitroaromatic HAPs. The activation was evaluated by incubating compound **31f** in phosphate buffer solution (10 mM, pH 7.4) at 37 °C with nitroreductase in the presence of NADPH as the cofactor. This latter doesn't induce enzyme-free reaction, as demonstrated by HPLC analysis. Aliquots were withdrawn at various time points and analyzed by reversed-phase HPLC. The half-lives were estimated on the basis of the disappearance of the prodrug at two nitroreductase concentrations (15 and 25 µg/mL) (Figure 4). For compound **31f**, the both estimated half-lives were below 1 min *vs* 5 and 15 min respectively for the lead ICF05016, used as a control in our experiment. Prodrugs **31f** was substrate for nitroreductase and was readily reduced with at least 90 % conversion after 3-5 min, showing proof of principle for this HAP strategy.

3. Conclusions

The aim of this work was to synthesize a series of QA-HAP conjugates differing by the nature of the PG-targeted QA function for effective chemotherapy of chondrosarcoma, a hypoxic and PG-rich tumour. The present study revealed that all prodrugs led to overall improvement in hypoxic selectivity compared with **ICF05016** and highlights the positive impact of a benzyl QA function on affinity to aggrecan, the most abundant PG in chondrosarcoma. Because of its attractive hypoxic selectivity (HCR = 24) and cytotoxicity in hypoxia compared to that of lead **ICF05016**, coupled with rapid *in vitro* reductive activation, compound **31f** emerged as the most effective HAP and will undergo further preclinical studies, including *in vivo* efficacy on the HEMC-SS xenograft model on mice.



Scheme 1 : Reagents and conditions : (a) EtOH, $SOCl_2$, -10 °C ,then 55 °C, overnight, **97%**; (b) ethyl formate, K_2CO_3, EtOH, rt, overnight, **85%**; (c) (1) ethylformate, NaH, THF anh., 0 °C, rt, overnight, (2) HCl 37%, EtOH, 110 °C, 2 h, (3) NH_2CN, EtOH/H_2O, pH 3, reflux, 1.5 h, **61%**; (d) $NaNO_2$, AcOH, -5 °C, then rt, overnight, **75%**; (e) $NaBH_4$, LiBr, THF/MeOH/H_2O, <10 °C, rt, 19 h, **68%**; (f) $POCl_3$, 130-140 °C, 80 h, **57%**; (g) HNR_1R_2, K_2CO_3, ACN or THF, reflux, 24 h, **35-93**%; (h) hydrazine hydrate (60% aqueous solution), EtOH, reflux, 12 h, **22-99%**; (i) (1) $LiN(TMS)_2$, THF anh., **8**, -78 °C, 15-80 min (duration determined by ^{31}P NMR monitoring); (2) amines **18-22** or commercial amines, THF anh., -78 °C, 5-75 min (duration determined by ^{31}P NMR monitoring), **22-78%**; (j) alkyl iodide, K_2CO_3, ACN anh., rt, 4-96 h, **60-100%.**

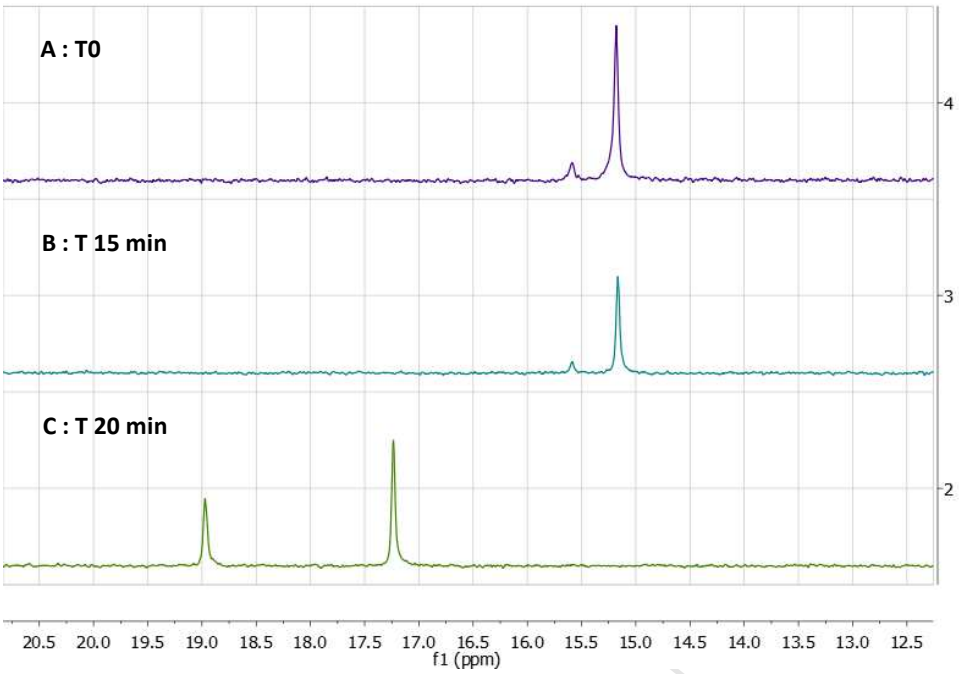


Figure 1. Example of NMR ^{31}P monitoring obtained for the synthesis of compound 24d.

A. Promply after addition of dichlorophosphoramidate **8**, two signals at 15.59 and 15.17 ppm appeared, relative respectively to dichloride **8** and monochloride intermediate formed;

B. After 15 min, phosphorylation reached an optimal ratio ~0.1/1 between the remaining dichlorophosphoramidate **8** and the formed intermediate.

C. After the addition of 2-(*N*,*N*-diethylamino)ethylamine, the two signals at 15.59 and 15.17 disappeared immediately, generating the corresponding tertiary amine prodrug **24d** with a signal at 17.24 ppm and an impurity at 18.97 ppm.

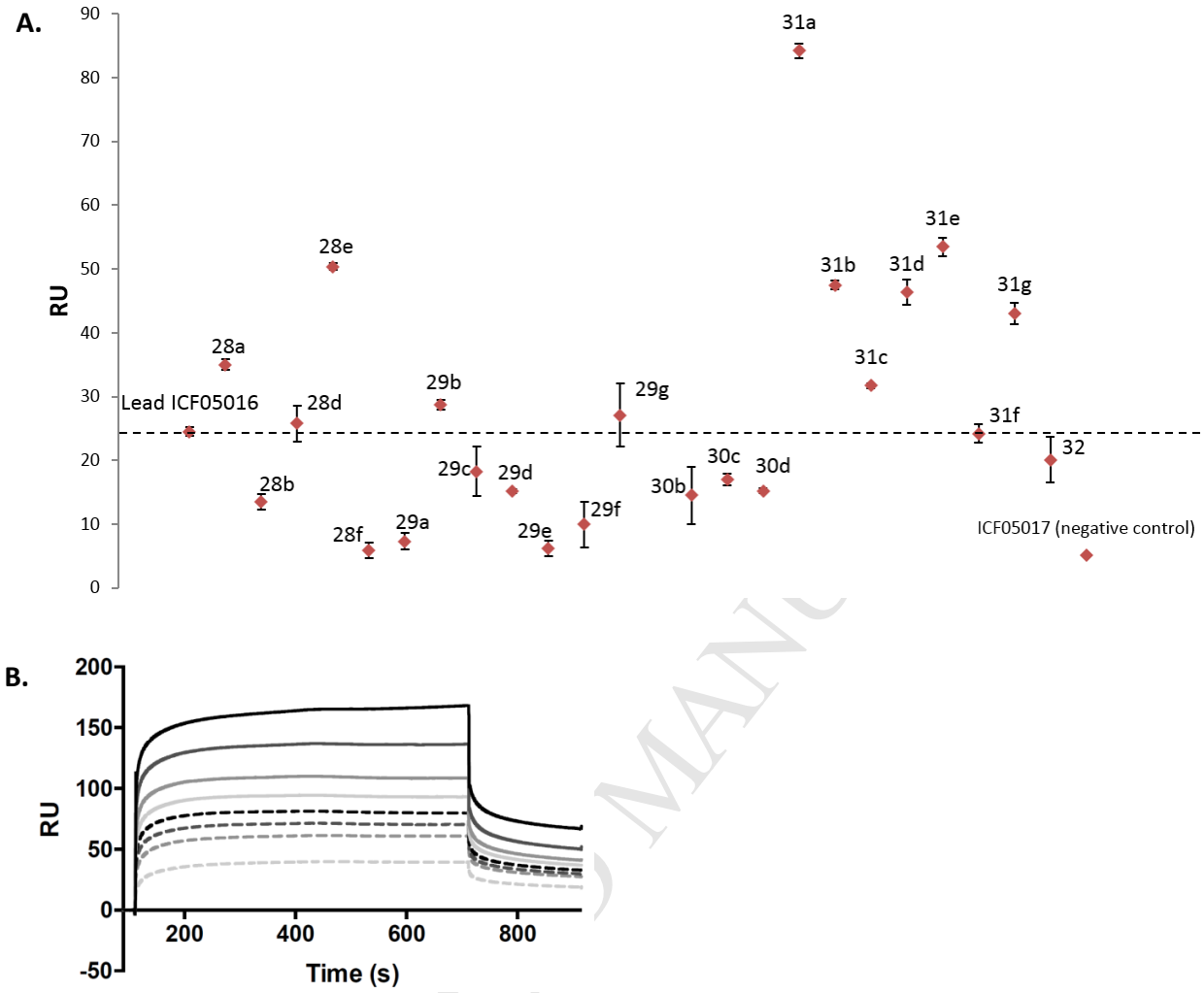
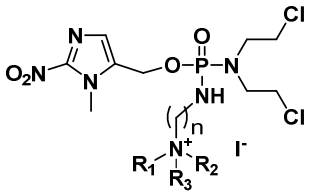


Figure 2. SPR response of compounds 28-32.

A. The SPR responses of the all QA-compounds from the first round of screening. **ICF05016** was used as positive control and its non PG-targeted equivalent **ICF05017** as negative control. Twelve compounds were selected, based on a Response Unit greater than or equal to the **ICF05016** value (dotted line).

B. Representative sensorgram showing binding of compound **31a** to immobilized aggrecan at height concentrations (0.2, 0.4, 0.5, 0.6, 0.8, 1, 1.5 and 2 mM from bottom to top) is presented (K_D = 2.3 mM; Chi^2 = 0.76).



28-32

Compd	n	R_1	R_2	R_3	Kd (mM)	Chi^2
ICF05016	3	Me	Me	Me	1.29 ± 0.92	0.56 ± 0.41
28a	5	Me	Me	Me	4.22 ± 1.25	0.39 ± 0.02
28b	4	Me	Me	Me	-	-
28c	2	Me	Me	Me	-	-
28d	5	Me	Me	Et	3.56 ± 0.78	0.36 ± 0.42
28e	4	Me	Me	Et	2.02 ± 0.49	0.24 ± 0.18
28f	3	Me	Me	Et	-	-
28g	2	Me	Me	Et	-	-
29a	5	Et	Et	Me	-	-
29b	4	Et	Et	Me	4.97 ± 1.41	0.40 ± 0.08
29c	3	Et	Et	Me	-	-
29d	2	Et	Et	Me	-	-
29e	5	Et	Et	Et	-	-
29f	4	Et	Et	Et	-	-
29g	3	Et	Et	Et	3.49 ± 0.45	0.35 ± 0.32
29h	2	Et	Et	Et	-	-
30a	5	*i*-Pr	*i*-Pr	Me	-	-
30b	4	*i*-Pr	*i*-Pr	Me	-	-
30c	3	*i*-Pr	*i*-Pr	Me	-	-
30d	2	*i*-Pr	*i*-Pr	Me	-	-
31a	5	Bn	Me	Me	2.55 ± 0.35	0.7 ± 0.09
31b	4	Bn	Me	Me	5.27 ± 0.21	1.50 ± 1.33
31c	3	Bn	Me	Me	2.99 ± 0.57	0.18 ± 0.06
31d	2	Bn	Me	Me	2.90 ± 0.28	1.30 ± 0.51
31e	5	Bn	Me	Et	3.47 ± 1.14	4.85 ± 2.92
31f	4	Bn	Me	Et	2.10 ± 0.13	0.62 ± 0.52
31g	3	Bn	Me	Et	ND	
31h	2	Bn	Me	Et	ND*	
32	3	piperidinyl		Me	-	-
Evofosfamide (TH-302)						2.9 ± 1.5

Table 1. Binding to immobilized aggrecan by SPR. Dissociation constants (K_D) and Chi^2 were determined for each derivative by "steady-state affinity analysis". All experiments were carried out in duplicate. ND: Not Determined. * no evaluation due to low degree of purity

Compd	n	R_1	R_2	R_3	IC_{50} (µM) Normoxia	IC_{50} (µM) Hypoxia	HCR
ICF05016	3	Me	Me	Me	29.3 ± 8.0	4.3 ± 1.2**	7
28a	5	Me	Me	Me	25.8 ± 6.3	2.4 ± 0.4**	11
28d	5	Me	Me	Et	15.8 ± 7.7	1.5 ± 0.8**	11
28e	4	Me	Me	Et	10.2 ± 0.3	1.5 ± 0.1**	7
29b	4	Et	Et	Me	13.4 ± 4.3	2.4 ± 0.7**	6
29g	3	Et	Et	Et	9.7 ± 3.5	1.4 ± 1.0**	7
31a	5	Bn	Me	Me	28.9 ± 2.6	2.8 ± 1.2**	10
31b	4	Bn	Me	Me	18.0 ± 2.6	1.8 ± 0.9**	10
31c	3	Bn	Me	Me	5.4 ± 0.9	0.4 ± 0.1**	14
31d	2	Bn	Me	Me	6.4 ± 2.1	1.1 ± 0.4**	6
31e	5	Bn	Me	Et	12.9 ± 3.5	0.8 ± 0.3**	16
31f	4	Bn	Me	Et	40.7 ± 8.8	1.7 ± 0.9**	24
31g	3	Bn	Me	Et	20.8 ± 8.7	2.1 ± 1.6**	10
Evofosfamide (TH-302)					2.9 ± 1.5	0.13 ± 0.02**	23

Table 2. Cytotoxicity of HAP derivatives on HEMC-SS cells in normoxic (21% O_2) and hypoxic (N_2, O_2 < 0.3%) conditions. Cultures were exposed to drugs for 24 h followed by incubation for 48 h in normoxia. Notes: ** significant difference *vs.* normoxic condition, *p<0.01*. Abbreviations: IC_{50}: Growth Inhibiting Concentration 50; HCR: Hypoxic-Cytotoxic Ratio = IC_{50} in normoxia/IC_{50} in hypoxia.

