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Article

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Pre-activated oxazaphosphorines designed for isophosphoramide mustard delivery as bulk form or nano-assemblies: synthesis and proof of concept.

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ABSTRACT: Oxazaphosphorines are alkylating agents used in routine clinical practices for treatment of cancer for many years. They are antitumor prodrugs that require cytochrome P450 bio-activation leading to 4-hydroxy derivatives. In the case of ifosfamide (IFO), the bio-activation produces two toxic metabolites; acrolein, an urotoxic compound, concomitantly generated with the isophosphoramide mustard and, chloroacetaldehyde, a neurotoxic and nephrotoxic compound, arising from the oxidation of the side chains. To improve the therapeutic index of IFO, we have designed pre-activated IFO derivatives with the covalent binding of several *O*- and *S*-alkyl moieties including poly-isoprenoid groups at the C-4 position of the oxazaphosphorine ring to avoid cytochrome bio-activation favoring the release of the active entity and limiting the chloroacetaldehyde release. Thanks to the grafted terpene moieties, some of these new conjugates demonstrated spontaneous self-assembling properties into nano-assemblies when dispersed in water. The cytotoxic activities on a panel of human tumor cell lines of these novel oxazaphosphorines, as bulk form or nano-assemblies, and the release of 4-hydroxy-IFO from these pre-activated IFO analogues in plasma are reported.

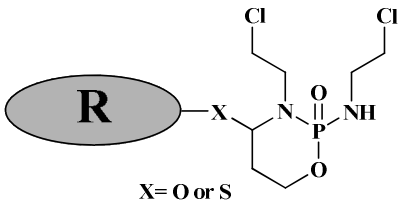
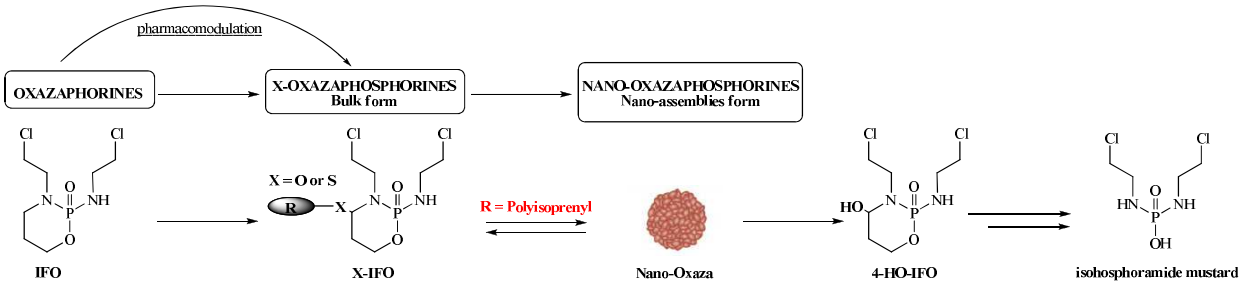


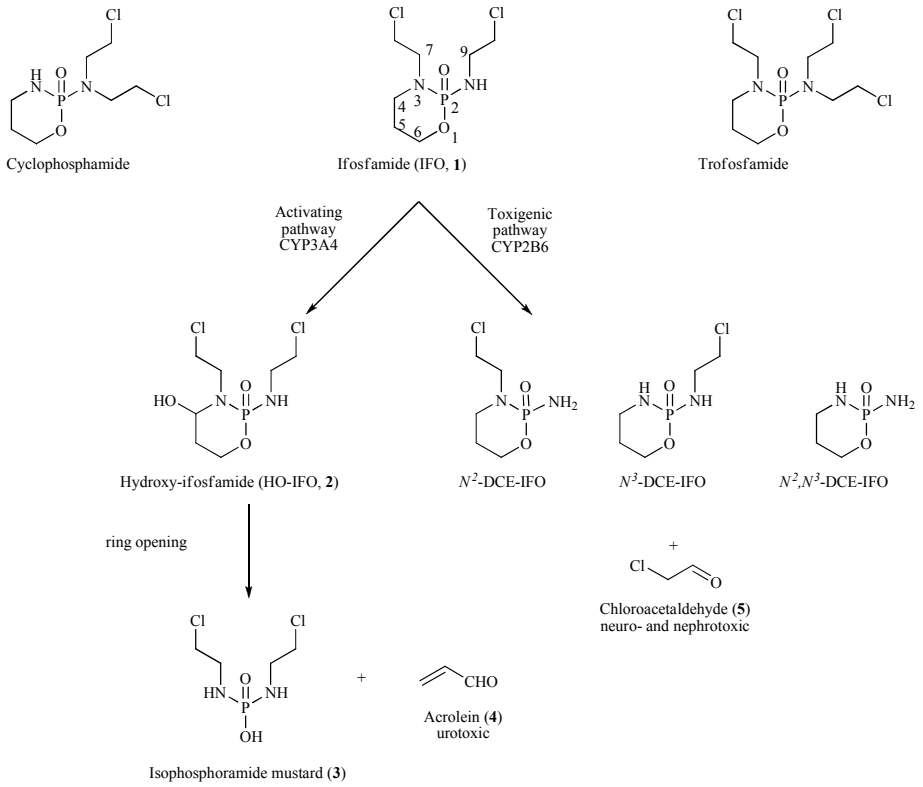
Table of contents graphic



1. Introduction.

Oxazaphosphorines belong to alkylating agents that have been widely used in routine clinical practices to treat several types of cancer from soft tissue tumor to lymphoma. Ifosfamide (IFO, **1**), cyclophosphamide and trofosfamide are oxazaphosphorines which contain two or three chloroethyl groups bounded to the nitrogen atoms (Scheme 1). As prodrugs, these compounds require a metabolic activation in position C-4 of the ring. This is fulfilled by specific liver cytochrome P450 (CYP)[1]. In the case of IFO, this leads to a hydroxylated intermediate, the 4-hydroxy-ifosfamide (4-HO-IFO, **2**), which after a ring opening mechanism can release the active isophosphoramide mustard (**3**) displaying cytotoxicity by DNA cross-links. This main activation pathway carried out by CYP3A4 leads concomitantly to the formation of acrolein (**4**) through tautomeric equilibrium and retro-Michael process. It is noteworthy that urotoxicity due to **4** and characterized by hemorrhagic cystitis, could be neutralized by co-administration of sodium mercaptoethanesulfonate (*mesna).*[2] A competitive CYP bio-activation of IFO by CYP2B6[1] (toxigenic pathway) occurring on the lateral chains (in positions C-7 and C-9) leads to the production of metabolites responsible for limiting side-effects. This secondary metabolism pathway, revealed during high dose protocols, oxidizes the chloroethyl side chains and leads to the release of *N*-deschlorethyl metabolites (N^2-DCE-IFO, N^3-DCE-IFO*,* N^2, N^3-DCE-IFO) along with chloroacetaldehyde (**5**) responsible for neuro- and nephrotoxicity which remain not countered until today.

Scheme 1: Oxazaphosphorines and metabolism of Ifosfamide.



To circumvent the toxicity related to the release of chloroacetaldehyde, pharmacomodulation of IFO have been investigated. Synthesis of analogues by engraftment of methyl groups on the side chains (to avoid chloroacetaldehyde release)[3] or replacement of the chlorine atoms by bromine[4] was described. Agents[5] that do not require *in vivo* biotransformation for activation including isophosphoramide mustard[6,7] and glufosfamide[8,9] have also been proposed, some of them reaching phase I clinical trial such as mafosfamide [10] or phase III for palifosfamide. Another strategy consists in the pre-activation of the oxazaphosphorine ring by chemical oxidation regioselectively centered on the C-4 position in order to bypass the cytochrome bio-activation. Many derivatives have already been prepared (4-OH,[11] 4-OOH,[12,13]4-OR, 4-SPh, 4-SCOR, 4-SCSR, 4-$ONHCONH_2$[14]). Although some of them showed improved *in vitro* activity, they were

found either too unstable for further development or with no advantages over the use of IFO even so the concept of activation of the position C-4 was not shown to be a drawback and needs to be pursued.

In this context, we assume that IFO analogues bearing long chain 4-alkoxy substituent might be valuable candidates since steric hindrance around the *N*-3 nitrogen atom should slow down the hydrolysis process to 4-HO-IFO and design more stable derivatives. However, the introduction of simple alkoxy derivatives would dramatically reduce the water solubility. To address the problem, we therefore chose to introduce various poly-isoprenoid chains at the C-4 position. Indeed, we have previously shown that the covalent linkage of squalene, a natural triterpene constituted of six isoprene units, to a drug belonging to various therapeutic classes, produced a bioconjugate that self-assembled into nano-assemblies (NAs) in aqueous media[15]. In most cases, the resulting NAs displayed an improved pharmaceutical profile compared to the parent compound[16,17,18,19]. The "squalenoylation" method, initially developed with highly hydrophilic nucleoside analogues has been further extended to more hydrophobic drugs such as penicillin G[20] or paclitaxel.[21] Furthermore, we recently showed that other poly-isoprenoid compounds derived from geraniol (2 isoprene units) and farnesol (3 isoprene units), were able to provide self-assembling properties to the conjugates with nucleoside analogues.[22] Based on these findings, we hypothesize that the covalent binding of a poly-isoprenoid chain of a suitable size to the C-4 carbon of IFO would deliver conjugates able to self-assemble into supramolecular objects in aqueous media. In order to modulate the release of 4-HO-IFO, we decided to use simple ether linker but also aminoethylsulfanyl linker. Thus, we report herein the synthesis of a set of lipophilic derivatives of pre-activated IFO bearing, either a geranyl, a farnesyl, or a squalene derived substituent at C-4 position (**9c-f**). To investigate the influence of the structural features within the lipophilic side chain, IFO analogs bearing a simple alkyl group (**9a**) and