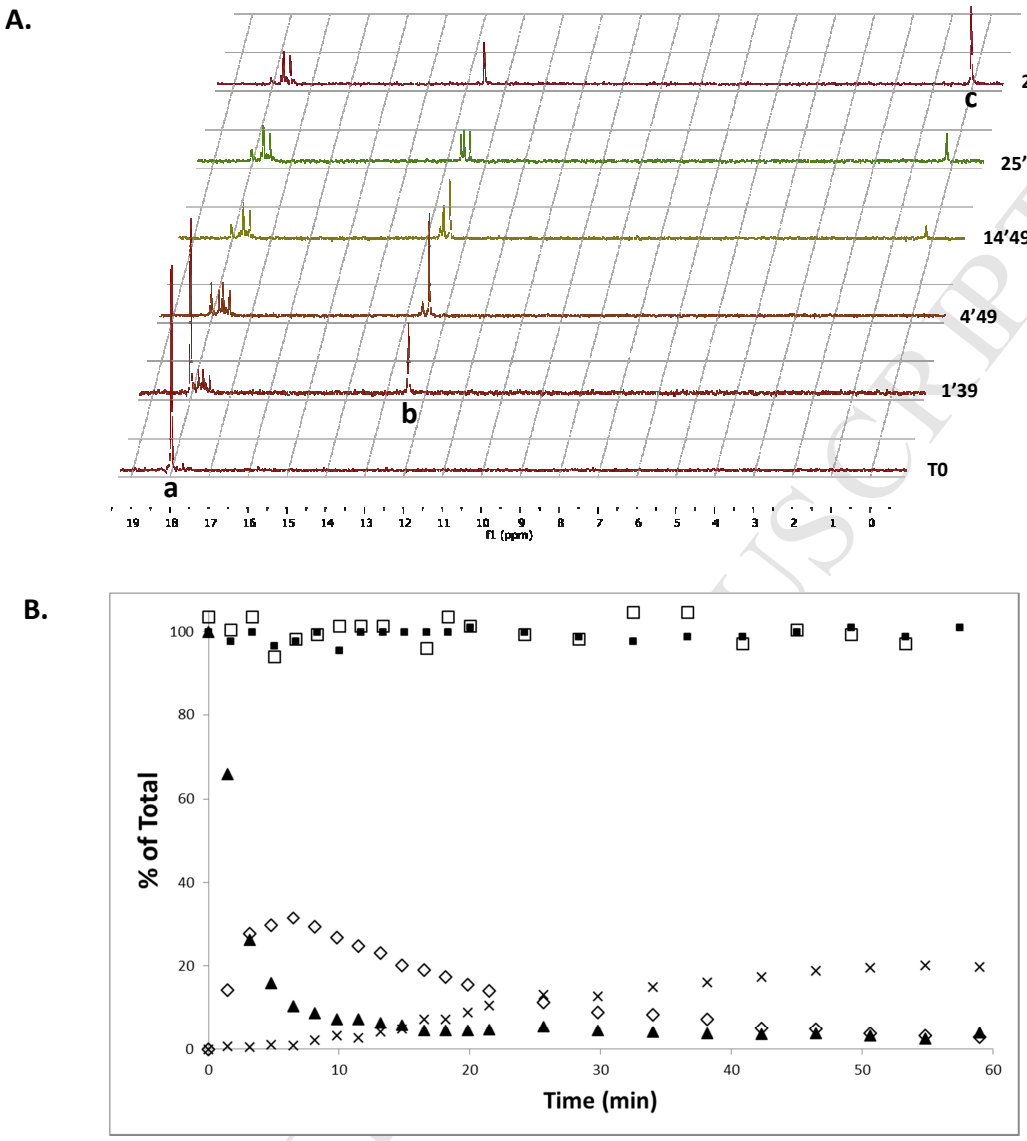


Figure 3. Drug release in reductive conditions (3 equiv. sodium dithionite, CH_3CN/0.1 M cacodylate buffer, pH 7.4, 37 °C) and stability in plasma assessed by NMR ^{31}P: A. Stacked NMR ^{31}P spectra; a. prodrug **31f**, 17.99 ppm; **b.** phosphoramidate anion, 12.39 ppm (proven by MS-HPLC, data not shown); c. inorganic phosphate, 0 ppm. Other signals are relatives to solvolysis, buffer and reductive adducts or transient unknown by-products. Chemical shifts are reported relative to the Ph_3PO reference. B. Representative time course of phosphoramide **31f** cleavage (▲), substituted bis(2-chloroethyl)phosphoramidate anion formation (◇) and inorganic phosphate formation (×) upon incubation of the prodrug with sodium dithionite in cacodylate buffer; Stability of phosphoramide **31f** in cacodylate buffer (■) and in rabbit plasma (□). Data points were measured from NMR ^{31}P areas (average of two runs).

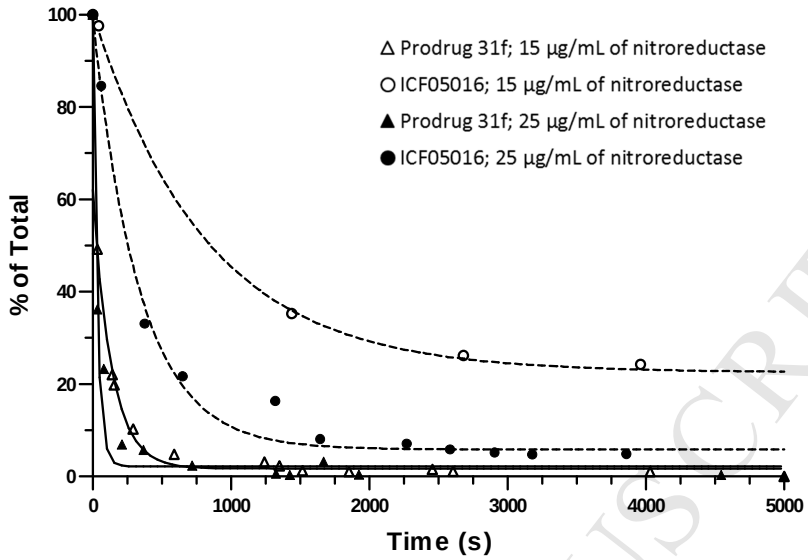


Figure 4. Percent of prodrugs **31f** and ICF05016 remaining after incubation in phosphate buffer (pH 7.4, 37 °C) with nitroreductase in the presence of NADPH. Reactions were followed by RP-HPLC at 322 nm. The lines represent the best-fit values calculated from GraphPadPrism.

4. Experimental protocols

4.1. Synthesis

All commercially available reagents and solvents were purchased at the following commercial suppliers: Sigma Aldrich (Saint-Quentin Fallavier, France), Acros Organics (Geel, Belgium), Fisher Scientific (Illkirch, France), Carlo Erba Reagents (Val de Reuil, France), VWR (Fontenay-sous-Bois, France) and Alfa Aesar (Karlsruhe, Germany) and were used without further purification. All solvents were dried using common techniques. Air and moisture sensitive reactions were carried out under anhydrous argon atmosphere. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel aluminium plates (60 F_{254}, 0.2 mm thick, Macherey or SDS) using the indicated solvent mixture expressed as volume/volume ratios. The plates were visualized with ultraviolet light (254 nm) and (or) by development with ninhydrine ethanolic solution (0.2%) or a 4-(4'-nitrobenzyl)pyridine (NBP)/potassium hydroxide dyeing reagent used for the determination of alkylating agents (the chromatography plate was immersed in the NBP solution (2.5% in acetone), heated for few min and then immersed in potassium hydroxide solution (10% in methanol)). Column chromatography was performed on silica gel 60A normal phase, 35-70 µm (Merck or SDS or Carlo Erba). Uncorrected melting points (mp) were measured on an electrothermal capillary Digital Melting Point Apparatus (IA9100, Bibby Scientific, Roissy, France). Infrared spectra (IR) were recorded in the range 4000-600 cm^{-1} on a IS10 with attenuated total reflectance (ATR) accessory Nicolet (Fisher Scientific). Nuclear magnetic resonance spectra (1H NMR and 13C NMR) were performed on a Bruker AM 200 spectrometer (200 MHz for 1H, 50 MHz for 13C), a Broker Avance DPX300 spectrometer (300 MHz for 1H, 75 MHz for 13C) or a Bruker DRX 500 spectrometer (500 MHz for 1H, 125 MHz for 13C) (Bruker Biospin SAS, Wissembourg, France). Chemical shift values (δ) are quoted in parts per million (ppm) and calibrated to the deuterated solvent reference peak for ^{1}H and ^{13}C spectra. ^{31}P NMR spectra (202 MHz) were recorded on a Bruker Avance 500 apparatus with broadband ^{1}H decoupling and chemical shifts were reported relative to a 1% phosphoric acid solution in deuterium oxide as a coaxial reference (0 ppm). Coupling constants (*J*) are quoted in Hz. To describe spin multiplicity, standard abbreviations such as s, d, dd, t, q, qt, hept, td, m, br.s referring to singlet, doublet, doublet of doublet, triplet, quartet, quintet, heptuplet, doublet of

triplet, multiplet, broad singlet respectively, are used. When necessary, chemical shifts assignments in ^{1}H and ^{13}C spectra were supported by two dimensional NMR experiments (COSY and HSQC). Compounds were analyzed by High-Resolution Mass Spectrometry in positive mode (HRMS, Waters® Micromass® Q-Tof micro™ Mass Spectrometer, UCA-Partner, Clermont Auvergne University, Clermont-Ferrand, France). Preparative high performance liquid chromatography was performed on a CombiflashEZprep (Teledyne ISCO). The purification of QA-derivatives was carried out on a C_{18} column using the following conditions: total experiment time: 30 min, flow rate = 15 mL/min, eluent mixture: H_2O/MeCN (v/v), gradient: 95/5 for 2 min, then 95/5 → 70/30 for 6 min, then 70/30 for 2 min, then 70/30 → 60/40 for 6 min, then 60/40 for 3 min, and 60/40 min → 10/90 for 11 min, λ = 254 and 323 nm.

Abbreviations: ACN, acetonitrile; DCM, dichloromethane; NBP, 4-(4'-nitrobenzyl)pyridine; TEA, triethylamine; THF, tetrahydrofuran; rt, room temperature.

4.1.1. Preparation of the common required 5-hydroxymethyl-1-*N*-methyl-2-nitro-1*H*-imidazole (6)

4.1.1.1. Ethyl *N*-methylglycinate hydrochloride salt (2)

Thionyle chloride (65 mL, 900 mmol) was added dropwise under stirring to a solution of sarcosine (**1**) (20.0 g, 224 mmol) in EtOH (250 mL) cooled in an ice-water bath, while maintaining temperature around -10 °C. Then the reaction mixture was gently heated at 55 °C overnight until the mixture became clear. Solvent and traces of thionyl chloride were removed by evaporation under reduced pressure and the solid residue was washed with Et_2O (3 × 50 mL). The remaining solid was well dried under vacuum to afford compound **2** (33.5 g, 218 mmol) as a white powder, which was used in the next step without further purification. **Yield** 97%; **mp** 126 °C (Lit.[52] mp 125-127 °C); **IR (ATR)** ν **cm^{-1}** 2970-2440, 1742, 1229; **^{1}H NMR (CDCl$_3$, 400 MHz)** δ 9.64 (br.s, 2H, NH_2^+), 4.24 (q, 2H, ^{3}J = 7.1 Hz, C$\underline{H}_2$CH$_3$), 3.84 (t, 2H, ^{3}J = 5.7 Hz, $NH_2^+C\underline{H}_2$), 2.80 (t, 3H, ^{3}J = 5.2 Hz, $NH_2^+C\underline{H}_3$), 1.26 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H}_3$); **^{13}C NMR (CDCl$_3$, 101 MHz) δ** 166.18 (CO_2), 62.62 ($\underline{C}H_2CH_3$), 48.94 ($NH_2^+CH_2$), 33.34 ($NH_2^+CH_3$), 14.03 ($CH_2\underline{C}H_3$).

4.1.1.2. Ethyl *N*-formyl-*N*-methylglycinate (3)

Ethyl *N*-methylglycinate hydrochloride salt (**2**) (30.0 g, 195 mmol) was suspended in a mixture of EtOH (200 mL) and ethyl formate (126 mL). Potassium carbonate (40.6 g, 294 mmol) was added under vigorous stirring and the suspension was stirred at rt overnight. The reaction mixture was then filtered and the precipitate was washed with EtOH (150 mL). The filtrate was concentrated and dissolved in a minimum amount of water (10 mL), followed by extraction with EtOAc (4 × 200 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford compound **3** (24.3 g, 167 mmol) as a pale yellow liquid. **Yield** 85%; **^{1}H NMR ($CDCl_3$, 500 MHz) δ** (two rotamers) 7.93 and 7.86 (s, 1H, CHO), 4.04 and 4.02 (q, 2H, ^3J = 7.2 Hz, $C\underline{H}_2CH_3$), 3.91 and 3.85 (s, 2H, NCH_2), 2.87 and 2.74 (s, 3H, NCH_3), 1.12 and 1.10 (t, 3H, ^3J = 7.1 Hz, $CH_2C\underline{H}_3$); **^{13}C NMR ($CDCl_3$, 75 MHz) δ** (two rotamers) 168.76, 168.31 (CO_2), 163.09, 162.85 (CHO), 61.62, 61.29 ($O\underline{C}H_2CH_3$), 50.93, 45.72 (NCH_2), 35.10, 30.76 (NCH_3), 14.09 ($OCH_2\underline{C}H_3$).