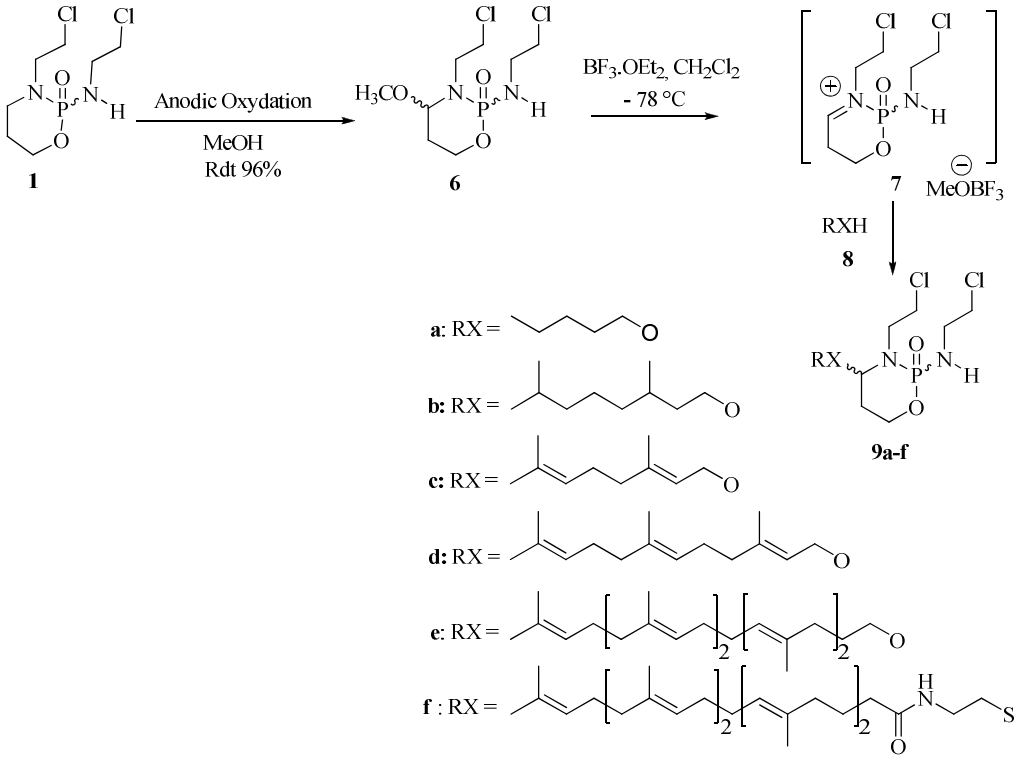
tetrahydrogeranyl group (**9b**) were also synthesized. The nano-assembly formulation of the pre-activated IFO derivatives and the *in vitro* cytotoxic activities on a panel of cancer lines of the NAs are also reported including *in vitro* investigations concerning the release of the key metabolite, i.e. 4-HO-IFO in mice plasma.

2. Results

2.1. Chemistry.

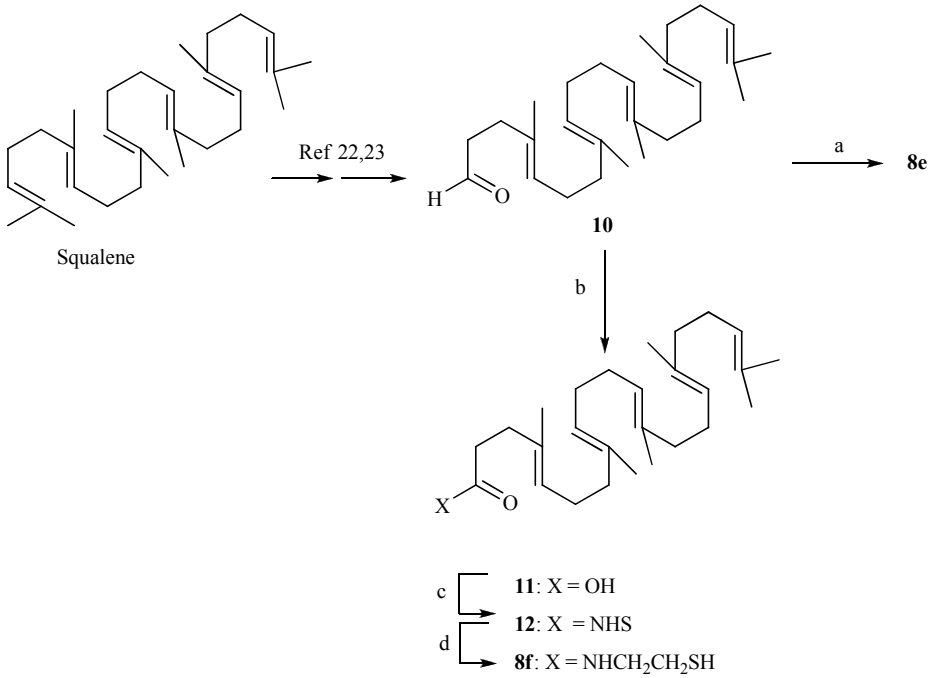
The synthesis of pre-activated conjugates **9a-f** is depicted in **Scheme 2**. According to our previous finding, anodic oxidation of IFO in methanol afforded the expected 4-methoxy-ifosfamide (MeO-IFO, **6)** as a mixture of isomers with an optimized yield of 96%.[11] The substitution of the methoxy group by the relevant alkyl chain took advantage of the formation of the iminium salt **7** upon Lewis acid treatment of **6**. A short screening of the Lewis acids (TMSOTf, $Ti(OEt)_4$, $TiCl_4$, BF_3**.**Et_2O) showed that BF_3**.**Et_2O was the most efficient reagent. Thus, treatment of **6** at -78 °C by one equivalent of BF_3**.**Et_2O followed by addition of a slight excess of nucleophiles **8a-f** gave compounds **9a-f** in 25-53% yield. All pre-activated IFO derivatives were stable enough to be purified by chromatography on silica gel. However a substantial loss of material was observed with the less lipophilic derivatives **9a-c** that accounted for their reduced yields. Conjugates **9a-f** were characterized by ^{1}H, ^{13}C, ^{31}P NMR, MS and HRMS. All compounds were shown to be obtained as diastereomeric mixtures due to the presence of the phosphorous stereogenic center together with the C-4 asymmetric center.

Scheme 2: Synthesis of the pre-activated conjugates **9a-f**.



Alcohols **8a-d** were commercially available, whereas squalene derivatives **8e,f** were obtained by synthesis from squalene and depicted in **Scheme 3**. The synthesis of 1,1',2-trisnorsqualenol (**8e**) was carried out from squalene via 1,1',2-trisnorsqualenic aldehyde (**10)** according to previously reported methods.[23,24] On the other hand, oxidation of aldehyde **10** gave the known 1,1',2-trisnorsqualenic acid (**11**).[23] Acid activation by NHS using DCC as coupling agent, followed by simple cysteamine treatment afforded **9f** in 72% overall yield.

Scheme 3: Synthesis of the squalenyl appendages **8e,f**[a].



[a] Reagents and conditions : (*a*) $NaBH_4$, EtOH, 20 °C, 0.5 h; (*b*) CrO_3, H_2SO_4, acetone 0°C; (*c*) NHS, DCC, THF, 20 °C, 24 h; (d) $HSCH_2CH_2NH_2.HCl$, Et_3N, CH_2Cl_2, 20 °C, 24h.

2.2. Preparation of supramolecular nano-assemblies.

NAs from prodrugs **9c-f** were prepared at a concentration of 2 mg/mL in a single step by nanoprecipitation, occurring spontaneously by addition of an acetonic solution of the bioconjugate (8 mg/ml) into MilliQ® water under vigourous stirring. Formation of NAs occurred immediately without the use of any surfactant. The organic solvent was then evaporated at room temperature (**9c-f**) under vacuum using a Rotavapor®. Then, NAs colloidal dispersions were stored at 4°C. These NAs that exhibited the characteristic Tyndall effect of colloidal systems had a polydispersity index lower than 0.12 as measured by quasi-elastic light scattering. The mean diameters of the obtained NAs are depicted in Table 1. Interestingly, prodrug **9c** with a short two-isoprene unit appendage gave satisfactory NAs. This finding is in agreement with our

previous results about poly-isoprenoyl conjugates of gemcitabine establishing that two-isoprene units are sufficient to provide conjugates with self-assembling properties.[21] No significant changes neither in the size nor in the polydispersity index of the NAs **9c-f** were detected over a three day storage period at 4°C. The colloidal stability of **9c-f** could be correlated with the fairly negative Zeta potentials observed ($\xi = -30$ mV for **9c**, $\xi = -20$ mV for **9d**, $\xi = -21$ mV for **9e** and $\xi = -34$ mV for **9f**). Furthermore, these highly hydrolysable compounds were found chemically unchanged by ^{1}H NMR and by MS after 24 h for **9c** and up to three days for **9d-f**. These results seem to indicate that, within the NAs, the polar head group was protected from the external aqueous medium by the hydrophobic isoprenoid core, and that longer the poly-isoprene chain is more stable the NAs are.

As expected, compounds **9a** and **9b** did not provide any auto-assembling properties, clearly demonstrating that the tri-substituted double bonds of the isoprenoid linker group played a crucial role to provide NAs. NAs of conjugates **9c-f** (2 mg/mL) were characterized using Dynamic Light Scattering (DLS) to determine their size, polydispersity index and zeta potential which are summarized in Table 1.

Table 1: Physico-chemical characteristics of the NAs of prodrugs **9c-f** at 2 mg/mL.

Compound	Mean diameter (nm)	Polydispersity Index (PdI)	Zeta potential (mV)	Drug Loading (%)
9c	126	0.10	-30	63.1
9d	195	0.16	-20	54.2
9e	147	0.12	-21	40.4
9f	167	0.06	-34	36.3

As no surfactant or any excipient was needed to obtain NAs from these materials, the drug loading could be directly deduced from respective molecular mass of the drugs and isoprenoyl moieties. This led to conclude that obtained NAs were characterized by high drug loadings compared to the currently available nano-carriers.

2.3. Transmission Electron Microscopy.

Transmission Electron Microscopy (TEM) allowed to characterize individual particles, their shape and their size. However we know that this characterization depends on their spreading onto the grids. We first studied nano-assemblies with conventional negative staining method and we observed spherical shape (150 - 200 nm) as shown in Figure 1), but unusually a few assemblies were present on the grids. We therefore explored others methods by varying the dye or the staining method. Hence, large field of particles could be observed with various sizes (90 – 300 nm) which are consistent with measurements by the nanosizer method.

Figure 1: Geranyloxy-IFO NAs suspended in water are observed by transmission electron microscopy using negative staining method by phosphotungstic acid solution. Magnification : A) 50,000; B) 85,000; C) and D) 140,000. scale bar : 100 nm.