4.1.1.3. Ethyl 2-amino-1-*N*-methyl-*1H*-imidazole-5-carboxylate (4)

To a solution of *N*-formyl sarcosine ethyl ester (**3**) (29.6 g, 204 mmol) in an equal mixture of ethyl formate and THF (190 mL) with cyclohexane (12 mL), was added slowly NaH (60% wt in mineral oil, 12.5 g, 313 mmol) at room temperature. After the addition was completed and hydrogen release stopped, the reaction mixture was allowed to stirred during 3.5 h. The reaction mixture was concentrated under vacuum. The obtained solid was suspended in a solution of EtOH (250 mL) containing concentrated aq. HCl 32 % (61 mL) and refluxed for 2 h. The hot reaction mixture was filtered and the resulting colourless solid was washed with boiling EtOH (2 × 150 mL). The filtrate was concentrated under vacuum and diluted with a mixture of EtOH/water (500 mL, 70/30, v/v). The pH of the solution was adjusted to 3, using an aqueous 5M solution of NaOH and cyanamide (17.5 g, 416 mmol) was added. The resulting mixture was refluxed for 1.5 h, then cooled to rt and concentrated under reduced pressure to approximately 1/8 of the initial volume. The pH of the remaining solution was adjusted to 9-10 with a saturated aqueous solution of potassium carbonate, after cooling in an ice-water bath. The precipitate formed was removed by filtration, washed with water (2 × 10 mL) and dried under vacuum at 40 °C overnight to afford compound **4** (16.9 g, 99.9 mmol) as a pale yellow to orange

solid. A supplementary fraction can be yielded after extraction of the remaining filtrate with ethyl acetate (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate/ethanol (95/5, v/v with 1% NH_4OH) as eluent to yield compound **4** (3.78 g, 22.3 mmol). **Yield** 61%; **Rf** 0.33 (SiO_2, EtOAc/EtOH, 9/1, v/v); **mp** 131 °C (Lit.[40] mp 130-133 °C); **IR (ATR) ν cm^{-1}** 3390, 3119, 1647, 1542, 1168, 750, 737; **^{1}H NMR (CDCl$_3$, 400 MHz) δ** 7.39 (s, 1H, CH_{Ar}), 4.66 (br.s, 2H, NH_2), 4.25 (q, 2H, ^3J = 7.1 Hz, $C\underline{H}_2CH_3$), 3.65 (s, 3H, NCH_3), 1.32 (t, 3H, ^3J = 7.1 Hz, $CH_2C\underline{H}_3$); **^{13}C NMR** (DMSO-d_6, 126 MHz) δ 159.68 (CO), 154.27 ($C_{Ar}NH_2$), 136.02 (CH_{Ar}), 116.88 ($\underline{C}_{Ar}CO$), 58.82 (OCH_2), 30.17 (NCH_3), 14.30 ($CH_2\underline{C}H_3$).

4.1.1.4. Ethyl 1-*N*-methyl-2-nitro-*1H*-imidazole-5-carboxylate (5)

To a solution of sodium nitrite (18.3 g, 266 mmol) in water (55 mL) cooled around -5 °C in an ice-salt bath, was added dropwise a solution of the amino ester **4** (6.42 g, 38.0 mmol) in acetic acid (42 mL). The temperature was allowed to rise gradually to rt and the reaction mixture was stirred overnight. The reaction mixture was extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using cyclohexane/ethyl acetate (7/3, v/v) as eluent. After concentration under vacuum, the residue was washed with water (2 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield nitro ester **5** as yellow crystals (5.67 g, 28.5 mmol). **Yield** 75%; **mp** 56-58 °C (Lit. 56-58 °C[40] and 65-66 °C[37]); **Rf** 0.40 (SiO_2, cyclohexane/ethyl acetate, 7/3, v/v); **IR (ATR) ν cm^{-1}** 1723, 1552, 1486, 1519, 1364, 1233, 767; **^{1}H NMR (CDCl$_3$, 300 MHz) δ** 7.70 (s, 1H, CH_{Ar}), 4.39 (q, 2H, ^3J = 7.1 Hz, $OC\underline{H}_2CH_3$), 4.31 (s, 3H, NCH_3), 1.39 (t, 3H, ^3J = 7.1 Hz, $OCH_2C\underline{H}_3$); **^{13}C NMR** (DMSO-d_6, 126 MHz) δ 158.71 (CO), 147.61 ($C_{Ar}NO_2$), 133.70 (CH_{Ar}), 126.07 ($C_{Ar}CO$), 61.34 (OCH_2), 35.14 (NCH_3), 13.91 ($CH_2\underline{C}H_3$).

4.1.1.5. (1-methyl-2-nitro-*1H*-imidazol-5-yl)methanol (6)

The ester **5** (11.0 g, 55.2 mmol) was dissolved in a mixture of anhydrous THF/MeOH (200 mL, 8/2, v/v) in a 1 L three-necked flask fitted with a mechanical stirrer, a 500 mL dropping funnel, a bubbler and maintained in an ice-water bath. A suspension of $NaBH_4$ (6.33 g, 167 mmol) in THF

(50 mL) and a solution of LiBr (14.6 g, 168 mmol) in THF (50 mL) were cooled at 0 °C, introduced in the same dropping funnel with water (50 m) and added dropwise to the nitroimidazole solution, at such a rate that the internal temperature did not exceed 10 °C. The reaction mixture was stirred at rt until total conversion of the starting nitroester **5**, according to TLC monitoring (19 h). Ammonium chloride (3 g) was then added at 0 °C and stirring was prolonged for 30 min. The precipitate was filtrated and washed with THF (3 × 50 mL). After concentration under vacuum, the yellow solid was taken up in a mixture of EtOAc/MeOH (98/2, v/v) and the resulting solution was passed through a pad of silica gel surmounted by a pad of Celite®545 to afford pure alcohol **6** as pale yellow to orange crystals (5.91 g, 37.7 mmol). **Yield** 68%; **Rf** 0.50 (SiO_2, EtOAc); **mp** 140 °C (lit.[40] 141-143 °C); **IR (ATR) ν cm^{-1}** 3231, 1491, 1358, 1186, 1040, 832; **^{1}H NMR (DMSO-*d*$_6$, 300 MHz) δ** 7.10 (s, 1H, CH_{Ar}), 5.40 (t, 1H, ^3J = 5.3 Hz, OH), 4.55 (d, 2H, ^3J = 5.3 Hz, $C\underline{H}_2OH$), 3.93 (s, 3H, NCH_3); **^{13}C NMR (DMSO-*d*$_6$,** 126 **MHz) δ** 145.63 ($C_{Ar}NO_2$), 138.60 ($\underline{C}_{Ar}CH_2$), 126.50 (CH_{Ar}), 52.96 (CH_2), 34.03 (NCH_3).

4.1.2. *N,N*-bis(2-chloroethyl)phosphoramidic acid dichloride (8)

N,N-bis(2-chloroethyl)amine HCl (**7**) (10.0 g, 56.0 mmol) was refluxed at 130-140 °C in the presence of an excess of $POCl_3$ (21 mL, 224 mmol) during a period of 80 h, until a clear and brown mixture was obtained. The excess of $POCl_3$ was removed under reduced pressure and the remaining brown residue purified by column chromatography on silica gel (EtOAc/cyclohexane, 20/80, v/v). The obtained solid was thoroughly washed with cyclohexane and dried under vacuum to afford pure compound **8** as white crystals (8.0 g, 31 mmol). **Yield** 55%; **Rf** 0.39 (SiO_2, cyclohexane/EtOAc, 8/2, v/v); **mp** 57 °C (Lit.[43] 57-59 °C); **IR (ATR) ν cm^{-1}** 1272, 1264 ($\nu_{P=O}$, ν_{C-N}), 1116, 1102 (ν_{P-O}, ν_{P-N}); **^{1}H NMR ($CDCl_3$, 200 MHz) δ** 3.841-3.51 (m, 8H); **^{13}C NMR ($CDCl_3$, 50 MHz) δ** 49.61 (d, 2C, $^2J_{C-P}$ = 4.1 Hz, $N\underline{C}H_2CH_2Cl$), 40.91 (d, 2C, $^3J_{C-P}$ = 2.7 Hz, $NCH_2\underline{C}H_2Cl$); **^{31}P NMR ($CDCl_3$, 202 MHz) δ** 17.42.

4.1.3. General procedure for the synthesis of non-targeted prodrugs 23 to 27

To a solution of (1-methyl-2-nitro-*1H*-imidazol-5-yl)methanol (**6**) (1 eq.) in freshly distilled anhydrous THF (10 mL for 3 mmol of alcohol **6**) was added dropwise lithium bis(trimethylsilyl)amide (1M in THF, 1.1 eq.) at −78 °C under an inert atmosphere. The reaction mixture was stirred around 5 min at −78 °C, and a solution of bis(2-chloroethyl)phosphoramidic dichloride (**8**) (1.1 eq.) in THF (3.3 mmol in 10 mL), previously cooled at −78 °C, was added all at once at the same temperature (T_0). The reaction mixture was stirred for 15 to 80 min, before the addition of a solution of the appropriate amine **18-22** (2-2.2 eq.) in THF (3 mL for 5 mmol of amine) and stirring was maintained at −78°C for 5 min to 75 min. These reaction times were determined by ^{31}P NMR monitoring for each compound. The reaction was stopped by addition of water (20 mL for 3 mmol of alcohol **6**), concentrated in vacuum and then extracted with EtOAc (3 × 50 mL for 3 mmol of alcohol **6**). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using an eluent gradient (EtOAc/EtOH with TEA or NH_4OH) to afford compounds **23-27** as yellow to orange oils.

(1-methyl-2-nitro-1*H*-imidazol-5-yl)methyl *N,N*-bis(2-chloroethyl)-*N'*-[5-(dimethylamino) pentyl]phosphorodiamidate (23a)

Starting from 500 mg (3.18 mmol) of alcohol **6**, the coupling reaction with bis(2-chloroethyl)phosphoramidic dichloride (**8**) was performed for 60 min before the addition of the prepared amine **18a** (826 mg, 6.34 mmol). The reaction was then stopped after 10 min of stirring. The crude product was purified by silica gel chromatography (EtOAc/EtOH/NH_4OH ranging from 90/10/1 to 50/50/3, v/v/v) to afford **23a** (457 mg, 0.965 mmol) as a yellow-orange oil. **Yield** 30%; **Rf** 0.35 (SiO_2, EtOAc/EtOH/TEA, 70/30/3, v/v/v); **IR (ATR) ν cm^{-1}** 3389, 3223, 1537, 1490, 1351, 1216, 1191, 1007, 979, 960, 834, 744; **^{1}H NMR (200 MHz, $CDCl_3$) δ** 7.19 (s, 1H, CH_{Ar}), 5.19 (dd, 1H, $^2J_{H-H}$ = 13.3 Hz, $^3J_{H-P}$ = 7.5 Hz, CH'O), 4.98 (dd, 1H, $^2J_{H-H}$ = 13.3 Hz, $^3J_{H-P}$ = 9.0 Hz, CH"O), 4.07 (s, 3H, NCH_3), 3.95 (td, 1H, $^3J_{H-H}$ = 6.8 Hz, $^2J_{H-P}$ = 11.3 Hz, N<u>H</u>P), 3.64-3.51 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.53-3.25 (m, 4H, $N(C\underline{H}_2CH_2Cl)_2$), 3.04-2.89 (m, 4H, $C\underline{H}_2NHP$, $C\underline{H}_2N(CH_3)_2$), 2.79 (s, 6H, $N(C\underline{H}_3)_2$), 1.90-1.53 (m, 6H, $CH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2$); **^{13}C NMR (50 MHz, $CDCl_3$) δ** 146.41 ($C_{Ar}NO_2$), 133.43 (d, $^3J_{C-P}$ = 7.1 Hz, $\underline{C}_{Ar}CH_2$), 129.11 (CH_{Ar}), 57.62 ($\underline{C}H_2N(CH_3)_2$), 56.44 (d, $^2J_{C-P}$ = 4.4

Hz, CH_2O), 49.33 (d, 2C, $^2J_{C\text{-}P}$ = 3.9 Hz, N($\underline{C}H_2CH_2Cl)_2$), 43.18 (2C, N($\underline{C}H_3)_2$), 42.81 (2C, N($CH_2\underline{C}H_2Cl)_2$), 40.17 ($CH_2NHP$), 34.78 ($NCH_3$), 30.12 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, $\underline{C}H_2CH_2NHP$), 23.92, 23.34 ($\underline{C}H_2\underline{C}H_2CH_2N(CH_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.33; **HRMS** (ESI) m/z 473.1607 $[M+H]^+$ (calculated for $[C_{16}H_{32}Cl_2N_6O_4P]^+$ 473.1594).

4.1.4. General procedure for the synthesis of targeted prodrugs 28 to 32

To a solution of amine **23-27** (1 eq.) in anhydrous ACN (5 mL for 50-100 mg of amine) under an inert atmosphere, was added potassium carbonate (2.5 eq) and appropriate alkyl iodide (5-24 eq.). Stirring was maintained at rt in a sealed flask for several hours until TLC monitoring showed total conversion of the initial product (further addition of alkyl iodide was sometimes necessary). The suspension was filtered and the precipitate was thoroughly washed with ACN. After evaporation of the filtrate, the residue was dissolved in acetone (10 mL for 50 mg of amine) and the mixture was filtered to remove the remaining potassium carbonate. After evaporation to dryness under reduced pressure, the corresponding quaternary ammoniums **28-32** were obtained analytically pure or purified by preparative high performance liquid chromatography and lyophilised.

4.1.4.1. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-trimethylpentane-1-aminium iodide (28a)

Alkylation of amine **23a** (34 mg, 71.8 µmol) with methyl iodide (24 eq.) using the standard procedure (reaction time: 4 h) afforded compound **28a** analytically pure, as a yellow hygroscopic solid (43 mg, 69.2 µmol). **Yield** 96%; **IR (ATR) ν cm^{-1}** 3426, 3230, 1537, 1489, 1349, 1219, 1190, 1007, 960, 835, 743; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.25 (s, 1H, CH_{Ar}), 5.16 (dd, 2H, $^2J_{H\text{-}H}$ = 13.3 Hz, $^3J_{H\text{-}P}$ = 8.2 Hz, CH'O), 5.12 (dd, 2H, $^2J_{H\text{-}H}$ = 13.3 Hz, $^3J_{H\text{-}H}$ = 8.8 Hz, CH"O), 4.07 (s, 3H, NCH_3), 3.67 (td, 4H, $^3J_{H\text{-}H}$ = 7.0 Hz, $^4J_{H\text{-}P}$ = 2.3 Hz, N($CH_2\underline{C}H_2Cl)_2$), 3.54-3.32 (m, 8H, N($\underline{C}H_2CH_2Cl)_2$, CH_2N^+), 3.19 (s, 9H, $N^+(CH_3)_3$), 2.96-2.84 (m, 2H, $\underline{C}H_2NHP$), 1.84-1.77 (m, 2H, $\underline{C}H_2CH_2N^+$), 1.59 (qt, 2H, $^3J_{H\text{-}H}$ = 7.2 Hz, $\underline{C}H_2CH_2NHP$), 1.41 (qt, 2H, $^3J_{H\text{-}H}$ = 7.5 Hz, $\underline{C}H_2CH_2CH_2NHP$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.64 ($C_{Ar}NO_2$), 135.10 (d, $^3J_{C\text{-}P}$ = 6.8 Hz, $\underline{C}_{Ar}CH_2$), 129.27 (CH_{Ar}), 67.81 (CH_2N^+), 57.73 (d, $^2J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 53.75, 53.72, 53.68 ($N^+(CH_3)_3$), 50.30 (d, 2C, $^2J_{C\text{-}P}$ = 4.6 Hz, N($\underline{C}H_2CH_2Cl)_2$),

43.24 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.37 ($CH_2NHP$), 35.23 ($NCH_3$), 32.19 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, $\underline{C}H_2CH_2NHP$), 24.37 ($\underline{C}H_2CH_2CH_2NHP$), 23.58 ($\underline{C}H_2CH_2N^+$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.28; **HRMS** (ESI) m/z 487.1744 $[M]^+$ (calculated for $[C_{17}H_{34}Cl_2N_6O_4P]^+$ 487.1751).

4.1.4.2. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-trimethylbutane-1-aminium iodide (28b)

Alkylation of amine **23b** (78 mg, 0.171 mmol) with methyl iodide (12 eq.) using the standard procedure (reaction time: 4 h), afforded compound **28b** analytically pure, as a yellow hygroscopic solid (95 mg, 0.158 mmol). **Yield** 92%; **IR (ATR) ν cm^{-1}** 3404, 3223, 1537, 1488, 1349, 1217, 1190, 1009, 960, 835, 743; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.25 (s, 1H, CH_{Ar}), 5.21-5.07 (m, 2H, OCH_2), 4.07 (s, 3H, NCH_3), 3.74-3.61 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.55-3.34 (m, 6H, $N(\underline{C}H_2CH_2Cl)_2$, CH_2N^+), 3.15 (s, 9H, $N^+(CH_3)_3$), 3.01-2.88 (m, 2H, $\underline{C}H_2NHP$), 1.89-1.77 (m, 2H, $\underline{C}H_2CH_2N^+$), 1.63-1.50 ($\underline{C}H_2CH_2NHP$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.64 ($C_{Ar}NO_2$), 135.06 (d, $^3J_{C\text{-}P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.26 (CH_{Ar}), 67.50 (CH_2N^+), 57.78 (d, $^2J_{C\text{-}P}$ = 4.8 Hz, OCH_2), 53.78, 53.76, 53.72 ($N^+(CH_3)_3$), 50.23 (d, 2C, $^2J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.23 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.05 ($CH_2NHP$), 35.25 ($NCH_3$), 29.41 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, $\underline{C}H_2CH_2NHP$), 21.36 ($\underline{C}H_2CH_2N^+$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.25; **HRMS** (ESI) m/z 473.1603 $[M]^+$ (calculated for $[C_{16}H_{32}Cl_2N_6O_4P]^+$ 473.1594).

4.1.4.3. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-trimethylethane-1-aminium iodide (28c)

Alkylation of amine **23d** (114 mg, 0.264 mmol) with methyl iodide (5 eq.) using the standard procedure (reaction time: 24 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **28c** as a hygroscopic lyophilisate (116 mg, 0.202 mmol). **Yield** 77%; **IR (ATR) ν cm^{-1}** 3436, 3196, 1537, 1488, 1350, 1222, 1190, 1005, 959, 835, 744; **^{1}H NMR (500 MHz, CD_3OD)** 7.27 (s, 1H, CH_{Ar}), 5.20 (dd, 2H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.4 Hz, CH'O), 5.17 (dd, 2H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}H}$ = 8.3 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.75-3.66 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.55-3.35 (m, 8H, $N(\underline{C}H_2CH_2Cl)_2$, $CH_2CH_2N^+$), 3.22 (s, 9H, $N^+(CH_3)_3$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.73 ($C_{Ar}NO_2$), 134.64 (d, $^3J_{C\text{-}P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.37 (CH_{Ar}),

67.47 (CH_2N^+), 58.08 (d, $^2J_{C-P}$ = 4.7 Hz, OCH_2), 54.35, 54.38, 54.41 ($N^+(CH_3)_3$), 50.23 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.23 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.05 ($CH_2NHP$), 35.25 ($NCH_3$), 29.41 (d, $^3J_{C-P}$ = 4.6 Hz, $\underline{C}H_2CH_2NHP$), 21.36 ($\underline{C}H_2CH_2N^+$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.72; **HRMS** (ESI) m/z 445.1279 $[M]^+$ (calculated for $[C_{14}H_{28}Cl_2N_6O_4P]^+$ 445.1281).

4.1.4.4. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-ethyl-*N*,*N*-dimethylpentane-1-aminium iodide (28d)

Alkylation of amine **23c** (38 mg, 81.5 µmol) with ethyl iodide (12 eq. initially followed by 12 eq after 24 h) using the standard procedure (reaction time: 36 h) afforded compound **28d** analytically pure, as a yellow hygroscopic solid (36 mg, 56.7 µmol). **Yield** 70%; **IR (ATR) ν cm^{-1}** 3420, 3235, 1537, 1489, 1350, 1216, 1190, 1007, 976, 960, 835, 770, 744; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.25 (s, 1H, CH_{Ar}), 5.16 (dd, 1H, $^2J_{H-H}$ = 13.5 Hz, $^3J_{H-P}$ = 8.3 Hz, OCH'), 5.12 (dd, 1H, $^2J_{H-H}$ = 13.5 Hz, $^3J_{H-P}$ = 8.7 Hz, OCH"), 4.08 (s, 3H, NCH_3), 3.71-3.62 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.51-3.33 (m, 6H, $N(\underline{C}H_2CH_2Cl)_2$, $\underline{C}H_2CH_3$), 3.34-3.28 (m, 2H, $\underline{C}H_2N^+(CH_3)_2CH_2CH_3$), 3.08 (s, 6H, $N^+(CH_3)_2$), 2.98-2.85 (m, 2H, $\underline{C}H_2NHP$), 1.83-1.72 (m, 2H, $\underline{C}H_2CH_2N^+$), 1.59 (qt, 2H, $^3J_{H-H}$ = 7.0 Hz, $\underline{C}H_2CH_2NHP$), 1.46-1.34 (m, 5H, $CH_2\underline{C}H_3$, $\underline{C}H_2CH_2CH_2NHP$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.64 ($C_{Ar}NO_2$), 135.11 (d, $^3J_{C-P}$ = 7.0 Hz, $\underline{C}_{Ar}CH_2$), 129.28 (CH_{Ar}), 64.87 ($CH_2\underline{C}H_2N^+$), 60.92 ($N^+(CH_3)_2\underline{C}H_2CH_3$), 57.73 (d, $^2J_{C-P}$ = 4.6 Hz, OCH_2), 50.80 (2C, $N^+(CH_3)_2$), 50.31 (d, 2C, $^2J_{C-P}$ = 4.6 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.25 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.39 ($CH_2NHP$), 35.24 ($NCH_3$), 32.20 (d, $^3J_{C-P}$ = 5.4 Hz, $\underline{C}H_2CH_2NHP$), 24.45 ($\underline{C}H_2CH_2CH_2NHP$), 23.17 ($\underline{C}H_2CH_2N^+$), 8.51 ($CH_2\underline{C}H_3$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.26; **HRMS** (ESI) m/z 501.1908 $[M]^+$ (calculated for $[C_{18}H_{36}Cl_2N_6O_4P]^+$ 501.1907).

4.1.4.5. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-ethyl-*N*,*N*-dimethylbutane-1-aminium iodide (28e)

Alkylation of amine **23b** (107 mg, 233 µmol) with ethyl iodide (12 eq.) using the standard procedure (reaction time: 4 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **28e** as a hygroscopic lyophilisate (99 mg, 161 µmol). **Yield** 69%; **IR (ATR) ν cm^{-1}** 3383, 3255, 1537, 1489, 1350, 1216, 1191, 1011, 980, 960, 835, 744; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.26 (s, 1H, CH_{Ar}), 5.16 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz,

$^{3}J_{H\text{-}P}$ = 8.3 Hz, OCH'), 5.12 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.5 Hz, OCH"), 4.07 (s, 3H, NCH_3), 3.72-3.63 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.52-3.35 (m, 6H, $N(\underline{C}H_2CH_2Cl)_2$), $\underline{C}H_2CH_3$), 3.34-3.29 (m, 2H, $\underline{C}H_2N^+(CH_3)_2CH_2CH_3$), 3.07 (s, 6H, $N^+(CH_3)_2$), 2.95 (td, 2H, $^{3}J_{H\text{-}P}$ = 11.4 Hz, $^{3}J_{H\text{-}P}$ = 6.8 Hz, $\underline{C}H_2NHP$), 1.85-1.76 (m, 2H, $\underline{C}H_2CH_2N^+$), 1.56 (qt, 2H, $^{3}J_{H\text{-}H}$ = 7.0 Hz, $\underline{C}H_2CH_2NHP$), 1.40-1.35 (m, 3H, $CH_2\underline{C}H_3$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.65 ($C_{Ar}NO_2$), 135.00 (d, $^{3}J_{C\text{-}P}$ = 7.1 Hz, $\underline{C}_{Ar}CH_2$), 129.19 (CH_{Ar}), 64.46 (CH_2N^+), 60.88 ($\underline{C}H_2CH_3$), 57.72 (d, $^{2}J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 50.72 (2C, $N^+(CH_3)_2$), 50.17 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.17 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.06 ($CH_2NHP$), 35.21 ($NCH_3$), 29.50 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, $\underline{C}H_2CH_2NHP$), 20.90 ($\underline{C}H_2CH_2CH_2NHP$), 8.46 ($CH_2\underline{C}H_3$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.25; **HRMS** (ESI) m/z 487.1747 $[M]^+$ (calculated for $[C_{17}H_{34}Cl_2N_6O_4P]^+$ 487.1751).

4.1.4.6. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-ethyl-*N*,*N*-dimethylpropane-1-aminium iodide (28f)

Alkylation of amine **23c** (55 mg, 123 µmol) with ethyl iodide (24 eq.) using the standard procedure (reaction time: 24 h) afforded compound **28f** analytically pure, as a yellow hygroscopic solid (68.0 mg, 113 µmol). **Yield** 92%; **IR (ATR) ν cm^{-1}** 3359, 1490, 1351, 1215, 1192, 1008, 961, 836, 744; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.28 (s, 1H, CH_{Ar}), 5.20 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.5 Hz, $^{3}J_{H\text{-}P}$ = 8.6 Hz, OCH'), 5.15 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.5 Hz, $^{3}J_{H\text{-}P}$ = 8.6 Hz, OCH"), 4.08 (s, 3H, NCH_3), 3.74-3.64 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.53-3.36 (m, 8H, $N(\underline{C}H_2CH_2Cl)_2$), $\underline{C}H_2N^+(CH_3)_2\underline{C}H_2CH_3$), 3.10 (s, 6H, $N^+(\underline{C}H_3)_2$), 3.02 (td, 2H, $^{3}J_{H\text{-}P}$ = 12.4 Hz, $^{3}J_{H\text{-}P}$ = 6.4 Hz, $\underline{C}H_2NHP$), 2.05-1.94 (m, 2H, $CH_2\underline{C}H_2CH_2$), 1.46-1.34 (m, 3H, $CH_2\underline{C}H_3$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.66 ($C_{Ar}NO_2$), 134.99 (d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.34 (CH_{Ar}), 62.83 ($CH_2\underline{C}H_2N^+$), 61.15 ($\underline{C}H_2CH_3$), 57.93 (d, $^{2}J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 51.03 (2C, $N^+(CH_3)_2$), 50.11 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.6 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.24 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 38.83 ($CH_2NHP$), 35.36 ($NCH_3$), 25.89 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, $CH_2\underline{C}H_2CH_2$), 8.58 ($CH_2\underline{C}H_3$); **^{31}P NMR (202 MHz, CD_3OD) δ (ppm)** 17.26; **HRMS** (ESI) m/z 473.1595 $[M]^+$ (calculated for $[C_{16}H_{32}Cl_2N_6O_4P]^+$ 473.1594).

4.1.4.7. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-ethyl-*N*,*N*-dimethylethane-1-aminium iodide (28g)

Alkylation of amine **23d** (114 mg, 0.264 mmol) with ethyl iodide (12 eq.) using the standard procedure (reaction time: 24 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **28g** as a hygroscopic lyophilisate (102 mg, 0.174 mmol). Yield 70%; **IR (ATR) ν cm^{-1}** 3400, 3192, 1537, 1488, 1350, 1223, 1191, 1005, 974, 959, 835, 744; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.28 (s, 1H, CH_{Ar}), 5.20 (dd, 2H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.4 Hz, CH'O), 5.17 (dd, 2H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}H}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.74-3.68 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.53-3.33 (m, 10H, $N(C\underline{H}_2CH_2Cl)_2$, $CH_2CH_2N^+CH_2$), 3.14 (s, 6H, $N^+(CH_3)_2$), 1.42-1.36 (m, 3H, $CH_2C\underline{H}_3$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.73 ($C_{Ar}NO_2$), 134.66 (d, $^{3}J_{C\text{-}P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.39 (CH_{Ar}), 64.36 ($CH_2\underline{C}H_2N^+$), 61.65 ($\underline{C}H_2CH_3$), 58.10 (d, $^{2}J_{C\text{-}P}$ = 4.9 Hz, OCH_2), 51.46, 51.44 ($N^+(CH_3)_2$), 49.95 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.8 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.15 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 35.74 ($CH_2NHP$), 35.29 ($NCH_3$), 8.53 ($CH_2\underline{C}H_3$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.73; **HRMS** (ESI) m/z 459.1427 $[M]^+$ (calculated for $[C_{15}H_{30}Cl_2N_6O_4P]^+$ 459.1438).

4.1.4.8. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N*-diethyl-*N*-methylpentane-1-aminium iodide (29a)

Alkylation of amine **24a** (148 mg, 295 µmol) with methyl iodide (12 eq.) using the standard procedure (reaction time: 12 h) afforded compound **29a** analytically pure, as a yellow hygroscopic solid (168 mg, 261 µmol). **Yield** 88%; **IR (ATR) ν cm^{-1}** 3410, 3216, 1537, 1489, 1349, 1216, 1190, 1008, 978, 959, 834, 769, 743; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.25 (s, 1H, CH_{Ar}), 5.16 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.3 Hz, CH'O), 5.12 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.7 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.71-3.62 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.50-3.35 (m, 5H, $N(C\underline{H}_2CH_2Cl)_2$, NHP), 3.39 (q, 4H, $^{3}J_{H\text{-}H}$ = 7.3 Hz, $N^+(C\underline{H}_2CH_3)_2$), 3.30-3.24 (m, 2H, $CH_2C\underline{H}_2N^+$), 3.00 (s, 3H, $N^+C\underline{H}_3$), 2.91 (tdd, 2H, $^{3}J_{H\text{-}P}$ = 11.2 Hz, $^{3}J_{H\text{-}H}$ = 6.8 Hz, $^{3}J_{H\text{-}H}$ = 1.5 Hz, $C\underline{H}_2NHP$), 1.80-1.70 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.59 (qt, 2H, $^{3}J_{H\text{-}H}$ = 7.2 Hz, $C\underline{H}_2CH_2NHP$), 1.47-1.38 (m, 8H, $C\underline{H}_2CH_2CH_2NHP$), 1.38-1.32 (m, 6H, $N^+(CH_2C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.65 ($C_{Ar}NO_2$), 135.11 (d, $^{3}J_{C\text{-}P}$ = 7.0 Hz, $\underline{C}_{Ar}CH_2$), 129.28 (CH_{Ar}), 61.78 ($CH_2\underline{C}H_2N^+$), 57.67 (2C, $N^+(\underline{C}H_2CH_3)_2$), 57.72 (d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, OCH_2), 50.31 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 47.83 ($N^+CH_3$), 43.25 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.40 ($C\underline{H}_2NHP$), 35.25 ($NCH_3$), 32.21 (d, $^{3}J_{C\text{-}P}$ = 5.5 Hz, $\underline{C}H_2CH_2NHP$), 24.51 ($C\underline{H}_2CH_2CH_2NHP$), 22.80

($\underline{C}H_2CH_2N^+$), 8.18 (2C, $N^+(CH_2\underline{C}H_3)_2$); **HRMS** (ESI) m/z 515.2073 $[M]^+$ (calculated for $[C_{19}H_{38}Cl_2N_6O_4P]^+$ 515.2064).