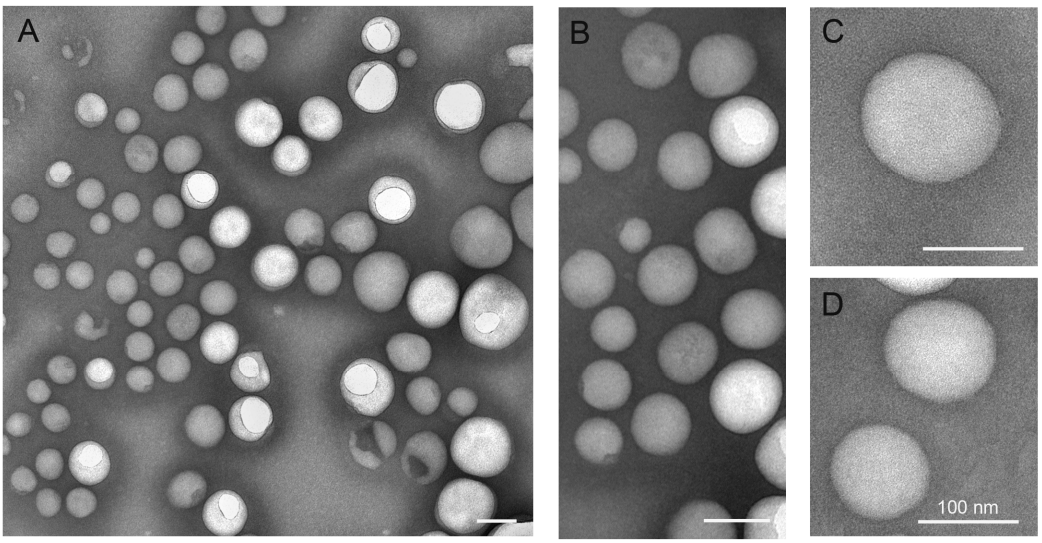
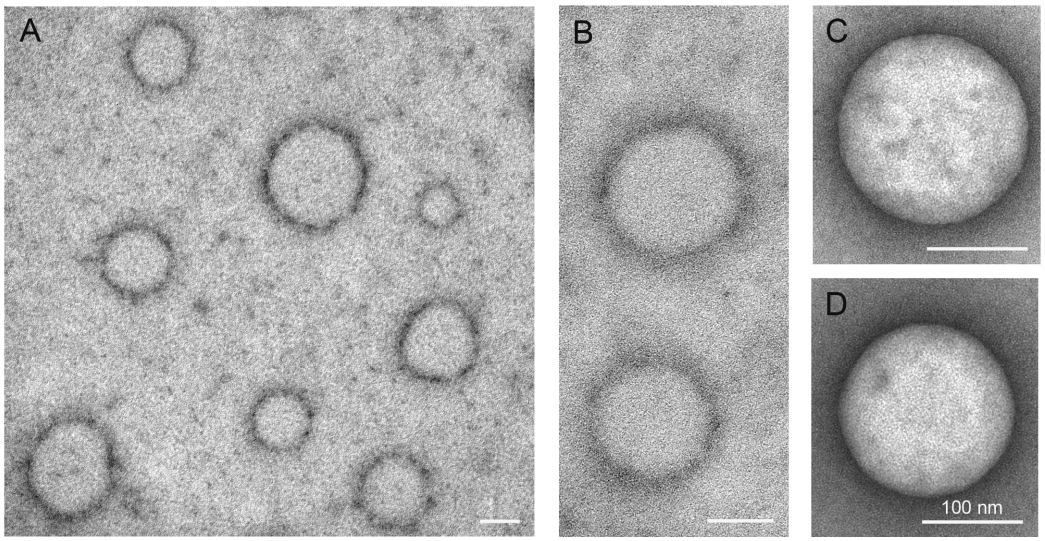


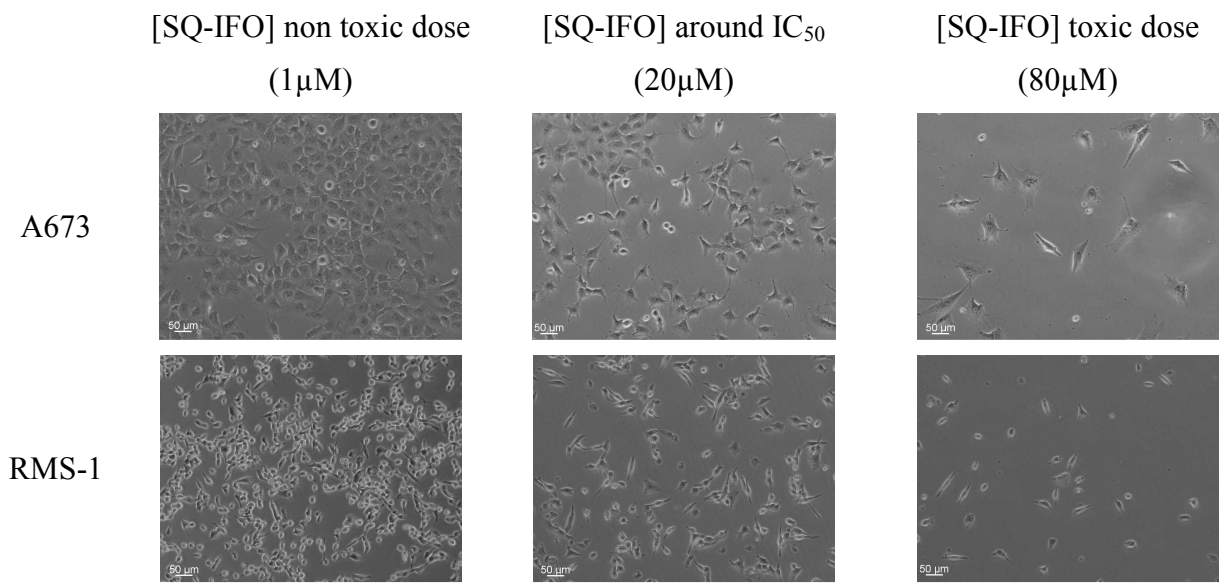
Figure 2: Farnesyloxy-IFO NAs suspended in water observed by transmission electron microscopy using positive staining method (A, B) or by negative staining method (C, D). Magnification: A) 50,000; B) 85,000; C) and D) 140,000 - scale bar: 100 nm.



2.4. In vitro cytotoxic evaluation

In vitro cytotoxicity of compounds **6, 9a-f** has been tested on Rhabdomyosarcoma (RMS-1) and Ewing sarcoma (A673) cell lines by MTS assay, using IFO as a control. In the absence of cytochrome, IFO showed no activity, whereas all the pre-activated compounds, either as bulk form or as NAs, demonstrated cytotoxic activities (Table 2) with IC_{50} ranging from 0.6 µM to 31.4 µM. This inhibition is shown in Figure 2 which depicts the action of different SQ-IFO concentrations on the cell proliferation of both cell lines after a 72h treatment. At a low concentration (1 µM) there is no inhibition of cell growth, whereas the concentrations of 20 and 80 µM show an inhibition cell of 50 and 80%, respectively.

Figure 3: Illustration of SQ-IFO (**9e**) cytotoxicity on RMS-1 and A673 cell lines after 72h incubation.



All these pre-activated compounds showed a cytotoxic activity after 72h incubation for the tested cell lines, except **9b**, which needed to be incubated as long as 96h to reach its IC_{50}. Surprisingly, for the two cell lines, the NAs formulation of compound **9c** led to a significant improved activity by comparing the IC_{50} obtained as bulk form (Table 2).

Table 2: Cytotoxic activities of IFO derivatives on Ewing sarcoma (A673) and Rhabdomyosarcoma (RMS-1) cell lines (n=3).

Compound	In vitro Activity (IC50 µM)	
	A673	RMS-1
6	1.5 ± 0.5	3.8 ± 1.4
9a	17.7 ± 2.3	5.3 ± 1.7
9b**	24.5 ± 2.1	11.3 ± 5.8
9c	25.3 ± 1.5	9.3 ± 4.2
9c NAs	14.7 ± 5.0	0.6 ± 0.2
9d NAs	18.7 ± 3.6	12.3 ± 2.0
9e NAs	22.0 ± 5.	11.4 ± 5.0
9f NAs	23.2 ± 1.3	31.4 ± 4.7*

(*) value obtained with the RD rhabdomyosarcoma cell line; (**) 96 h incubation

The NAs squalenoyl prodrugs **9e** and **9f** were previously evaluated on a panel of cancer cell lines (Table 3). As usually observed with other squalene conjugates, owing to their prodrug nature, **9e** and **9f** displayed variable activities on most cell lines.

Table 3: Cytotoxic activities of poly-isoprenoyl prodrugs NAs **(9e,f)** on various cancer cell lines (n=4).

Cell line	*In vitro* Activity (IC_{50} µM)	
	9e NAs	**9f** NAs
RD	37.0 ± 1.0	31.4 ± 4.7
SK-N-MC	31.5 ± 1.1	23.1 ± 1.1
UW-479	65.2 ± 1.1	30.5 ± 1.1
SAOS-2	139.1 ± 0.9	31.1 ± 4.0
MCF-7 MDR	157.0 ± 52.2	119.0 ± 27.1
MCF-7	160.9 ± 78.2	45.6 ± 23.2
A549	158.1 ± 83.8	38.6 ± 22.3
M109	>200	22.0 ± 8.6
KB 3.1	128.5 ± 52.7	35.4 ± 21.6
B16F10	134.5 ± 41.9	23.0 ± 4.3
MiaPaCA-2	132.1 ± 67.9	15.5 ± 8.4

Regarding cytotoxic activities of SQ-IFO (**9e**) and SQ-thio-IFO (**9f**), both compounds were active on rhabdomyosarcoma (RD) and neuroblastoma (SK-N-MC). SQ-IFO (**9e**) activity on the other cell lines was poor as IC_{50} were above 50.0 µM (Table 3). However, it can be underline that SQ-thio-IFO (**9f**) showed interesting activities on glioma (UW-479), osteosarcoma (SAOS-2), lung cancer (M109) melanoma (B16F10) and pancreatic cancer (MiaPaCa-2) cell lines with similar activities ranging between 15.5 to 23.0 µM (Table 3). Moreover, SQ-thio-IFO (**9f**)

showed intermediate cytotoxic activities on the other cell lines but reverse the multidrug resistance on the breast cell line (MCF-7 MDR) as shown in Table 3.

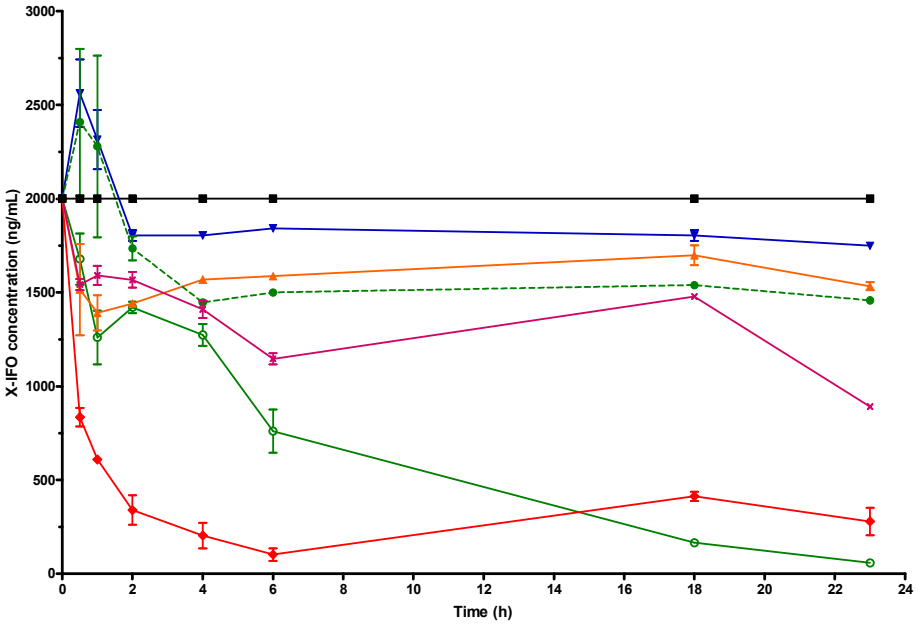
2.5. In vitro release of 4-HO-IFO from X-IFO prodrugs.