4.1.4.9. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N*-diethyl-*N*-methylbutane-1-aminium iodide (29b)

Alkylation of amine **24b** (27 mg, 55.4 µmol) with methyl iodide (12 eq.) using the standard procedure (reaction time: 24 h) afforded compound **29b** analytically pure, as a yellow hygroscopic solid (30 mg, 47.7 µmol). **Yield** 86%; **IR (ATR) ν cm^{-1}** 3419, 3208, 1537, 1490, 1350, 1217, 1191, 1012, 836, 744; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.25 (s, 1H, CH_{Ar}), 5.16 (dd, 1H, $^2J_{H\text{-}H}$ = 13.3 Hz, $^3J_{H\text{-}P}$ = 8.3 Hz, CH'O), 5.12 (dd, 1H, $^2J_{H\text{-}H}$ = 13.3 Hz, $^3J_{H\text{-}P}$ = 8.6 Hz, CH"O), 4.07 (s, 3H, NCH_3), 3.68 (td, 4H, $^3J_{H\text{-}H}$ = 7.1 Hz, $^4J_{H\text{-}P}$ = 2.7 Hz, $N(CH_2\underline{C}H_2Cl)_2$), 3.51-3.33 (m, 8H, $N(\underline{C}H_2CH_2Cl)_2$, $N^+(\underline{C}H_2CH_3)_2$), 3.29-3.22 (m, 2H, $CH_2\underline{C}H_2N^+$), 3.00 (s, 3H, $N^+\underline{C}H_3$), 2.94 (td, 2H, $^3J_{H\text{-}H}$ = 6.8 Hz, $^3J_{H\text{-}P}$ = 11.6 Hz, CH_2NHP), 1.82-1.71 (m, 2H, $\underline{C}H_2CH_2N^+$), 1.55 (qt, 2H, $^3J_{H\text{-}H}$ = 7.8 Hz, $\underline{C}H_2CH_2NHP$), 1.34 (t, 6H, $^3J_{H\text{-}H}$ = 7.2 Hz, $N^+(CH_2\underline{C}H_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.67 ($C_{Ar}NO_2$), 135.03 (d, $^3J_{C\text{-}P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.25 (CH_{Ar}), 61.53 (CH_2N^+), 57.76 (d, $^2J_{C\text{-}P}$ = 4.9 Hz, OCH_2), 57.70 (2C, $N^+(\underline{C}H_2CH_3)_2$), 50.24 (d, 2C, $^2J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 47.81 ($N^+CH_3$), 43.22 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.12 ($CH_2NHP$), 35.22 ($NCH_3$), 29.54 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, $\underline{C}H_2CH_2NHP$), 20.62 ($\underline{C}H_2CH_2N^+$), 8.15 (2C, $N^+(CH_2\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.32; **HRMS** (ESI) m/z 501.1906 $[M]^+$ (calculated for $[C_{18}H_{36}Cl_2N_6O_4P]^+$ 501.1907).

4.1.4.10. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N*-diethyl-*N*-methylpropane-1-aminium iodide (29c)

Alkylation of amine **24c** (52 mg, 109 µmol) with methyl iodide (6 eq.) using the standard procedure (reaction time: 4 h) afforded compound **29c** analytically pure, as a yellow hygroscopic solid (63 mg, 102 mmol); **Yield** 93%; **IR (ATR) ν cm^{-1}** 3338, 3210, 1537, 1489, 1350, 1219, 1190, 1008, 978, 960, 835, 745; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.28 (s, 1H, CH_{Ar}), 5.19 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.2 Hz, CH'O), 5.15 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.73-3.66 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.50-3.35 (m, 10H, $N(\underline{C}H_2CH_2Cl)_2$,

$C\underline{H}_2N^+(C\underline{H}_2CH_3)_2$), 3.05-2.97 (m, 2H, CH_2NHP), 3.02 (s, 3H, $N^+C\underline{H}_3$), 2.00-1.90 (m, 2H, $CH_2C\underline{H}_2CH_2$), 1.36 (t, 6H, $^3J_{H\text{-}H}$ = 7.2 Hz, $N^+(CH_2C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.65 ($C_{Ar}NO_2$), 134.94 (d, $^3J_{C\text{-}P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.32 (CH_{Ar}), 59.75 ($CH_2\underline{C}H_2N^+$), 57.92 (2C, $N^+(\underline{C}H_2CH_3)_2$), 57.89 ($OCH_2$), 50.09 (d, 2C, $^2J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 47.99 ($N^+CH_3$), 43.22 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 38.84 ($CH_2NHP$), 35.32 ($NCH_3$), 25.51 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, $CH_2\underline{C}H_2CH_2$), 8.22 (2C, $N^+(CH_2\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.03; **HRMS** (ESI) m/z 487.1750 $[M]^+$ (calculated for $[C_{17}H_{34}Cl_2N_6O_4P]^+$ 487.1751).

4.1.4.11. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N*-diethyl-*N*-methylethane-1-aminium iodide (29d)

Alkylation of amine **24d** (100 mg, 218 µmol) with methyl iodide (12 eq.) using the standard procedure (reaction time: 25 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **29d** as a hygroscopic lyophilisate (104 mg, 173 µmol). **Yield** 79%; **IR (ATR) ν cm^{-1}** 3433, 3195, 1537, 1489, 1350, 1223, 1190, 1006, 977, 960, 835, 772, 745; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.29 (s, 1H, CH_{Ar}), 5.20 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.4 Hz, CH'O), 5.17 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.75-3.65 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.52-3.32 (m, 12H, $N(C\underline{H}_2CH_2Cl)_2$, $C\underline{H}_2C\underline{H}_2N^+(C\underline{H}_2CH_3)_2$), 3.06 (s, 3H, N^+CH_3), 1.36 (t, 6H, $^3J_{H\text{-}H}$ = 7.2 Hz, $N^+(CH_2C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.73 ($C_{Ar}NO_2$), 134.70 (d, $^3J_{C\text{-}P}$ = 6.8 Hz, $\underline{C}_{Ar}CH_2$), 129.40 (CH_{Ar}), 61.25 ($\underline{C}H_2N^+(CH_2CH_3)_2CH_3$), 58.37 (2C, $N^+(\underline{C}H_2CH_3)_2$), 58.08 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, OCH_2), 49.86 (d, 2C, $^2J_{C\text{-}P}$ = 4.5 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 48.55 ($N^+CH_3$), 43.14 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 35.40 ($CH_2NHP$), 35.25 ($NCH_3$), 8.15 (2C, $N^+(CH_2\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.70; **HRMS** (ESI) m/z 473.1599 $[M]^+$ (calculated for $[C_{16}H_{32}Cl_2N_6O_4P]^+$ 473.1594).

4.1.4.12. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-triethylpentane-1-aminium iodide (29e)

Alkylation of amine **24a** (124 mg, 247 µmol) with ethyl iodide (6 eq. initially followed by 6 eq at T 4h and 6 eq at T 6h) using the standard procedure (reaction time: 16 h) afforded compound **29e** analytically pure, as a yellow hygroscopic solid (146 mg, 222 µmol). **Yield** 90%; **IR (ATR) ν**

cm^{-1} 3416, 3210, 1537, 1488, 1349, 1217, 1189, 1004, 978, 959, 835, 772, 744; **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.26 (s, 1H, CH_{Ar}), 5.17 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.3 Hz, CH'O), 5.12 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.6 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.70-3.62 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.50-3.32 (m, 4H, $N(C\underline{H}_2CH_2Cl)_2$), 3.35 (q, 6H, $^{3}J_{H\text{-}H}$ = 7.2 Hz, $N^+(C\underline{H}_2CH_3)_3$), 3.24-3.20 (m, 2H, $CH_2C\underline{H}_2N^+$), 2.91 (td, 2H, $^{3}J_{H\text{-}P}$ = 11.1 Hz, $^{3}J_{H\text{-}H}$ = 6.8 Hz, $C\underline{H}_2NHP$), 1.75-1.68 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.60 (qt, 2H, $^{3}J_{H\text{-}H}$ = 7.1 Hz, $C\underline{H}_2CH_2NHP$), 1.50-1.38 (m, 2H, $C\underline{H}_2CH_2CH_2NHP$), 1.31 (m, 9H, $N^+(CH_2C\underline{H}_3)_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.61 ($C_{Ar}NO_2$), 135.12 (d, $^{3}J_{C\text{-}P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.29 (CH_{Ar}), 58.15 ($CH_2\underline{C}H_2N^+$), 57.73 (d, $^{2}J_{C\text{-}P}$ = 4.6 Hz, OCH_2), 54.04 (3C, $N^+(\underline{C}H_2CH_3)_3$), 50.31 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.29 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.42 ($CH_2NHP$), 35.30 ($NCH_3$), 32.20 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, $\underline{C}H_2CH_2NHP$), 24.55 ($\underline{C}H_2CH_2CH_2NHP$), 22.43 ($\underline{C}H_2CH_2N^+$), 7.89 (3C, $N^+(CH_2CH_3)_3$); **^{31}P NMR (202 MHz, CD$_3$OD) δ** 18.40; **HRMS** (ESI) m/z 529.2231 $[M]^+$ (calculated for $[C_{20}H_{40}Cl_2N_6O_4P]^+$ 529.2220).

4.1.4.13. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-triethylbutane-1-aminium iodide (29f)

Alkylation of amine **24b** (28 mg, 57.8 µmol) with ethyl iodide (18 eq. initially followed by 18 eq after 15 h) using the standard procedure (reaction time: 56 h) afforded compound **29f** analytically pure, as a yellow hygroscopic solid (28 mg, 43.5 µmol). **Yield** 78%; **IR (ATR) ν cm^{-1}** 3414, 3255, 1537, 1488, 1350, 1221, 1190, 1007, 835, 744; **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.26 (s, 1H, CH_{Ar}), 5.21-5.09 (m, 2H, CH_2O), 4.08 (s, 3H, NCH_3), 3.73-3.61 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.52-3.32 (m, 4H, $N(C\underline{H}_2CH_2Cl)_2$), 3.35 (q, 6H, $^{3}J_{H\text{-}H}$ = 7.3 Hz, $N^+(C\underline{H}_2CH_3)_3$), 3.24-3.19 (m, 2H, $CH_2C\underline{H}_2N^+$), 2.96 (td, 2H, $^{3}J_{H\text{-}P}$ = 11.4 Hz, $^{3}J_{H\text{-}H}$ = 6.7 Hz, $C\underline{H}_2NHP$), 1.79-1.72 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.63-1.53 (m, 2H, $C\underline{H}_2CH_2NHP$), 1.36-1.28 (m, 9H, $N^+(CH_2C\underline{H}_3)_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.65 ($C_{Ar}NO_2$), 135.04 (d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.26 (CH_{Ar}), 57.92 ($CH_2\underline{C}H_2N^+$), 57.76 (d, $^{2}J_{C\text{-}P}$ = 4.6 Hz, OCH_2), 54.07 (3C, $N^+(\underline{C}H_2CH_3)_3$), 50.24 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.6 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.25 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.19 ($CH_2NHP$), 35.27 ($NCH_3$), 29.56 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, $\underline{C}H_2CH_2NHP$), 20.29 ($\underline{C}H_2CH_2N^+$), 7.85 (3C, $N^+(CH_2\underline{C}H_3)_3$); **^{31}P NMR (202 MHz, CD$_3$OD) δ** 18.34; **HRMS** (ESI) m/z 515.2049 $[M]^+$ (calculated for $[C_{19}H_{38}Cl_2N_6O_4P]^+$ 515.2064).

4.1.4.14. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-triethylpropane-1-aminium iodide (29g)

Alkylation of amine **24c** (19 mg, 39.7 µmol) with ethyl iodide (12 eq. initially followed by 12 eq at T 24h and 12 eq at T 36h) using the standard procedure (reaction time: 72 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **29g** as a hygroscopic lyophilisate. **Yield** 23%; **IR (ATR) ν cm^{-1}** 3431, 3229 (ν_{NH}), 1537 (δ_{NH}), 1489 (ν_{asNO2}), 1351 (ν_{sNO2}), 1215, 1190 ($\nu_{P=O}$, ν_{C-N}), 1008, 982, 960 (ν_{P-O}, ν_{C-O}, ν_{P-N}), 836 (δ_{NH}), 745 (δ_{CH}); **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.26 (s, 1H, CH_{Ar}), 5.18 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.4 Hz, CH'O), 5.14 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.5 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.74-3.64 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.63-3.25 (m, 12H, $N(C\underline{H}_2CH_2Cl)_2$, $C\underline{H}_2N^+(C\underline{H}_2CH_3)_3$), 3.00 (td, 2H, $^3J_{H-P}$ = 12.2 Hz, $^3J_{H-H}$ = 6.4 Hz, $C\underline{H}_2NHP$), 1.98-1.80 (m, 2H, $C\underline{H}_2CH_2NHP$), 1.31 (t, 9H, $^3J_{H-H}$ = 6.2 Hz, $N^+(CH_2C\underline{H}_3)_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.68 ($C_{Ar}NO_2$), 134.89 (d, $^3J_{C-P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.27 (CH_{Ar}), 57.85 (d, $^2J_{C-P}$ = 4.6 Hz, OCH_2), 56.14 ($CH_2\underline{C}H_2N^+$), 54.16 (3C, $N^+(\underline{C}H_2CH_3)_3$), 50.07 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.18 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 38.81 ($C\underline{H}_2NHP$), 35.23 ($NCH_3$), 25.15 (d, $^3J_{C-P}$ = 5.5 Hz, $\underline{C}H_2CH_2NHP$), 7.82 (3C, $N^+(CH_2CH_3)_3$); **NMR ^{31}P (202 MHz, CD$_3$OD) δ** 18.03; **HRMS** (ESI) m/z 501.1895 $[M]^+$ (calculated for $[C_{18}H_{36}Cl_2N_6O_4P]^+$ 501.1907).