The release kinetic study of the 4-HO-IFO (**2**) from the pre-activated compounds (**6, 9b-f**) was performed over 24 h in mice plasma incubated at 37°C. All pre-activated IFO compounds (**6, 9b-f**) were studied using stock solutions in ACN. The concentrations of 4-HO-IFO and of the pre-activated compounds were assessed in mice plasma using a quantitative HPLC-MS/MS assay and allowed to display the decrease profile of the pre-activated compounds (**6, 9b-f**) and the appearance profile of the 4-HO-IFO metabolite concentrations, both expressed in ng/mL, taking into account that the 4-HO-IFO compound was analyzed as the semicarbazone derivative. The kinetics curves show that 4-HO-IFO was indeed produced from all these pre-activated derivatives, except for **9f**. The Figure 4 shows the results obtained with the bulk form of the pre-activated compounds. On one hand, it was observed that concentrations of **9b** (13,17-dimethyloctyloxy-IFO), **9c** (geranyloxy-IFO), **9d** (farnesyloxy-IFO) and **9e** (SQ-IFO) decreased especially during the first two hours and then remained stable, as shown in Figure 4A. On another hand, the concentration of compound **6** (MeO-IFO) decreased quickly during the first two hours and became very low after the 6th hour. Moreover, for compound **9f** (SQ-thio-IFO), the concentration decreased gradually to reach a low value after 23h of incubation. The Figure 4B shows the appearance curves of released 4-HO-IFO from each X-IFO compound. We observed that the compound **6** provided increasing 4-HO-IFO concentrations after 2h with a maximum value of 1250 ng/mL, and then slowly decreased until 23h. After 0.5h, compounds **9b** and **9d** produced maximum 4-HO-IFO concentrations of 200 and 700 ng/mL, respectively.

Compounds **9c** and **9e** displayed 4-HO-IFO concentrations of 350 and 400 ng/mL after 4 and 6h, respectively. By contrast, concentration lower than 30 ng/mL (at the bottom of Figure 4B) were detected with compound 9f in which the alkoxy group is replaced by an alkylsulfanyl residue.

Figure 4: Release study of 4-HO-IFO from X-IFO as bulk form in mice plasma incubated at 37°C for 24h. (♦ **6** (MeO-IFO), ▼ **9b** (13, 17-dimethyloctyloxy-IFO), ▲ **9c** (geranyloxy-IFO), × **9d** (farnesyloxy-IFO), ● **9e** (SQ-IFO), ○ **9f** (SQ-thio-IFO and ■ **1** (IFO)): Time-concentration profiles of X-IFO A) and of 4-HO-IFO B).

A)



B)

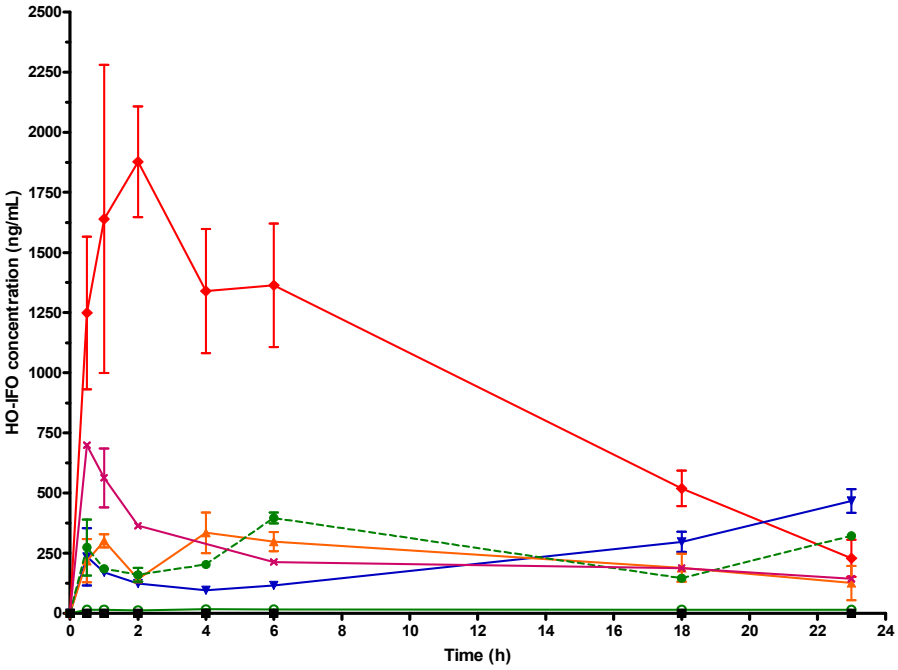
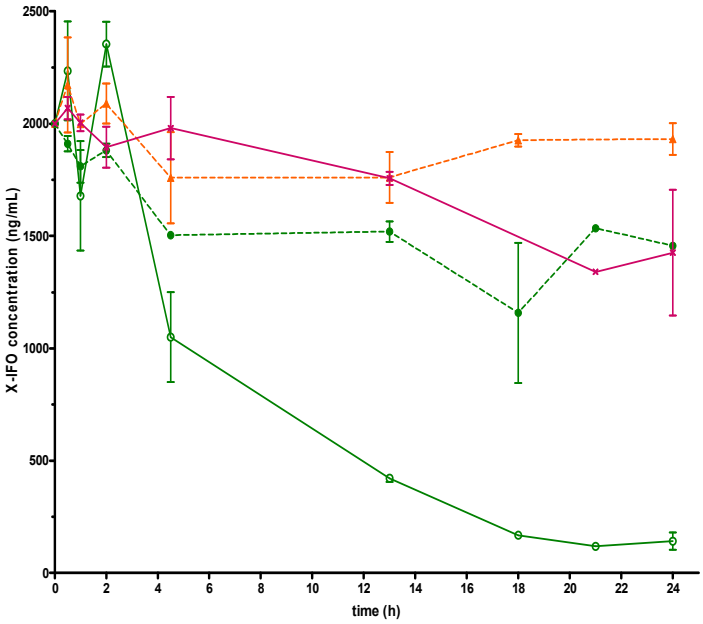


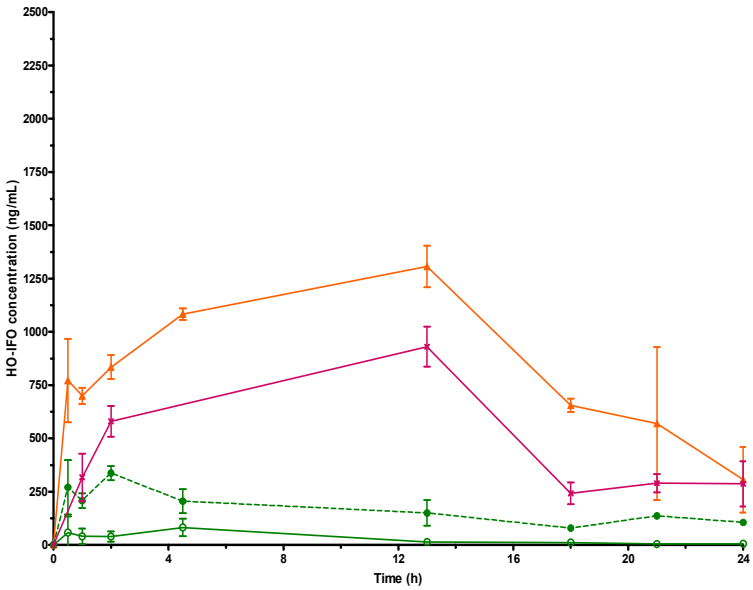
Figure 5A) shows the time-concentration profile obtained for the compounds **9c-f** prepared as NAs. Concentrations of compounds **9c-e** first decreased for 4.5 h and then remained stable for 24h. Regarding compound **9f**, its concentration decreased gradually and reached a minimum value after 24h. Figure 5B) shows the formation of released 4-HO-IFO from the NAs of the pre-activated compounds. It was observed that both compounds **9c** and **9d** produced after 13h incubation a maximum 4-HO-IFO concentration of 1300 and 900 ng/mL respectively. On the other hand, compounds **9e** and **9f** could release low concentrations of 4-HO-IFO with concentration of 350 ng/mL after 2h and 100 ng/mL after 4.5h, respectively.

Figure 5: Release study of 4-HO-IFO from X-IFO as NAs in mice plasma incubated at 37°C for 24h. (▲ **9c** (geranyloxy-IFO), × **9d** (farnesyloxy-IFI)**,** ● **9e** (SQ-IFO) and ○ **9f** (SQ-thio-IFO): Time-concentration profiles of X-IFO A) and of 4-HO-IFO B).

A)



B)



3. Discussion and Conclusion.

The pharmacomodulation strategy described herein of IFO has led to the synthesis of seven new pre-activated IFO derivatives (MeO-IFO **6**, pentyloxy-IFO **9a**, 13,17-dimethyloctyloxy-IFO **9b**, geranyloxy-IFO **9c**, farnesyloxy-IFO **9d**, SQ-IFO **9e** and SQ-thio-IFO **9f**). The engraftment of geraniol, farnesol, squalenol or squalenecysteamide to IFO allowed obtaining four compounds, which were able to self-assemble into NAs.

As described previously,[11] the *in vitro* cytotoxicity of MeO-IFO (**6**) in absence of cytochrome was similar to IFO with CYP bio-activation. Concerning the other pre-activated IFO derivatives, the *in vitro* cytotoxic studies demonstrated their activity in the same range, with IC_{50} between 0.6 and 31.4 µM, as expected. Indeed, the aim of this strategy was not to improve their intrinsic cytotoxic activity as the released entity (4-HO-IFO) is the same for all these pre-activated compounds. The aim was to control the delivery of the active agent through the modulation of its release depending on the properties of the engrafted moieties.

It was shown that 72h-incubation were needed for the determination of the IC_{50}, except for 13,17-dimethyloctyloxy-IFO (**9b**), which seemed to act more slowly, as IC_{50} was reached after 96h. This may be tentatively explained by the increased steric hindrance due to the branched methyl group in the 3' position of the alkyl appendage. Such configuration could significantly modify the release kinetics of the key entity (4-HO-IFO). Concerning the *in vitro* kinetic study of 4-HO-IFO release, we demonstrated that all the pre-activated IFO derivatives produced the key entity leading to the isophosphoramide mustard, as they were designed for this purpose. Nevertheless, SQ-thio-IFO (**9f)** was not hydrolyzed into 4-HO-IFO, likely due to the different type of bound linkage between the squalenecysteamide and IFO: a sulfide rather than an ether bound is present in the case of the other compounds (**6, 9a-e**). The relative hydrolytic stability of

9f in respect to the *O*-alkyl derivatives **9a-e** could be attributed to the unfavorable protonation of the soft sulfur center, *S*-ethers being weaker bases than *O*-ether.