4.1.4.15. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N,N,N*-triethylethane-1-aminium iodide (29h)

Alkylation of amine **24d** (70 mg, 153 µmol) with ethyl iodide (12 eq. initially followed by 12 eq after 26 h) using the standard procedure (reaction time: 50 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **29 h** as a hygroscopic lyophilisate (57 mg, 92 µmol). **Yield** 60%; **IR (ATR) ν cm^{-1}** 3438, 3182, 1537, 1489, 1350, 1222, 1190, 1001, 958, 834, 770; **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.27 (s, 1H, CH_{Ar}), 5.20 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.4 Hz, CH'O), 5.17 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.74-3.65 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.53-3.34 (m, 4H, $N(C\underline{H}_2CH_2Cl)_2$), 3.38 (q, $^3J_{H-H}$ = 7.3 Hz, $N^+(C\underline{H}_2CH_3)_3$), 3.33-3.25 (m, 4H, $CH_2CH_2N^+$), 1.33 (t, 9H, $^3J_{H-H}$ = 7.2 Hz, $N^+(CH_2C\underline{H}_3)_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.75 ($C_{Ar}NO_2$), 134.67 (d, $^3J_{C-P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 129.43 (CH_{Ar}), 58.11 (d, $^2J_{C-P}$ = 4.6 Hz, OCH_2), 57.79 ($CH_2\underline{C}H_2N^+$), 54.58 (3C, $N^+(\underline{C}H_2CH_3)_3$), 49.95 (d, 2C, $^2J_{C-P}$ = 4.7

Hz, N(CH$_2$CH$_2$Cl)$_2$), 43.17 (d, 2C, $^3J_{C\text{-}P}$ = 1.6 Hz, N(CH$_2$CH$_2$Cl)$_2$), 35.02 (CH$_2$NHP), 35.21 (NCH$_3$), 7.82 (3C, N$^+$(CH$_2$CH$_3$)$_3$); **NMR ^{31}P (202 MHz, CD$_3$OD) δ** 17.88; **HRMS** (ESI) m/z 487.1760 [M]$^+$ (calculated for [C$_{17}$H$_{34}$Cl$_2$N$_6$O$_4$P]$^+$ 487.1751).

4.1.4.16. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*,*N*-diisopropyl-*N*-methyl-pentane-1-aminium iodide (30a)

Alkylation of amine **25a** (67 mg, 126 µmol) with methyl iodide (6 eq. initially followed by 6 eq at T 20h, 6 eq at T 24h and 6 eq at T 28h) using the standard procedure (reaction time: 48 h) afforded in quantitative yield, compound **30a** analytically pure, as a yellow hygroscopic solid (85 mg, 126 mmol). **IR (ATR) ν cm^{-1}** 3411, 1537, 1489, 1351, 1190, 1014, 961, 835, 743; **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.26 (s, 1H, CH$_{Ar}$), 5.17 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.2 Hz, CH'O), 5.13 (dd, 1H, $^2J_{H\text{-}H}$ = 13.4 Hz, $^3J_{H\text{-}P}$ = 8.6 Hz, CH"O), 4.08 (s, 3H, NCH$_3$), 3.99 (hept, 2H, $^3J_{H\text{-}H}$ = 6.6 Hz, CH(CH$_3$)$_2$), 3.74-3.62 (m, 4H, N(CH$_2$CH$_2$Cl)$_2$), 3.51-3.34 (m, 4H, N(CH$_2$CH$_2$Cl)$_2$), 3.33-3.25 (m, 2H, CH$_2$N$^+$), 2.96-2.87 (m, 2H, CH$_2$NHP), 2.94 (s, 3H, N$^+$CH$_3$), 1.88-1.77 (m, 2H, CH$_2$CH$_2$N$^+$), 1.60 (qt, 2H, $^3J_{H\text{-}H}$ = 7.1 Hz, CH$_2$CH$_2$NHP), 1.47 (d, $^3J_{H\text{-}H}$ = 6.3 Hz, CH(CH$_3$)$_2$), 1.44-1.38 (m, 2H, CH$_2$CH$_2$CH$_2$NHP); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.60 (C$_{Ar}$NO$_2$), 135.12 (d, $^3J_{C\text{-}P}$ = 7.1 Hz, C$_{Ar}$CH$_2$), 129.29 (CH$_{Ar}$), 64.92 (2C, CH(CH$_3$)$_2$), 58.34 (CH$_2$N$^+$), 57.74 (d, $^2J_{C\text{-}P}$ = 4.7 Hz, OCH$_2$), 50.31 (d, 2C, $^2J_{C\text{-}P}$ = 4.7 Hz, N(CH$_2$CH$_2$Cl)$_2$), 44.17 (N$^+$CH$_3$), 43.30 (2C, N(CH$_2$CH$_2$Cl)$_2$), 41.45 (CH$_2$NHP), 35.32 (NCH$_3$), 32.13 (d, $^3J_{C\text{-}P}$ = 5.6 Hz, CH$_2$CH$_2$NHP), 25.11 (CH$_2$CH$_2$CH$_2$NHP), 24.79 (CH$_2$CH$_2$N$^+$), 17.96, 17.69 (4C, CH(CH$_3$)$_2$); **^{31}P NMR (202 MHz, CD$_3$OD) δ** 18.39; **HRMS** (ESI) m/z 543.2384 [M]$^+$ (calculated for [C$_{21}$H$_{42}$Cl$_2$N$_6$O$_4$P]$^+$ 543.2377).

4.1.4.17. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*,*N*-diisopropyl-*N*-methyl-butane-1-aminium iodide (30b)

Alkylation of amine **25b** (72 mg, 141 µmol) with methyl iodide (12 eq. initially followed by 12 eq after 3 h) using the standard procedure (reaction time: 72 h) afforded compound **30b** analytically pure, as a yellow hygroscopic solid (77 mg, 117 µmol). **Yield** 83%; **IR (ATR) ν cm^{-1}** 3424, 3202, 1537, 1489, 1350, 1219, 1190, 1007, 980, 960, 835, 743; **^{1}H NMR (200 MHz,**

CD_3OD) δ (ppm) 7.26 (s, 1H, CH_{Ar}), 5.25-5.04 (m, 2H, CH_2O), 4.08 (s, 3H, NCH_3), 4.02-3.92 (m, 2H, $C\underline{H}(CH_3)_2$), 3.75-3.60 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.55-3.34 (m, 6H, $N(C\underline{H}_2CH_2Cl)_2$, CH_2N^+), 3.05-2.86 (m, 2H, $C\underline{H}_2NHP$), 2.93 (s, 3H, $N^+C\underline{H}_3$), 1.93-1.73 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.64-1.40 (m, 2H, $C\underline{H}_2CH_2NHP$), 1.47 (d, 12H, $^3J_{H-H}$ = 6.5 Hz, $CH(C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ (ppm)** 147.62 ($C_{Ar}NO_2$), 135.06 (d, $^3J_{C-P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.27 (CH_{Ar}), 65.21, 64.97 ($C\underline{H}(CH_3)_2$), 58.06 (CH_2N^+), 57.75 (d, $^2J_{C-P}$ = 4.7 Hz, OCH_2), 50.24 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 44.19 ($N^+CH_3$), 43.26 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.12 ($CH_2NHP$), 35.30 ($NCH_3$), 30.06 (d, $^3J_{C-P}$ = 5.4 Hz, $\underline{C}H_2CH_2NHP$), 22.59 ($\underline{C}H_2CH_2N^+$), 17.95, 17.67 (4C, $CH(\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ (ppm)** 18.26; **HRMS** (ESI) m/z 529.2222 $[M]^+$ (calculated for $[C_{20}H_{40}Cl_2N_6O_4P]^+$ 529.2220).

4.1.4.18. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*,*N*-diisopropyl-*N*-methyl-propane-1-aminium iodide (30c)

Alkylation of amine **25c** (50 mg, 100 µmol) with methyl iodide (12 eq. initially followed by 12 eq after 72 h) using the standard procedure (reaction time: 96 h) afforded compound **30c** analytically pure, as a yellow hygroscopic solid (61 mg, 95.3 mmol). **Yield** 96%; **IR (ATR) ν cm^{-1}** 3413, 3209, 1537, 1490, 1350, 1217, 1190, 1007, 980, 960, 835, 743; **^{1}H NMR (500 MHz, CD_3OD) δ (ppm)** 7.27 (s, 1H, CH_{Ar}), 5.20 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.1 Hz, CH'O), 5.15 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 3.98 (hept, 2H, $^3J_{H-H}$ = 6.5 Hz, $C\underline{H}(CH_3)_2$), 3.74-3.63 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.54-3.33 (m, 6H, $N(C\underline{H}_2CH_2Cl)_2$, CH_2N^+), 3.04-2.94 (m, 2H, $C\underline{H}_2NHP$), 2.94 (s, 3H, $N^+C\underline{H}_3$), 2.06-1.92 (m, 2H, $CH_2C\underline{H}_2CH_2$), 1.47 (d, 12H, $^3J_{H-H}$ = 6.4 Hz, $CH(C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.59 ($C_{Ar}NO_2$), 134.95 (d, $^3J_{C-P}$ = 7.4 Hz, $\underline{C}_{Ar}CH_2$), 129.31 (CH_{Ar}), 65.17, 65.11 (2C, $\underline{C}H(CH_3)_2$), 57.89 (d, $^2J_{C-P}$ = 4.8 Hz, OCH_2), 56.32 (CH_2N^+), 50.07 (d, 2C, $^2J_{C-P}$= 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 44.37 ($N^+CH_3$), 43.24 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 39.14 ($CH_2NHP$), 35.37 ($NCH_3$), 27.26 (d, $^3J_{C-P}$ = 4.3 Hz, $CH_2\underline{C}H_2CH_2$), 17.96, 17.69 (4C, $CH(\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.94; **HRMS** (ESI) m/z 515.2067 $[M]^+$ (calculated for $[C_{19}H_{38}Cl_2N_6O_4P]^+$ 515.2064).

4.1.4.19. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*,*N*-diisopropyl-*N*-methyl-ethane-1-aminium iodide (30d)

Alkylation of amine **25d** (28 mg, 57.5 µmol) with methyl iodide (24 eq. initially followed by 24 eq after 15 h) using the standard procedure (reaction time: 96 h) afforded compound **30d** analytically pure, as a yellow hygroscopic solid (30 mg, 47.7 mmol). **Yield** 83%; **IR (ATR) ν cm^{-1}** 3425, 3210, 1537, 1490, 1351, 1220, 1190, 1008, 960, 836, 743; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.29 (s, 1H, CH_{Ar}), 5.21 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.4 Hz, CH'O), 5.12 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.4 Hz, $^{3}J_{H\text{-}P}$ = 8.4 Hz, CH"O), 4.08 (s, 3H, NCH_3), 4.03-3.89 (m, 3H, NHP, C$\underline{H}(CH_3)_2$), 3.70 (td, 4H, $^{3}J_{H\text{-}H}$ = 6.5 Hz, $^{4}J_{H\text{-}P}$ = 3.4 Hz, N($CH_2C\underline{H}_2Cl)_2$), 3.52-3.26 (m, 8H, N($C\underline{H}_2CH_2Cl)_2$, $C\underline{H}_2C\underline{H}_2$NHP), 2.96 (s, 3H, N^+CH_3), 1.43-1.50 (m, 12H, CH($C\underline{H}_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.69 ($C_{Ar}NO_2$), 134.70 (d, $^{3}J_{C\text{-}P}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 129.45 (CH_{Ar}), 65.60, 65.54 ($\underline{C}H(CH_3)_2$), 58.16 (d, $^{2}J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 57.85 (d, $^{3}J_{C\text{-}P}$ = 3.1 Hz, CH_2N^+), 49.92 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.7 Hz, N($\underline{C}H_2CH_2Cl)_2$), 43.75 ($N^+CH_3$), 43.22 (2C, N($CH_2\underline{C}H_2Cl)_2$), 36.61 ($CH_2$NHP), 35.33 ($NCH_3$), 17.87, 17.50 (4C, CH($\underline{C}H_3)_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.69; **HRMS** (ESI) m/z 501.1894 $[M]^+$ (calculated for $[C_{18}H_{36}Cl_2N_6O_4P]^+$ 501.1907).

4.1.4.20. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*,*N*-dimethylpentane-1-aminium (31a)

Alkylation of amine **26a** (33 mg, 61.3 µmol) with methyl iodide (6 eq.) using the standard procedure (reaction time: 20 h) afforded compound **31a** analytically pure, as a yellow hygroscopic solid (40 mg, 57.9 µmol). Yield 94%; **IR (ATR) ν cm^{-1}** 3421, 3220, 1652, 1537, 1488, 1350, 1219, 1190, 1005, 960, 834, 729, 703; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.65-7.50 (m, 5H, CH_{Ar}), 7.25 (s, 1H, CH_{Ar}), 5.16 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.5 Hz, $^{3}J_{H\text{-}P}$ = 8.4 Hz, CH'O), 5.12 (dd, 1H, $^{2}J_{H\text{-}H}$ = 13.5 Hz, $^{3}J_{H\text{-}P}$ = 8.9 Hz, CH"O), 4.57 (s, 2H, $C\underline{H}_2$Ph), 4.07 (s, 3H, NCH_3), 3.74-3.61 (m, 4H, N($CH_2C\underline{H}_2Cl)_2$), 3.51-3.33 (m, 7H, N($C\underline{H}_2CH_2Cl)_2$, $CH_2C\underline{H}_2N^+$, NHP), 3.05 (s, 6H, $N^+(C\underline{H}_3)_2$), 2.91 (tdd, 2H, $^{3}J_{H\text{-}P}$ = 11.1 Hz, $^{3}J_{H\text{-}H}$ = 6.7 Hz, $^{3}J_{H\text{-}H}$ = 2.0 Hz, $C\underline{H}_2$NHP), 1.94-1.87 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.60 (qt, 2H, $^{3}J_{H\text{-}H}$ = 7.1 Hz, $C\underline{H}_2CH_2$NHP), 1.47-1.37 (m, 2H, $C\underline{H}_2CH_2CH_2$NHP); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.64 ($C_{Ar}NO_2$), 135.10 (d, $^{3}J_{C\text{-}P}$ = 6.9 Hz, $\underline{C}_{Ar}CH_2O$), 134.13 ($\underline{C}_{Ar}CH_2N$), 131.95, 130.38, 129.27, 128.89 (6C, CH_{Ar}), 68.98 ($\underline{C}H_2$Ph), 65.78 ($CH_2\underline{C}H_2N^+$), 57.72 (d, $^{2}J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 50.50 (2C, $N^+(\underline{C}H_3)_2$), 50.31 (d, 2C, $^{2}J_{C\text{-}P}$ = 4.7 Hz, N($\underline{C}H_2CH_2Cl)_2$), 43.24 (2C, N($CH_2\underline{C}H_2Cl)_2$), 41.40 ($CH_2$NHP), 35.22 ($NCH_3$), 32.21 (d, $^{3}J_{C\text{-}P}$ = 5.5 Hz, $\underline{C}H_2CH_2$NHP), 24.50 ($C\underline{H}_2CH_2CH_2$NHP), 23.35

($\underline{C}H_2CH_2N^+$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.27; **HRMS** (ESI) m/z 563.2047 $[M]^+$ (calculated for $[C_{23}H_{38}Cl_2N_6O_4P]^+$ 563.2064).