All studied prodrugs were obtained as diasteromeric mixtures as evidenced by ^{31}P and ^{13}C NMR. Although each isomer could potentially release the parent drug at different rates, we chose to evaluate the mixture as racemic without separation for two reasons: one of the reasons was that these acid-sensitive prodrugs do not crystallize and must be separated by chromatography leading to a substantial loss of material. However, the main reason was that the mechanism leading to the formation of the 4-OH-IFO metabolite does not involve any enzymatic process but is a simple acid catalyzed hemiaminal ether hydrolysis via protonation, leading to the release of the 4-*O*-alkyl side-chain moiety with formation of an iminium ion and water addition. Thus, the C-4 asymmetric center disappears in this process and the subsequent water addition on the iminium ion would be expected to give 4-OH-IFO. Moreover, the produg stereochemistry corresponds to the one observed in the metabolites resulting of CYP3A4 mediated bioactivation of IFO (see Scheme 1) because 4-OH-IFO is in equilibrium with the ring-opened aldo-isophosphoramide which leads to epimerization of the C-4 center in the more stable stereochemistry. The hypothesis that the C-4 stereogenic center of these prodrugs displays only a small influence on the cytotoxicity has been reinforced by the observation that both 4-MeO-IFO isomers were found to have very similar cytotoxicity on carcinoma KB cell line (20 and 22 μM respectively).[11] This result could probably be related to the axial conformation of the methoxy group in both diastereomers differing only by the conformation of the phosphoryl (P=O) bond. Similarly, we can postulate that the *O*-alkyl groups engrafted in the C-4 position of these prodrugs are in the same axial position due to stereoelectronic control in the addition step. Thus, the same unique relationship with the nitrogen lone pair in both isomers should lead to roughly

similar rate of iminium formation when hydrolyzed. Finally, we have chosen to examine these prodrugs in their racemic form since IFO used in the clinic is racemic and because it was demonstrated that there is no difference in efficacy through the release of 4-HO-IFO between racemic IFO and its enantiomers.[25] In addition, when *in vivo* pharmacokinetic (PK) studies were performed, no statistically differences of therapeutic concentrations and PK parameters were shown between them.[26]

Taking into account the results of both studies, all the designed pre-activated compounds were active without any CYP bio-activation, contrary to IFO. This observation confirms the proof of concept concerning the design of these new pre-activated isoprenic IFO derivatives, as these compounds generated directly the key entity leading to the release of the isophosphoramide mustard, as it was previously described by Hohorst[12] and Takamizawa[13] in the mid 70s. This will allow further investigations on the administered dose reduction in order to limit the toxic side effects occurring in high dose protocols. Moreover, the NAs formulation of the drug could lead to nanomedecines designed for a controlled release of the key entity.[15]

Among the designed compounds, four seemed to stand out by their interesting results. As our main goal was to administer a safer drug, the high activity and fast 4-HO-IFO release profile of MeO-IFO (**6**) were an impediment for controlling optimal pharmacokinetics development of this compound. Due to the lack of stability, the compound MeO-IFO released 4-HO-IFO very quickly (Figure 4B), the use of this compound in chemotherapy may be considered as equivalent to the direct administration of 4-HO-IFO, implying that **6** may never reach its target and could lead to harm the patient. However, compounds **9c-f** have interesting properties as they self-assemble into NAs with important drug loading. They also show a gradual 4-HO-IFO release

kinetic profile which could be useful for a future active targeting strategy using nano-assembly functionalization.

The next step of this pharmacomodulation strategy will be first to investigate and to explain the reason of the increased activity of **9c** as NAs, which could be imaged by cell internalization investigations using labelled NAs. Then, the *in vivo* efficacy using human tumor xenografted mice and the toxicological impact of these new pre-activated oxazaphosphorine prodrugs (bulk and NAs) will be further investigated.

4. Experimental section.

4.1. Chemistry

4.1.1. General

The structure of the synthesized X-IFO products was characterized by ^{1}H, ^{13}C and ^{31}P NMR spectra, which were recorded on Bruker Avance 400 (400, 100 and 160MHz, for ^{1}H, ^{13}C and ^{31}P, respectively) spectrometers **(**Bruker Daltonik, Bremen, Germany). Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C NMR spectra rests on the *J*-modulated spin-echo sequence and DEPT135 experiments. Positive electrospray mass spectra (+ESI-MS) were performed using a QuattroLC® mass spectrometer (Waters, Manchester, UK) and high resolution mass spectra (HR-MS) were achieved with ±3 ppm accuracy with an Agilent 6520 series Q-Tof mass spectrometer (Agilent Technologies, Santa Clara, USA) operating in positive electrospray ionization. The size of the obtained NAs was measured using a Malvern particle size analyzer (Zetasizer®). Analytical grade solvents were provided by Carlo Erba (Val-de-Reuil, France). Ifosfamide (Holoxan®) was supplied from Baxter (Baxter Healthcare Ltd, Auckland, New Zealand) with 99.5% purity. Borontrifluoride diethyl etherate, squalene, geraniol, pentanol, farnesol trans-trans and 3,7-dimethyloctanol were purchased to Alfa Aesar (Schiltigheim, France). Dichloromethane was distilled over calcium hydride, under nitrogen. All reactions involving air- or water-sensitive compounds were performed under positive nitrogen pressure. Analytical thin-layer chromatography was performed on silica gel $60F_{254}$ (Merck) and the preparative chromatography was performed using column filled with Silica Gel 60A (Merck, Darmstadt, Germany).

4.1.2. HPLC-MS/MS assay of X-IFO purity

The six synthesized and purified X-IFO products **9a-f** (pentyloxy-IFO, geranyloxy-IFO, 13,17-dimethyloctyloxy-IFO, farnesyloxy-IFO, SQ-IFO, SQ-thio-IFO) could contain the residual product MeO-IFO (**6**) and showed a batch purity of 99%. Purity of each X-IFO batch was calculated using a quantitative HPLC-MS/MS assay of the concentration of MeO-IFO impurity. Based on the percentage ratio of MeO-IFO and X-IFO concentrations, purity (%) of X-IFO was expressed as: P = 100 x ((1- c MeO-IFO/c X-IFO)).

Briefly, HPLC-MS/MS analyses of reference MeO-IFO standards (10-400 ng/mL) and X-IFO samples (diluted at 10 µg/mL) were performed using a 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) fitted with an Uptisphere® C18 5µm column, (2.1 × 100 mm). Isocratic elution was achieved with a flow rate of 0.25 mL/min using the mobile phase (acetonitrile/ammonium formate 5mM, 95:5, v/v). The total run time was 16.0 min. Analytes were detected using a QuattroLC® triple quadrupole mass spectrometer (Waters, Manchester, UK), operating in the positive electrospray ionization (capillary voltage, 3.5kV and cone voltage, 25V) using tandem mass spectrometry with their respective MRM transitions (as example, *m/z* 291 → 259 for MeO-IFO (**6**) using argon as collision gas and collision energy of 20 eV. The unknown concentration of MeO-IFO in X-IFO samples was quantified using calibration curve of MeO-IFO standards, thanks to Masslynx® software. In the same way, the concentration of the IFO impurity was quantified in the initial MeO-IFO product using HPLC-MS/MS (MRM) using the transition m/z 261 → 154 for IFO.

4.2. Pre-activated drug synthesis

*4.2.1 Synthesis of MeO-IFO (**6**)*

A solution of ifosfamide ***(1)*** (200 mg, 0.766 mmol,) and sodium tetrafluoroborate ($NaBF_4$) (170 mg, 1.54 mmol) in methanol (10 mL) was introduced into an undivided cell equipped with two

graphite rod electrodes at room temperature. The anodic oxidation was followed by checking the full consumption of the starting material with thin-layer chromatography ($CH_2Cl_2/(CH_3)_2CO$ (50:50, v/v). After which 3.1 F/mol of electricity was passed into the solution at an intensity of 20 mA, sodium carbonate (100 mg, 1 mmol) was added and the solvent was removed under reduced pressure. The residue was then filtered after dissolution in CH_2Cl_2. After concentration *in vacuo*, we obtained an oil residue corresponding to 4-methyloxy-ifosfamide (4-MeO-IFO) (215 mg, 96% yield). This product was then used as raw material without further purification. ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.25 (m, 1 H, $H_{5\alpha}$), 2.72 (m, 1 H, $H_{5\beta}$), 3.42 (m, 4 H, H_7 and H_9), 3.52 (m, 4 H, H_{10} and H_8), 3.84 (m, 3 H, $H_{6\alpha}$ and H_{11}), 4.26 (m, 1 H, $H_{6\beta}$), 4.62 (td, 1 H, J=3 and 21 Hz, H_4). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 29.50 (C_5), 43.29 (C_7), 44.23 (C_8), 45.73 (C_{10}), 49.67 (C_9) 54.79 (C_{11}) 62.36 (C_6), 90.75 (C_4). ^{31}P NMR ($(CD_3)_2CO$, 160 MHz): δ 7.73 (s) and 8.40 (s).

MS (+ESI): *m/z* 291.1 (100%) $[M+H]^+$; 293.1 (70%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 291.0426 ($[M+H]^+$ calcd for $C_8H_{17}N_2O_3P_1Cl_2$: 290.0354.

*4.2.2. Synthesis of pentyloxy-IFO (**9a**)*

To a stirring solution of 4-MeO-IFO (**6**) (200 mg, 0.69 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.69 mmol) under inert atmosphere at -78 °C. After 45 minutes, a solution of pentanol (148µL, 1.37 mmol) in CH_2Cl_2 (0.5 mL) was added dropwise. The mixture was stirred for an additional 1h at -78 °C and 10 mL of a saturated Na_2CO_3 solution were added. After extraction with CH_2Cl_2 (3×20 mL), the organic phases were dried over $MgSO_4$ and concentrated using reduced pressure. The obtained residue was purified by column chromatography (silica gel, $CH_2Cl_2/(CH_3)_2CO/Et_3N$ (95:5:0.5, v/v/v)) to yield **9a** as a yellow oil (90 mg, yield 33%). ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 0.92 (t, 3H, J = 7.1 Hz, $O(CH_2)_4CH_3$),

1.30-1.45 (m, 4H, $CH_3CH_2CH_2$), 1.62 (m, 2H, OCH_2CH_2), 2.02-2.07 (m, 3H, H_5 and NH), 3.16-3.30 (m, 2H, OCH_2CH_2), 3.47-3.55 (m, 4H, NCH_2CH_2Cl and $NHCH_2CH_2Cl$), 3.60-3.65 (m, 2H, NCH_2CH_2Cl), 3.70-3.75 (m, 2H, $NHCH_2CH_2Cl$), 4.05-4.20 (m, 1H, H_6), 4.35-4.50 (m, 1H, H_6), 4.70-4.80 (dd, 1H, J = 20.5 Hz and = 2.9 Hz, H_4). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 14.3 (CH_3, $O(CH_2)_4CH_3$), 23.2 (CH_2, $O(CH_2)_3CH_2CH_3$), 29.3 (CH_2, $O(CH_2)_2CH_2CH_2CH_3$), 30.4 (CH_2, $OCH_2CH_2(CH_2)_2CH_3$), 30.9 (CH_2, C_5), 43.6 (CH_2, NCH_2CH_2Cl), 44.0 (CH_2, $NHCH_2CH_2Cl$), 46.6 (CH_2, $NHCH_2CH_2Cl$), 50.2 (CH_2, NCH_2CH_2Cl), 63.2 (CH_2, C_6), 68.6 (CH_2, $OCH_2(CH_2)_3CH_3$), 89.8 (CH, C_4). ^{31}P NMR ($(CD_3)_2CO$, 160 MHz): δ 7.60 (s) and 8.46 (s).

MS (+ESI): *m/z* 347.1 (100%) $[M+H]^+$; 349.1 (70%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 347.1068 ($[M+H]^+$ calcd for $C_{12}H_{25}N_2O_3P_1Cl_2$: 347.1058).

*4.2.3. Synthesis of 13,17-dimethyloctyloxy-IFO (**9b**)*

To a stirring solution of 4-MeO-IFO (**6**) (200 mg, 0.69 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.69 mmol) under inert atmosphere at -78°C. After 45 minutes, 3, 7-dimethyloctanol (262 µL, 1.37 mmol) dissolved in a small volume of CH_2Cl_2 (0.5 mL) was added dropwise, the mixture was continuously stirred for an additional 1 h. The reaction was then stopped by addition at low temperature of 10 mL of a saturated Na_2CO_3 solution. After extraction with CH_2Cl_2, the organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was subject to column chromatography purification (silica gel, $CH_2Cl_2/(CH_3)_2CO/Et_3N$ (95:5:0.5, v/v/v). The different fractions containing the compound of interest were pooled and concentrated under reduced pressure to yield a yellow to brown oily residue corresponding to the compound of interest **9c** (90 mg, yield 28%). ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 0.74 (m, 9H, $O(CH_2)_2CH(CH_3)(CH_2)_2CH(CH_3)_2$), 1.05-1.20 (m, 4H, $(CH_2)_2CH(CH_3)_2$), 1.60-1.70 (m,

2H,NCH_2CH_2Cl), 2.01-2.07 (m, 2H, H_6), 2.91 (s,1H, NH), 3.15-3.30 (m, 4H, NCH_2CH_2Cl and $NHCH_2CH_2Cl$), 3.45-3.60 (m, 4H, NCH_2CH_2Cl and $NHCH_2CH_2Cl$), 3.64 (m, 2H, H_{11}),4.05-4.19 (m, 1H, H_5), 4.35-4.50 (m, 1H, H_5), 4.70 (m, 1H, H_4). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 20.0 (CH_3, $CH(CH_3)_2$), 23.0 (CH_3, $CH(CH_3)_2$), 25.4 (CH_3, $CH_2CH(CH_3)$), 28.7 (CH_3, $CH_2(CH_2)_2CH(CH_3)_2$), 30.5 (CH_2, C_5), 40.0 (CH_2, $NHCH_2CH_2Cl$), 43.6 (CH_2, $CH_2CH_2CH(CH_3)_2$), 44.0 (CH_2, NCH_2CH_2Cl), 46.7 (CH_2, NCH_2CH_2Cl), 49.7 (CH_2, $OCH_2CH_2CH(CH_3)$), 50.3 (CH_2, $NHCH_2CH_2Cl$), 63.2 (CH_2, C_6), 66.7 (CH_2, $OCH_2CH_2CH(CH_3)$), 89.8 (CH, C_4). ^{31}P NMR ($(CD_3)_2CO$, 160 MHz): δ 8.47 (s) and 7.55 (s).

MS (+ESI): *m/z* 417.2 (100%) $[M+H]^+$; 419.2 (70%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 417.1828 ($[M+H]^+$ Calcd for $C_{17}H_{35}N_2O_3P_1Cl_2$: 417.1840)

*4.2.4. Synthesis of geranyloxy-IFO (**9c**)*

To a stirring solution of 4-MeO-IFO **(6)** (200 mg, 0.68 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.68 mmol) at -78°C. After 45 minutes, a solution of geraniol (237 µL, 1.37 mmol) in CH_2Cl_2 (0.5 mL) was added dropwise, the mixture was stirred for an additional 1 h at –78 °C and quenched by addition of 10 mL of a saturated Na_2CO_3 solution. After extraction with CH_2Cl_2 (3×20 mL), the combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography (silica gel, $CH_2Cl_2/(CH_3)_2CO/Et_3N$ (95:5:0.5, v/v/v)) to yield **9c** as a yellow oil (80 mg, yield 25%). ^1H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.62 (s, 3H, $HC=C(CH_3)$), 1.68 (s, 3H, $HC=C(CH_3)_2$), 1.72 (s, 3H, $HC=C(CH_3)_2$), 2.01-2.09 (m, 6H, $(CH_2)_2CH=C(CH_3)_2$ and $CH_2CH=(CH_3)$), 2.09-2.16 (m, 2H, H_5), 2.85 (m, 1H, NH), 3.15-3.28 (m, 4H, NCH_2CH_2Cl and $NHCH_2CH_2Cl$), 3.62-3.67 (m, 2H, NCH_2CH_2Cl), 3.70-3.75 (m, 2H, $NHCH_2CH_2Cl$), 4.08-4.12 (m, 2H, $OCH_2CH=CH(CH_3)$), 4.12-4.19 (m, 1H, H_6), 4.39-4.49 (m, 1H, H_6), 4.75 (dt, 1H, *J* =

20.9 Hz and J = 3.2 Hz, H_4), 5.12 (tt, 1H, J = 1.2 Hz and J = 6.8 Hz, C*H*=C(CH_3)$_2$), 5.39 (td, 1H, J = 1.2 Hz and J = 6.6 Hz, OCH_2C*H*=C(CH_3)). ^{13}C NMR ((CD_3)$_2$CO, 100 MHz): δ 16.7 (CH_3, OCH_2CH=C(*C*H_3)), 17.8 (CH_3, HC=C(*C*H_3)$_2$), 25.9 (CH_3, HC=C(*C*H_3)$_2$), 27.2 (CH_2, CH_2*C*H_2HC=C(CH_3)$_2$), 31.1 (CH_2, C_5), 40.2 (CH_2, NHCH_2*C*H_2Cl), 43.6 (CH_2, *C*H_2CH_2HC=C(CH_3)$_2$), 44.1 (CH_2, NCH_2*C*H_2Cl), 46.5 (CH_2, N*C*H_2CH_2Cl), 50.2 (CH_2, NH*C*H_2CH_2Cl), 63.3 (CH_2, C_6), 64.9 (CH_2, O*C*H_2), 88.8 (CH, C_4), 121.7 (CH, OCH_2*C*H=C(CH_3)), 124.8 ((CH, *C*H=C(CH_3)$_2$), 132.1 (C, CH=*C*(CH_3)$_2$), 140.9 (C, OCH_2CH=*C*(CH_3)). ^{31}P NMR ((CD_3)$_2$CO, 160 MHz): δ 8.49 (s) ppm.

MS (+ESI): *m/z* 413.2 (100%) $[M+H]^+$; 415.2 (65%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 413.1522 ($[M+H]^+$ Calcd for $C_{17}H_{31}N_2O_3P_1Cl_2$: 413.1527).

*4.2.5. Synthesis of farnesyloxy-IFO (**9d**)*

To a stirring solution of 4-MeO-IFO **(6)** (200 mg, 0.68 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.68 mmol) at -78°C. After 45 minutes, a solution of farnesol (343 µL, 1.37 mmol) in CH_2Cl_2 (0.5 mL) was added dropwise, the mixture was stirred for an additional 1h at –78 °C and quenched by addition of 10 mL of a saturated Na_2CO_3 solution. After extraction with CH_2Cl_2 (3×20 mL), the combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography (silica gel, CH_2Cl_2/(CH_3)$_2$CO/Et_3N (95:5:0.5, v/v/v)) to yield **9d** as a yellow oil (110 mg, yield 33%). ^{1}H NMR ((CD_3)$_2$CO, 400 MHz): δ 1.60 (s, 3H, HC=C(C*H*$_3$)), 1.62 (s, 3H, HC=C(C*H*$_3$)$_2$), 1.66 (s, 3H, HC=C(C*H*$_3$)), 1.71 (s, 3H, HC=C(C*H*$_3$)$_2$), 1.96-2.19 (m, 10H, (C*H*$_2$)$_2$CH=C(CH_3) (C*H*$_2$)$_2$CH=C(CH_3)$_2$ and H_5), 3.15-3.28 (m, 4H, NC*H*$_2CH_2$Cl and NHC*H*$_2CH_2$Cl), 3.61-3.68 (m, 2H, NCH_2C*H*$_2$Cl), 3.70-3.77 (m, 2H, NHCH_2C*H*$_2$Cl), 4.02-4.29 (m, 3H, OC*H*$_2$CH=CH(CH_3)) and H_6), 4.39-4.49 (m, 1H, H_6), 4.72-4.81 (dt, 1H, J = 20.9 Hz and

J = 3.2 Hz, H_4), 5.12 (m, 2H, C*H*=C(CH_3)CH_2CH_2C*H*=C$(CH_3)_2$), 5.37-5.43 (td, 1H, *J* = 1.2 Hz and *J* = 6.6 Hz, OCH_2C*H*=C(CH_3)). ^{13}C NMR ($(CD_3)_2CO$,100 MHz): δ 16.8 (CH_3, HC=C(*C*H_3)), 17.9 (CH_3, HC=C(*C*H_3)), 23.7 (CH_3, HC=C(*C*H_3)$_2$), 26.0 (CH_3, HC=C(*C*H_3)$_2$), 27.6 (CH_2, CH_2*C*H_2HC=C$(CH_3)_2$), 30.7 (CH_2, NHCH_2*C*H_2Cl), 31.2 (CH_2, C_5), 32.7 (CH_2, *C*H_2CH_2HC=C(CH_3)), 40.3 (CH_2, CH_2*C*H_2HC=C(CH_3), 40.6 (CH_2, NHCH_2*C*H_2Cl), 43.7 (CH_2, *C*H_2CH_2HC=C$(CH_3)_2$), 44.2 (CH_2, NCH_2*C*H_2Cl), 46.6 (CH_2, N*C*H_2CH_2Cl), 50.2 (CH_2, NH*C*H_2CH_2Cl), 63.3 (CH_2, C_6), 65.0 (CH_2, O*C*H_2), 88.9 (CH, C_4), 121.8 (CH, OCH_2*C*H=C(CH_3)), 124.9 (CH, *C*H=C(CH_3)), 125.2 ((CH, *C*H=C(CH_3)), 131.8 (C, CH=*C*$(CH_3)_2$), 136.0 (C, CH=*C*(CH_3)), 141.1 (C, OCH_2CH=*C*(CH_3)). ^{31}P NMR ($(CD_3)_2CO$, 160 MHz): δ 8.52 (s) and 7.77 (s).