4.1.4.21. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*,*N*-dimethylbutane-1-aminium iodide (31b)

Alkylation of amine **26b** (31 mg, 56.4 µmol) with methyl iodide (6 eq.) using the standard procedure (reaction time: 20 h) afforded in quantitative yield, compound **31b** analytically pure, as a yellow hygroscopic solid (38 mg, 56.1 µmol). **IR (ATR) ν cm^{-1}** 3419, 3231, 1537, 1489, 1350, 1216, 1190, 1003, 980, 960, 835, 729, 703; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.61-7.51 (m, 5H, CH_{Ar}), 7.25 (s, 1H, CH_{Ar}), 5.18 (dd, 1H, $^2J_{H\text{-}H}$ = 13.7 Hz, $^3J_{H\text{-}P}$ = 8.5 Hz, CH'O), 5.13 (dd, 1H, $^2J_{H\text{-}H}$ = 13.7 Hz, $^3J_{H\text{-}P}$ = 8.9 Hz, CH"O), 4.57 (s, 2H, $C\underline{H}_2Ph$), 4.07 (s, 3H, NCH_3), 3.70-3.66 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.51-3.34 (m, 6H, $N(C\underline{H}_2CH_2Cl)_2$, $CH_2C\underline{H}_2N^+$), 3.06 (s, 6H, $N^+(C\underline{H}_3)_2$), 2.97 (td, 2H, $^3J_{H\text{-}P}$ = 11.4 Hz, $^3J_{H\text{-}H}$ = 6.8 Hz, $C\underline{H}_2NHP$), 1.99-1.91 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.58 (qt, 2H, $^3J_{C\text{-}P}$ = 7.0 Hz, $C\underline{H}_2CH_2NHP$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.66 ($C_{Ar}NO_2$), 135.02 (d, $^3J_{C\text{-}P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2O$), 134.13 ($\underline{C}_{Ar}CH_2N$), 131.97, 130.39, 129.26, 128.86 (6C, CH_{Ar}), 68.98 ($\underline{C}H_2Ph$), 65.45 ($CH_2\underline{C}H_2N^+$), 57.79 (d, $^2J_{C\text{-}P}$ = 4.7 Hz, OCH_2), 50.53 (2C, $N^+(CH_3)_2$), 50.26 (d, 2C, $^2J_{C\text{-}P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.24 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.08 ($CH_2NHP$), 35.25 ($NCH_3$), 29.52 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, $\underline{C}H_2CH_2NHP$), 21.18 ($\underline{C}H_2CH_2N^+$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.26; **HRMS** (ESI) m/z 549.1895 $[M]^+$ (calculated for $[C_{22}H_{36}Cl_2N_6O_4P]^+$ 549.1907).

4.1.4.22. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*,*N*-dimethylpropane-1-aminium iodide (31c)

Method A: Alkylation of amine **26c** (28 mg, 53.7 µmol) with methyl iodide (6 eq) using the standard procedure (reaction time: 20 h) afforded compound **31c** analytically pure, as a yellow hygroscopic solid (33 mg, 49.7 µmol). **Yield** 93%.

Method B: Alkylation of amine **23c** (52 mg, 117 µmol) was carried out with 24 equivalents of benzyl iodide[44] (351 µL, 2.82 mmol) in the presence of potassium carbonate (97 mg, 702 µmol). The work up of the reaction mixture according to the general procedure allowed to obtain a crude product that was dissolved in a minimum amount of water and washed with DCM (10

mL). The aqueous layer was decanted and then lyophilised, to afford compound **31c**, analytically pure, as a yellow hygroscopic solid (23 mg, 34.7 mmol). **Yield** 30%. **IR (ATR) ν cm^{-1}** 3427, 3214 (ν_{NH}), 1645, 1537 (δ_{NH}), 1489 (ν_{asNO2}), 1350 (ν_{sNO2}), 1217, 1190 ($\nu_{P=O}$, ν_{C-N}), 1005, 977, 960 (ν_{P-O}, ν_{C-O}, ν_{P-N}), 835 (δ_{NH}), 729, 702 (δ_{CH}); **^{1}H NMR (500 MHz, CD_3OD) δ** 7.61-7.51 (m, 5H, CH_{Ar}), 7.25 (s, 1H, CH_{Ar}), 5.17 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.3 Hz, CH'O), 5.13 (dd, 1H, $^2J_{H-H}$ = 13.4 Hz, $^3J_{H-P}$ = 8.4 Hz, CH"O), 4.58 (s, 2H, C$\underline{H}_2$Ph), 4.07 (s, 3H, NCH_3), 3.73-3.63 (m, 4H, N($CH_2C\underline{H}_2Cl)_2$), 3.52-3.35 (m, 6H, N($C\underline{H}_2CH_2Cl)_2$, $CH_2C\underline{H}_2N^+$), 3.07 (s, 6H, $N^+(C\underline{H}_3)_2$), 3.03 (td, 2H, $^3J_{H-P}$ = 12.5 Hz, $^3J_{H-H}$ = 6.5 Hz, C$\underline{H}_2$NHP), 2.13-2.05 (m, 2H, $CH_2C\underline{H}_2CH_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.68 ($C_{Ar}NO_2$), 134.89 (d, $^3J_{C-P}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2O$), 134.23 ($\underline{C}_{Ar}CH_2N$), 131.99, 130.40, 129.33, 128.78 (6C, CH_{Ar}), 69.31 ($\underline{C}H_2$Ph), 63.67 ($CH_2\underline{C}H_2N^+$), 57.92 (d, $^2J_{C-P}$ = 4.8 Hz, OCH_2), 50.67, 50.65 ($N^+(\underline{C}H_3)_2$), 50.09 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, N($\underline{C}H_2CH_2Cl)_2$), 43.21 (2C, N($CH_2\underline{C}H_2Cl)_2$), 38.89 ($CH_2$NHP), 35.31 ($NCH_3$), 26.03 ($CH_2\underline{C}H_2CH_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.01; **HRMS** (ESI) m/z 535.1750 $[M]^+$ (calculated for $[C_{21}H_{34}Cl_2N_6O_4P]^+$ 535.1751).

4.1.4.23. 2-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*,*N*-dimethylethane-1-aminium iodide (31d)

Alkylation of amine **26d** (24 mg, 47.3 µmol) with methyl iodide (12 eq.) using the standard procedure (reaction time: 12 h) afforded in quantitative yield, compound **31d** analytically pure, as a yellow hygroscopic solid (30 mg, 47.3 µmol). **IR (ATR) ν cm^{-1}** 3421, 3203, 1537, 1489, 1350, 1217, 1191, 1000, 980, 959, 835, 765, 744, 703; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.63-7.51 (m, 5H, CH_{Ar}), 7.25 (s, 1H, CH_{Ar}), 5.19 (m, 2H, OCH_2), 4.60 (s, 2H, C$\underline{H}_2$Ph), 4.06 (s, 3H, NCH_3), 3.69 (td, 4H, $^3J_{H-H}$ = 6.6 Hz, $^3J_{H-P}$ = 3.1 Hz, N($CH_2C\underline{H}_2Cl)_2$), 3.52-3.34 (m, 8H, N($C\underline{H}_2CH_2Cl)_2$, $C\underline{H}_2C\underline{H}_2$NHP), 3.11 (s, 6H, $N^+(CH_3)_2$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.70 ($C_{Ar}NO_2$), 134.65 (d, $^3J_{C-P}$ = 7.1 Hz, $\underline{C}_{Ar}CH_2O$), 134.24 ($\underline{C}_{Ar}CH_2N$), 132.11, 130.44, 129.39, 128.57 (6C, CH_{Ar}), 70.01 ($\underline{C}H_2$Ph), 65.35 ($CH_2\underline{C}H_2N^+$), 58.08 (d, $^2J_{C-P}$ = 4.9 Hz, OCH_2), 50.96, 50.94 ($N^+(\underline{C}H_3)_2$), 49.85 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, N($\underline{C}H_2CH_2Cl)_2$), 43.12 (2C, N($CH_2\underline{C}H_2Cl)_2$), 35.91 ($CH_2$NHP), 35.24 ($NCH_3$); **^{31}P NMR (202 MHz, CD_3OD) δ** 17.80; **HRMS** (ESI) m/z 521.1595 $[M]^+$ (calculated for $[C_{20}H_{32}Cl_2N_6O_4P]^+$ 521.1594).

4.1.4.24. 5-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*-ethyl-*N*-methylpentane-1-aminium iodide (31e)

Alkylation of amine **26a** (32 mg, 58.2 mmol) with ethyl iodide (18 eq. initially followed by 18 eq at T 16h and 18 eq at T 24h) using the standard procedure (reaction time: 72 h) afforded in quantitative yield, compound **31e** analytically pure, as a yellow hygroscopic solid (40 mg, 56.7 µmol). **IR (ATR) ν cm^{-1}** 3425, 3240, 1537, 1489, 1350, 1215, 1190, 1008, 960, 835, 765, 744, 702; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.64-7.50 (m, 5H, CH_{Ar}), 7.24 (s, 1H, CH_{Ar}), 5.15 (dd, 1H, $^{2}J_{H-H}$ = 13.3 Hz, $^{3}J_{H-P}$ = 8.3 Hz, CH'O), 5.11 (dd, 1H, $^{2}J_{H-H}$ = 13.1 Hz, $^{3}J_{H-P}$ = 8.1 Hz, CH"O), 4.54 (s, 2H, $C\underline{H}_2Ph$), 4.07 (s, 3H, NCH_3), 3.74-3.61 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.50-3.33 (m, 6H, $N(C\underline{H}_2CH_2Cl)_2$, $N^+C\underline{H}_2CH_3$), 3.29-3.18 (m, 2H, $CH_2C\underline{H}_2N^+$), 2.96 (s, 3H, N^+CH_3), 2.94-2.87 (m, 2H, $C\underline{H}_2NHP$), 1.85-1.77 (m, 2H, $C\underline{H}_2CH_2N^+$), 1.60 (qt, 2H, $^{3}J_{H-H}$ = 6.9 Hz, $C\underline{H}_2CH_2NHP$), 1.48-1.30 (m, 5H, $C\underline{H}_2CH_2CH_2NHP$, $N^+CH_2C\underline{H}_3$); **^{13}C NMR (126 MHz, CD_3OD) δ** 147.65 ($C_{Ar}NO_2$), 135.09 (d, $^{3}J_{C-P}$ = 7.0 Hz, $\underline{C}_{Ar}CH_2O$), 134.09 ($\underline{C}_{Ar}CH_2N$), 131.88, 130.40, 129.26, 128.78 (6C, CH_{Ar}), 66.42 ($\underline{C}H_2Ph$), 61.75 ($CH_2\underline{C}H_2N^+$), 57.71 (d, $^{2}J_{C-P}$ = 4.3 Hz, OCH_2), 57.70 ($N^+\underline{C}H_2CH_3$), 50.30 (d, 2C, $^{2}J_{C-P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 47.86 ($N^+CH_3$), 43.24 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 41.43 ($CH_2NHP$), 35.20 ($NCH_3$), 32.23 (d, $^{3}J_{C-P}$ = 5.5 Hz, $\underline{C}H_2CH_2NHP$), 24.53 ($\underline{C}H_2CH_2CH_2NHP$), 23.01 ($\underline{C}H_2CH_2N^+$), 8.47 ($N^+CH_2\underline{C}H_3$); **NMR ^{31}P (202 MHz, CD_3OD) δ** 18.40; **HRMS** (ESI) m/z 577.2204 $[M]^+$ (calculated for $[C_{24}H_{40}Cl_2N_6O_4P]^+$ 577.2220).

4.1.4.25. 4-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*-ethyl-*N*-methylbutane-1-aminium iodide (31f)

Alkylation of amine **26b** (49 mg, 90.8 µmol) with ethyl iodide (25 eq.) using the standard procedure (reaction time: 48 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **31f** as a hygroscopic lyophilisate (29 mg, 42.5 µmol). **Yield** 47%; **IR (ATR) ν cm^{-1}** 3412, 3214, 1537, 1489, 1350, 1215, 1190, 1005, 979, 959, 834, 767, 744, 702; **^{1}H NMR (500 MHz, CD_3OD) δ** 7.60-7.50 (m, 5H, CH_{Ar}), 7.25 (s, 1H, CH_{Ar}), 5.17 (dd, 2H, $^{2}J_{H-H}$ = 13.4 Hz, $^{3}J_{H-P}$ = 8.5 Hz, CH'O), 5.13 (dd, 2H, $^{2}J_{H-H}$ = 13.4 Hz, $^{3}J_{H-P}$ = 8.5 Hz, CH"O), 4.55 (s, 2H, $C\underline{H}_2Ph$), 4.07 (s, 3H, NCH_3), 3.72-3.64 (m, 4H, $N(CH_2C\underline{H}_2Cl)_2$), 3.52-3.19 (m, 8H, $N(C\underline{H}_2CH_2Cl)_2$, $C\underline{H}_2N^+C\underline{H}_2CH_3$), 3.01-2.93 (m, 2H, $C\underline{H}_2NHP$), 2.97 (s, 3H, N^+CH_3), 2.00-1.82

(m, 2H, C<u>H</u>$_2$CH$_2$N$^+$), 1.57 (qt, 2H, $^3J_{H-H}$ = 7.2 Hz, C<u>H</u>$_2$CH$_2$NHP), 1.44 (t, 3H, $^3J_{H-H}$ = 7.3 Hz, N$^+$CH$_2$C<u>H</u>$_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.62 (C$_{Ar}$NO$_2$), 135.05 (d, $^3J_{C-P}$ = 7.3 Hz, <u>C</u>$_{Ar}$CH$_2$O), 134.12 (<u>C</u>$_{Ar}$CH$_2$N), 131.83, 130.37, 129.29, 128.76 (6C, CH$_{Ar}$), 66.36 (<u>C</u>H$_2$Ph), 61.48 (CH$_2$<u>C</u>H$_2$N$^+$), 57.81 (d, $^2J_{C-P}$ = 4.6 Hz, OCH$_2$), 57.79 (N$^+$<u>C</u>H$_2$CH$_3$), 50.27 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, N(<u>C</u>H$_2$CH$_2$Cl)$_2$), 47.94 (N$^+$CH$_3$), 43.30 (2C, N(CH$_2$<u>C</u>H$_2$Cl)$_2$), 41.11 (CH$_2$NHP), 35.36 (NCH$_3$), 29.50 (d, $^3J_{C-P}$ = 5.2 Hz, <u>C</u>H$_2$CH$_2$NHP), 20.97 (<u>C</u>H$_2$CH$_2$N$^+$), 8.57 (N$^+$CH$_2$<u>C</u>H$_3$); **^{31}P NMR (202 MHz, CD$_3$OD) δ** 18.29; **HRMS** (ESI) m/z 563.2048 [M]$^+$ (calculated for [C$_{23}$H$_{38}$Cl$_2$N$_6$O$_4$P]$^+$ 563.2064).