MS (+ESI): *m/z* 481.3 (100%) $[M+H]^+$; 483.3 (75%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 481.2143 ($[M+H]^+$ Calcd for $C_{22}H_{39}N_2O_3P1Cl2$:481.2150).

*4.2.6. Synthesis of trisnorsqualenol (**8e**)*

To a stirring solution of 1,1',2-trisnor-squalénic aldehyde (**10**) (1,5 g; 3,9 mmol) in ethanol (9 mL) was added portionwise $NaBH_4$ (145 mg; 3,8 mmol), the mixture was continuously stirred for 30 minutes at room temperature. The reaction was then stopped by addition dropwise of a solution of HCl 2N until pH=3. The solution was concentrated under vaccue and the residue was diluted in water (15 mL). After extraction with AcOEt, the organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography (silica gel, Petroleum spirit/Et_2O (90:10, v/v). yield a colorless oil of **8d** (1,32 g; 88 %). ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.60 (s, 15H, CH=C(C*H*$_3$)), 1.68 (s, 3H, (C*H*$_3$)$_2$C=CH), 2.11-1.95 (m, 20H, CHC*H*$_2$C*H*$_2$C(CH_3)=CH), 3.61 (t, 2H, *J* = 7 Hz, CH_2C*H*$_2$OH), 5.20-5.06 (m, 5H, C(CH_3)=C*H*CH_2). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 16.0 (2CH_3,

CH=C(CH$_3$), 16.2 (2CH$_3$, CH=C(CH$_3$)), 17.8 (CH$_3$, CH=C(CH$_3$)), 25.8 (CH$_3$, CH=C(CH$_3$)$_2$), 26.7 (CH$_2$, CHCH$_2$$CH_2$C(CH$_3$)), 26.8 (CH$_2$, CH$_2$$CH_2$C(CH$_3$)), 26.9 (CH$_2$, CH$_2$$CH_2$C(CH$_3$)), 28.4 (2CH$_2$, CH$_2$$CH_2$C(CH$_3$)), 30.9 (CH$_2$, CH$_2$$CH_2$C(CH$_3$)), 36.2 (CH$_2$, CH$_2$$CH_2$C(CH$_3$)), 39.8 (CH$_2$, CH$_2$$CH_2$C(CH3)), 39.9 (2CH$_2$, CH$_2$$CH_2$C(CH3)), 63.0 (CH$_2$, CH$_2$$CH_2$OH)), 124.4 (3CH$_2$, CH$_2$$C$H=C(CH$_3$)), 124.6 (CH$_2$, CH$_2$$C$H=C(CH$_3$)), 125.0 (CH$_2$, CH$_2$$C$H=C(CH$_3$)), 131.4 (C, CH$_2$CH=$C$(CH$_3$)$_2$), 134.7 (C, CH$_2$$C$(CH$_3$)=CH), 135.0 (C, CH$_2$$C$(CH$_3$)=CH), 135.1 (C, CH$_2$$C$(CH$_3$)=CH), 135.3 (C, CH$_2$$C$(CH$_3$)=CH).

*4.2.7. Synthesis of SQ-IFO (**9e**)*

To a stirring solution of 4-MeO-IFO (**6**) (200 mg, 0.68 mmol) in distilled CH_2Cl_2 (0.5 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.68 mmol) under inert atmosphere at -78 °C. After 10 minutes, trisnorsqualenol (**8d**) (200 mg, 1.37 mmol) dissolved in a small volume of CH_2Cl_2 (0.5 mL) was added dropwise, the mixture was continuously stirred for an additional 1h. The reaction was then stopped by addition at low temperature of 5 mL of a saturated Na_2CO_3 solution. After extraction with CH_2Cl_2 the organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was subject to column chromatography purification (silica gel, CH_2Cl_2/MeOH/Et_3N (98:2:0.1, v/v/v)). To yield **9d** as a yellow oil (120 mg, yield 27%). ^{1}H NMR ((CD$_3$)$_2$CO, 400 MHz): δ 1.61 (s, 3H, CH=C(CH_3)), 1.64 (s, 12H, CH=C(CH_3)), 1.68 (s, 3H, (CH_3)$_2$C=CH), 1.78-1.72 (m, 2H, CH$_2$CH$_2$CH$_2$O), 2.19-1.97 (m, 20H, 9CH$_2$ and H$_5$), 3.33-3.16 (m, 3H, NHCH$_2$CH_2Cl and H$_7$), 3,58-3,43 (m, 3H, H$_7$, CH$_2$CH$_2$CH$_2$O), 3.66 (t, J = 6 Hz, 2H, NHCH_2CH$_2$Cl), 3.80-3.71 (m, 2H, NCH2CH_2Cl), 4.00-3.90 (m, 1H, NH), 4.21-4.09 (m, 1H, H$_6$), 4.50-4.38 (m, 1H, H$_6$), 4.75 and 4.70 (2 t, J = 4 Hz, 1H, H$_4$), 5.24-5.09 (m, 5H, (CH$_3$)C=CHCH$_2$). ^{13}C NMR ((CD$_3$)$_2$CO, 100 MHz): δ 16.1 (2CH$_3$, CH$_2$CH=C(CH$_3$)), 16.2 (2CH$_3$, CH$_2$CH=C(CH$_3$)), 17.8 (CH$_3$, CH$_2$CH=C(CH$_3$)), 25.9 (CH$_3$, (CH$_3$)$_2$C=CH), 27.3 (CH$_2$),

27.4 (CH_2),27.5 (CH_2), 28.9 ($3CH_2$),32.2 (CH_2), 36.7 (CH_2),40.5 ($3CH_2$), 43.5 (CH_2, $NHCH_2\mathit{C}H_2Cl$), 44.0 (CH_2, $NCH_2\mathit{C}H_2Cl$), 46.6 (CH_2,$NH\mathit{C}H_2CH_2Cl$), 50.2 (CH_2,$N\mathit{C}H_2CH_2Cl$), 63.2 (CH_2, C_6), 68.1 (CH_2), 89.7 (CH, C_4), 124.9 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 125.0 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 125.1 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 125.2 (2CH, $(CH_3)C{=}\mathit{C}HCH_2$), 131.6 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.0 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.4 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.6 (2C, $(CH_3)\mathit{C}{=}CHCH_2$). ^{31}P NMR ($(CD_3)_2CO$, 160 MHz): δ 10.65 (s), 10.25 (s).

MS (+ESI): *m/z* 645.3 (100%) $[M+H]^+$; 647.3 (72%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 645.3710 ($[M+H]^+$ Calcd for $C_{34}H_{59}N_2O_3P_1Cl_2$: 645.3718).

*4.2.8. Synthesis of N-(mercaptoethyl)-squalenamide (**8f**)*

To a solution of 1,1',2- trisnorsqualenic acid **11** (910 mg ; 2.02 mmol) in THF (16 mL) were added NHS (240 mg, 2.08 mmol) and DCC (416 mg ; 2.02 mmol). The reaction mixture was stirred at room temperature for 24h and filtered. The filtrate was concentrated under reduced pressure and the residue was taken up into CH_2Cl_2 (12 mL). Triethylamine (560 mg, 5.80 mmol) and cysteamine hydrochloride (660 mg; 5.80 mmol) were then added and the reaction mixture was stirred for 24h. Water (5 mL) was added and the mixture was extracted with ethyl acetate (3×20 mL). The combined organic layers were washed with 1N HCl (2×10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, petroleum spirit/AcOEt, (3:1, v/v)) to give **8e** as a colorless oil (660 mg, 72 %). ^1H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.60 (s, 12H, $CH_2CH{=}C(C\mathit{H_3})$), 1.62 (s, 3H, $CH_2CH{=}C(C\mathit{H_3})$, 1.68 (s, 3H, $(CH_3)_2C{=}CH$), 2.11-1.96 (m, 16H, $C\mathit{H_2}C\mathit{H_2}C(CH_3){=}$), 2.29 (s, 4H, $NH(O{=})CC\mathit{H_2}C\mathit{H_2}C(CH_3){=}$), 2.62 (td, 2H, J = 7 Hz and J = 2 Hz, $NHCH_2C\mathit{H_2}SH$), 3.40 (m, 2H, $NHC\mathit{H_2}CH_2SH$), 5.20-5.05 (m, 5H, $(CH_3)C{=}C\mathit{H}CH_2$), 5.94 (s large, 1H, N*H*). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 16.1 (2 CH_3, $CH_2CH{=}C(\mathit{C}H_3)$), 16.2 (2$CH_3$,

$CH_2CH{=}C(\mathit{C}H_3)$), 17.8 ($CH_3$, $CH_2CH{=}C(\mathit{C}H_3)$), 24.9 ($CH_2$), 25.9 ($CH_3$, $CH{=}C(\mathit{C}H_3)_2$), 26.8 ($2CH_2$), 26.9 (CH_2), 28.4 ($2CH_2$), 35.3 (CH_2), 35.4 (CH_2), 39.7 (CH_2), 39.9 ($2CH_2$), 42.4 (CH_2), 124.5 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 124.6 (CH, $(CH_3)C{=}\mathit{C}HCH_2$),125.7 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 125.7 (CH, $(CH_3)C{=}\mathit{C}HCH_2$), 131.4 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 133.6 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.0 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.1 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 135.3 (C, $(CH_3)\mathit{C}{=}CHCH_2$), 173.0 (C, NH*C*=O).