4.1.4.26. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-benzyl-*N*-ethyl-*N*-methylpropane-1-aminium iodide (31g)

Alkylation of amine **26c** (32 mg, 60.7 µmol)) with ethyl iodide (24 eq. initially followed by 24 eq at T 24h and 24 eq at T 48 h) using the standard procedure (reaction time: 72 h) and purification by preparative high performance liquid chromatography (gradient ACN in water) afforded compound **31g** as a hygroscopic lyophilisate. **Yield** 32%; **IR (ATR) ν cm^{-1}** 3397, 3213, 1537, 1488, 1350, 1214, 1190, 1006, 959, 835, 761, 702; **^{1}H NMR (500 MHz, CD$_3$OD) δ** 7.61-7.49 (m, 5H, CH$_{Ar}$), 7.25 (2s, 1H, CH$_{Ar}$), 5.19-5.09 (m, 2H, CH$_2$O), 4.54 (s, 2H, CH$_2$Ph), 4.06 (2s, 3H, NCH$_3$), 3.72-3.63 (m, 4H, N(CH$_2$<u>C</u>H$_2$Cl)$_2$), 3.51-3.33 (m, 8H, N(<u>C</u>H$_2$CH$_2$Cl)$_2$, <u>C</u>H$_2$N$^+$<u>C</u>H$_2$CH$_3$), 3.06-2.96 (m, 2H, C<u>H</u>$_2$NHP), 2.97 (s, 3H, N$^+$CH$_3$), 2.13-1.93 (m, 2H, CH$_2$C<u>H</u>$_2$CH$_2$), 1.45 (t, 3H, $^3J_{H-H}$ = 7.3 Hz, N$^+$CH$_2$C<u>H</u>$_3$); **^{13}C NMR (126 MHz, CD$_3$OD) δ** 147.69 (C$_{Ar}$NO$_2$), 134.85 (d, $^3J_{C-P}$ = 7.3 Hz, <u>C</u>$_{Ar}$CH$_2$O), 134.16 (<u>C</u>$_{Ar}$CH$_2$N), 131.94, 130.43, 129.28, 128.64 (6C, CH$_{Ar}$), 66.67 (<u>C</u>H$_2$Ph), 59.69 (CH$_2$<u>C</u>H$_2$N$^+$), 57.84 (d, $^2J_{C-P}$ = 4.6 Hz, OCH$_2$), 50.05 (d, 2C, $^2J_{C-P}$ = 4.7 Hz, N(<u>C</u>H$_2$CH$_2$Cl)$_2$), 47.99 (N$^+$CH$_3$), 43.17 (2C, N(CH$_2$<u>C</u>H$_2$Cl)$_2$), 38.87 (CH$_2$NHP), 35.21 (NCH$_3$), 25.68 (d, $^3J_{C-P}$ = 4.6 Hz, CH$_2$<u>C</u>H$_2$CH$_2$), 8.46 (N$^+$CH$_2$<u>C</u>H$_3$); **^{31}P NMR (202 MHz, CD$_3$OD) δ** 18.10, 18.09; **HRMS** (ESI) m/z 549.1893 [M]$^+$ (calculated for [C$_{22}$H$_{36}$Cl$_2$N$_6$O$_4$P]$^+$ 549.1907).

4.1.4.27. 3-({[bis(2-chloroethyl)amino][(1-methyl-2-nitro-1*H*-imidazol-5-yl)methoxy]phosphoryl}amino)-*N*-methyl-*N*-piperidinylpropane-1-aminium iodide (32)

Alkylation of amine **27** (37 mg, 76.2 µmol) with methyl iodide (6 eq. initially followed by 6 eq after 20 h) using the standard procedure (reaction time: 24 h) afforded compound **32**

analytically pure, as a yellow hygroscopic solid (47 mg, 75.0 µmol). **Yield** 99%; **IR (ATR) ν cm^{-1}** 3420, 3213, 1652, 1537, 1489, 1349, 1217, 1190, 1007, 980, 959, 835, 744; **^{1}H NMR (500 MHz, $CDCl_3$) δ** 7.28 (s, 1H, CH_{Ar}), 5.20 (dd, 1H, $^{2}J_{H-H}$ = 13.4 Hz, $^{3}J_{H-P}$ = 8.3 Hz, CH'O), 5.16 (dd, 1H, $^{2}J_{H-H}$ = 13.5 Hz, $^{3}J_{H-P}$ = 8.7 Hz, CH"O), 4.09 (s, 3H, NCH_3), 3.74-3.64 (m, 4H, $N(CH_2\underline{C}H_2Cl)_2$), 3.54-3.36 (m, 10H, $N(\underline{C}H_2CH_2Cl)_2$, $\underline{C}H_2N^+(\underline{C}H_2CH_2)_2CH_2$), 3.09 (s, 3H, N^+CH_3), 3.02 (td, 2H, $^{3}J_{H-P}$ = 12.2 Hz, $^{3}J_{H-H}$ = 6.2 Hz, $\underline{C}H_2NHP$), 2.04-1.87 (m, 6H, $\underline{C}H_2CH_2N^+(CH_2\underline{C}H_2)_2CH_2$), 1.76-1.65 (m, 2H, $N^+(CH_2CH_2)_2\underline{C}H_2$); **^{13}C NMR (126 MHz, $CDCl_3$) δ** 146.67 ($C_{Ar}NO_2$), 134.94 (d, $^{3}J_{C-P}$ = 7.0 Hz, $\underline{C}_{Ar}CH_2$), 129.33 (CH_{Ar}), 62.51 (3C, $\underline{C}H_2N^+(\underline{C}H_2CH_2)_2CH_2$), 57.96 (d, $^{2}J_{C-P}$ = 4.9 Hz, OCH_2), 50.11 (d, 2C, $^{2}J_{C-P}$ = 4.7 Hz, $N(\underline{C}H_2CH_2Cl)_2$), 43.23 (2C, $N(CH_2\underline{C}H_2Cl)_2$), 38.90 ($CH_2NHP$), 35.34 ($NCH_3$), 25.20 ($\underline{C}H_2CH_2NHP$), 22.01, 21.02 (3C, $N^+(CH_2\underline{C}H_2)_2\underline{C}H_2$); **^{31}P NMR (202 MHz, CD_3OD) δ** 18.03; **HRMS** (ESI) m/z 499.1752 $[M]^+$ (calculated for $[C_{18}H_{34}Cl_2N_6O_4P]^+$ 499.1751).

4.2. Biology

4.2.1. Surface plasmon resonance (SPR) binding assay

SPR assay was carried out on a Biacore T200 instrument (GE Healthcare) with a CM4 sensor chip (GE Healthcare). First flow cell was left empty for background signal substraction and second flow cell was used for immobilization of aggrecan from bovine articular cartilage (Sigma-Aldrich). To immobilize aggrecan, the sensor chip was first activated using a 1:1 mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.5 M *N*-hydroxysuccinimide (amine coupling kit, GE Healthcare) at a flow rate of 5 µL/min for 10 min. Aggrecan was then coated on the sensor chip at 400 µg/mL in running buffer HBS-P$^+$ 1X (0.01 M HEPES, 0.15 M NaCl, 0.05% v/v surfactant p20, pH 7.4) with 6 mM hexadecyltrimethylammoniumbromide (CTAB) at 5 µL/min to a level of ~500 response units (RU). Unoccupied binding sites were blocked using 1 M ethanolamine (pH 8.5) (Amine coupling kit, GE Healthcare) at 5 µL/min for 10 min. The studied molecules (analytes) were diluted in running buffer HBS-P$^+$ 1X before application to the sensor chip at 30 µL/min with 600 seconds association time and 200 seconds dissociation time. Regeneration of the sensor chips was achieved using 2 M aqueous NaCl solution for 150 seconds. A primary screen of different compounds was performed by binding level analysis at 500 µM. The SPR responses were

obtained by subtracting the responses measured in the aggrecan immobilized flow cell with the response measured on the negative control flow cell (without immobilized aggrecan) for potential non-specific binding and 1mM. Dissociation constants (K_D) were determined for a shortlist of compounds after injection of height different concentrations of analytes (0.2, 0.4, 0.5, 0.6, 0.8, 1, 1.5, 2 mM) and using BioEvaluation software with a "steady-state affinity analysis".

4.2.2. *In vitro* anti-proliferation assay

Human HEMC-SS chondrosarcoma cell line was obtained from the European Collection of Authenticated Cell Cultures and cultured in DMEM/F12 medium (Life Technologies) supplemented with 10% fetal calf serum (Dutscher) and 4 µg/mL gentamicin.

After trypsinization, HEMC-SS cells were seeded in 96-well plates at a density of 20.10^3 cells in 150 µL of the corresponding culture medium and allowed to adhere overnight. Increasing concentrations of drugs or evofosfamide (TH-302, Abmol) diluted in DMSO (maintaining final DMSO concentration at 0.5% (v/v)) were added. After plate incubation for 24 h in normoxic (21% O_2, 5% CO_2, 37°C) or hypoxic (N_2, O_2<0.3%, 37°C) conditions, the culture media were removed and cell layers were washed with PBS. Cells were then left to grow for 48 h in normoxic conditions. Viability of cells cultured was quantified by AlamarBlue assay. Cytotoxic activity was expressed as the drug concentration that inhibited cell growth by 50% (IC_{50}). Experiments were performed at least in triplicates. Data are presented as means ± SD. Statistical significance was determined using Student's a t-Test. Results were considered significant at $p < 0.001$.

4.3. Stability measurements in aqueous buffer. Prodrug **31f** was dissolved in sodium phosphate buffer (10 mM, pH 7.4, 37 °C) containing 0.13% DMSO at a concentration of 17 µM and incubated at 37 °C. Aliquots were withdrawn at various time points over a 24 h period and

directly subjected to RP-HPLC analysis. The HPLC chromatogram peak area at 322 nm was used to calculate the concentration of the remaining prodrug. Analytical RP-HPLC measurements were performed on a HP1100 (Agilent, Palo Alto, CA, USA). The separation was carried out on a C_{18} column (Phenomenex, Luna C18, 3.0 × 150 mm, 3 µm) using the following conditions: C_{18} guard column from Phenomenex (Le Pecq, France) total experiment time: 20 min, flow rate = 0.4 mL/min, eluent mixture: H_2O/ACN (v:v) containing trifluoro acetic acid (15 mM), gradient: 80/20 at 0 min → 75/25 at 3 min, then 75/25 → 60/40 for 2 min, then 60/40 → 50/50 for 5 min, then 50/50 → 80/20 for 1 min, then 80/20 for 9 min.

4.4. ^{31}P NMR Kinetics of chemical activation. The prodrug **31f** (≈ 10 mg) was dissolved in CH_3CN (90 µL) and cacodylate buffer (300 µL, 0.1 M, pH 7.4), and sodium dithionite was dissolved in cacodylate buffer. 30 µL of the solution containing 3 eq. of sodium dithionite were added to the prodrug solution, and the reaction mixture was transferred to a 5 mm NMR tube, and the data acquisition was started. Spectra were taken at different time intervals over a 24 h period, and time points for each spectrum were assigned from the initiation of the reaction. Chemical shifts were reported relative to a Ph_3PO solution (5 % in DMSO-d_6) as a coaxial reference (26 ppm). The temperature of the probe was maintained at 37 °C using the Bruker variable temperature unit. The disappearance of the starting material as well as the appearance of the phosphoramidate anion and their relative concentrations were determined by measuring peak areas.

4.5. Stability measurements in plasma. Rabbit blood was collected in heparin anticoagulant. The blood was centrifuged at 4°C (1300 g for 10 min) to collect the plasma. The prodrug **31f** (≈ 10 mg) was dissolved in CH_3CN (90 µL) and plasma (330 µL), the mixture was transferred to a 5 mm NMR tube, and the data acquisition was started as previously described for kinetics of chemical activation.

4.6. Nitroreductase assay. Recombinant *E. coli* nitroreductase and dihydronicotinamide adenine dinucleotide (NADPH, reduced form, tetrasodium salt) were purchased from Sigma–Aldrich. 10 µL of a prodrug stock solution (1.7 mM) in ultra pure water with 13 % DMSO were added to sodium phosphate buffer (1000 µL, 10 mM, pH 7.4, 37°C) containing NADPH. The

reaction was initiated by addition of *E. coli* nitroreductase (25 or 15 µL, 1.0 mg/mL in sodium phosphate buffer; final concentrations: prodrug: 17 µM; NADPH: 0.9 mM; nitroreductase: 25 or 15 µg/mL). Aliquots were withdrawn at various time points over a 2 h period and the reactions were followed by RP-HPLC as a function of time. The separation was carried out in the same conditions as previously described for phosphate buffer stability. The HPLC chromatogram peak area at 322 nm was used to calculate the concentration of the remaining prodrug. Between the time points, all solutions were incubated at 37 °C. Reference solutions containing 10 µL of compound stock solution and 100 µL NADPH solution (9 mM) completed with phosphate buffer solution (1000 µL final volume), were prepared and analyzed under the same conditions in order to distinguish between nitroreductase-based activation and enzyme-free reaction.

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Available Supporting Information: Synthesis and characterization data of compounds **9-27** and **ICF05016.** ^{1}H NMR, ^{13}C NMR and ^{31}P NMR Spectroscopic Data for all synthesized compounds **1-32.**

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Highlights

- Proteoglycan-targeting applied to hypoxia-activated prodrug in chondrosarcoma
- A series of hypoxia-activated prodrugs was synthesized
- Screening by surface plasmon resonance technology to assess affinity to aggrecan
- Hypoxic selectivity was maintained and even increased, compared with the lead
- Positive impact of benzyle derivatives on affinity to aggrecan and hypoxic selectivity