*4.2.9. Synthesis of SQ-thio-IFO (**9f**)*

To a stirring solution of 4-MeO-IFO (**6**) (200 mg, 0.68 mmol) in distilled CH_2Cl_2 (0.5 mL) was added dropwise $BF_3.OEt_2$ (86 µL, 0.68 mmol) under inert atmosphere at -78°C. After 10 minutes, *N*-(mercaptoethyl)-squalenamide **8e** (660 mg, 1.37 mmol) dissolved in a small volume of CH_2Cl_2 (0.7 mL) was added dropwise, the mixture was continuously stirred for an additional 1h. The reaction was then stopped by addition at low temperature of 5 mL of a saturated Na_2CO_3 solution. After extraction with CH_2Cl_2 the organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH/Et_3N (98:2:0.1, v/v/v)). The different fractions containing the compound of interest were pooled and concentrated under reduced pressure to yield a yellow to brown oily residue corresponding to the compound of interest **9e** (98 mg, yield 20%). ^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 1.59 (s, 3H, $CH_2CH{=}C(C\mathit{H}_3)$, 1.61 (s, 12H, $CH_2CH{=}C(C\mathit{H}_3)$), 1.66 (s, 3H, $(C\mathit{H}_3)_2C{=}CH$), 2.14-1.96 (m, 17H, $CH_2(C\mathit{H}_3)C{=}$, $CH_2CH{=}C(C\mathit{H}_3),\mathit{H}_5$), 2.26 (s, 4H, $(CH_3)CC\mathit{H}_2CH_2C(=O)NH$), 2.50-2.38 (m, 1H, H_5), 2.70-2.58 (m, 2H, $NHC\mathit{H}_2CH_2S$), 3.41-3.18 (m, 4H, $NHCH_2C\mathit{H}_2Cl$, $NHCH_2C\mathit{H}_2S$), 3.53-3.44 (m, 2H, $NC\mathit{H}_2CH_2Cl$), 3.62 (t, J = 7 Hz, 2H, $NHC\mathit{H}_2CH_2Cl$), 3.87-3.76 (m, 2H, $NCH_2C\mathit{H}_2Cl$), 4.34-4.15 (m, 2H, H_6, NH), 4.80-4.66 (m, 2H, H_4, H_6), 5.24-5.07 (m, 5H, $(CH_3)C{=}CHCH_2$), 7.32 (s large, 1H, CON*H*). ^{13}C NMR ($(CD_3)_2CO$, 100 MHz): δ 16.1 ($2CH_3$, $CH{=}C(\mathit{C}H_3)$), 16.2 ($2CH_3$, $CH{=}C(\mathit{C}H_3)$), 17.8 (CH_3, $CH{=}C(\mathit{C}H_3)$),

25.9 (CH_3, (CH$_3$)$_2$C=CH), 27.3 (CH_2), 27.4 (CH_2), 27.5 (CH_2),28.9 (3CH_2), 32.6 (CH_2),31.8 (CH_2), 33.4 (CH_2, C_5), 36.3 (CH_2), 35.7 (CH_2),40.5 (3CH_2), 39.9 (CH_2, NHCH$_2$CH$_2$S), 44,2 (CH_2, NHCH$_2$$CH_2$Cl), 42.6 ($CH_2$, NCH$_2$$CH_2$Cl), 45.8 ($CH_2$, NH$CH_2CH_2$Cl), 48.6 ($CH_2$, N$CH_2CH_2$Cl), 66.3 (CH, C_4), 124.9 (CH, (CH$_3$)C=CHCH$_2$), 125.0 (CH, (CH$_3$)C=CHCH$_2$), 125.1 (CH, (CH$_3$)C=CHCH$_2$), 125.2 (2CH, (CH$_3$)C=CH), 131.6 (C, (CH$_3$)C=CH), 135.0 (C, (CH$_3$)C=CH), 135.5 (C, (CH$_3$)C=CH), 135.6 (2C, (CH$_3$)C=CH), 172.8 (C, CONH), 63.9 (CH_2, C_6). ^{31}P NMR ((CD$_3$)$_2$CO, 160 MHz): δ 8.47 (s) and 7.55 (s).

MS (+ESI): *m/z* 718.3 (100%) $[M+H]^+$; 720.3 (80%) $[M+2+H]^+$.

HRMS (+ESI): *m/z* 718.3692 ($[M+H]^+$ Calcd for $C_{36}H_{62}N_3O_3P_1S_1Cl_2$:718.3705).

4.3. Preparation and characterization of pre-activated drug nano-assemblies.

Geranyloxy-IFO (**9c**), farnesyloxy-IFO (**9d**), SQ-IFO (**9e**) and SQ-thio-IFO (**9f**) NAs were prepared by nanoprecipitation as follows. The purified product was dissolved in acetone at a concentration of 8 mg/mL. This solution was then added dropwise into Milli-Q water under magnetic stirring to obtain a final 2 mg/mL concentration. The acetone was then evaporated under reduced pressure using a Rotovapor to obtain an aqueous suspension of the pre-activated drug NAs.

The hydrodynamic diameter of these NAs was measured at 20 °C by Dynamic Light scattering using a Nanosizer ZS (Malvern Instrument Ltd, France). An amount of 20 µL of the suspension was diluted in 980 µL of Milli-Q water for the analysis of the NAs properties. The results give the mean hydrodynamic diameter of the dispersed NAs from three independent series of ten measurements. The standard deviation and the polydispersity index were also given, moreover the Zeta (ξ) potential was also determined.

4.4.Transmission Electron Microscopy processing.

3 µl of sample, were deposited on a 300-mesh copper grid covered with a thin carbon film, activated by conventional glow-discharge (adapted for negative staining) or in the presence of pentylamine adapted for positive staining.[27] Grids were washed with aqueous uranyl acetate 2% (w/v) or with aqueous phosphotungstic acid 0.3% (w/v), dried and observed in zero loss *bright field* mode , using a Zeiss 902 transmission electron microscope. Images were captured at various magnifications (50,000, 85,000 or 140,000) with a Veleta CCD camera (2k x2k) and analyzed with iTEM software (Olympus Soft Imaging Solution).

4.5. Cell lines and cell culture.

Several cancerous cell lines have been used for the *in vitro* evaluation. These cell lines were chosen based on IFO's therapeutic indications. Thirteen cancer cell lines were selected for the cytotocity assay as follows: RD (Rhabdomyosarcoma), A673 (human Ewing sarcoma), SK-N-MC (Ewing sarcoma), UW-479 (Glioma), SAOS-2 (Osteosarcoma), MCF-7 (Breast Cancer), MCF-7 MDR (Breast Cancer), A549 (Lung carcinoma), M109 (Lung Carcinoma), KB3.1 (Lung Cancer), B16F10 (Melanoma), MiaPaCa-2 (Pancreatic Cancer) and RMS-1 (human Rhabdomyosarcoma). The cells were grown in DMEM (Dulbecco's Modified Eagle Medium) or RPMI (Roswell Park Memorial Institute medium) supplemented with 10% of fetal calf serum (FCS) and 100 U/mL penicilline and 100 µg/mL streptomycin at 37 °C in presence 5% CO_2 and 95% hygrometry.

4.6. Cytotoxicity assay.

Cytotoxicity of the different pre-activated drugs has been investigated using the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) method (Promega). The compounds (**6, 9a-d**) MeO-, pentyloxy-, 13,17-dimethyloctyloxy- geranyloxy-

and franesyloxy-IFO have been tested on A673 and RMS-1 cell lines, corresponding to respectively human Ewing Sarcoma and Rhabdomyosarcoma cell lines. The pre-activated prodrugs MeO-IFO (**6**), pentyloxy-IFO (**9a**) and 13, 17-dimethyloctyloxy-IFO (**9b**) were tested under bulk form geranyloxy-IFO (**9c**) was tested under bulk and NAs forms and farnesyloxy-IFO (**9d**) only as NAs, as the free form was to lipophilic and insoluble in the media to be tested. The compounds SQ-IFO (**9e**) and SQ-thio-IFO (**9f**) were tested on 12 other human cell lines: (RD, A673, SK-N-MC, UW-479, SAOS-2, MCF-7, MCF-7 MDR, A549, M109 KB3.1, B16F10 and MiaPaCa-2) and their cytotoxic activities were evaluated under NAs form.

The cells were seeded in 96-well plates at optimal cell density determined previously and incubated with 100 µL of DMEM or RPMI containing 10% of FBS and 100 U/mL penicilline and 100 µg/mL streptomycin at 37 °C in presence 5% CO_2 and 95% hygrometry. After 24h, the cells were treated with 100 µL of the different pre-activated prodrugs at different concentrations (i.e, 0.1, 0.5, 1, 5, 10, 50, 100, 200 µM) for all the cell lines. After 72h, or 96h for the compound 13,17-dimethyloctyloxy-IFO (**9b**), 20 µL (1/10) of MTS reagent were added in each well. Depending on the cell line, 2 to 5h incubations were needed to obtain the optimum optical density which was measured at 490 nm wavelength using a microplate reader (EL808, Biotek Instrument). Untreated cells were used as control. Each pre-activated drug concentration was tested in six replicates. Results show the percentage of living cells compared to the control, and the IC_{50} of each compound regarding the tested cell line was determined using Prism 4 (Graph Pad Software, San Diego).

4.7. In vitro release of 4-HO-IFO from X-IFO prodrugs by HPLC-MS/MS.

Stock solutions of 4-HO-IFO were produced as described from hydroperoxy-ifosfamide (4-HOO-IFO) (Niomech, Germany)[28]. During sample incubation at 37°C, the 4-HO-IFO compound

was stabilized in mice plasma (Euromedex, Strasbourg) samples and prepared according to the derivatization method using hydrochloride semi-carbazide reagent (SCZ, Fluka)[29].Briefly, 1 mL plasma in a 2 mL polypropylene tube and 100 µL solution (SCZ 2M in pH 7.4 buffer) were mixed. A volume of 50 µL of internal standard (100 ng/mL in acetonitrile) was added to a sample aliquot of 55 µL. Liquid/liquid extraction was then performed with 1 mL of methyl *ter*-butyl ether. After vortex-mixing, the supernatant was then transferred in another 1.5 mL conic tube and evaporated at 35 °C under vacuum. Finally, the extract was dissolved with 200 µL of HPLC solvent and analysed (20 µL) by HPLC-MS/MS. The RX-IFO compounds and the 4-HO-IFO metabolite were quantified simultaneously in mice plasma, over a concentration range from 50 to 5000 ng/mL, by HPLC-MS/MS using the QuattroLC tandem mass spectrometer (Waters, Manchester, UK) operating with positive electrospray ionization and detected with the following MRM transitions: *m/z* 261.0 → m/z 154.0 for IFO, m/z 334.0 → 221.0 for semicarbazone derivative (4-HO-IFO-SCZ), *m/z* 180.0 → 135.0 for hexamethyl phosphoramide as internal standard; *m/z* 291.0 → 259.0 for methyloxy-IFO, *m/z* 413.0 → 259.0 for geranyloxy-IFO, *m/z* 417.1 → 259.0 for 13,17-dimethyloctyloxy-IFO, *m/z* 481.0 → 259.0 for farnesyloxy-IFO, *m/z* 645.5 → 259.0 for SQ-IFO and m/z 718.3 → 259.0 for SQ-thio-IFO. They were analyzed using the isocratic eluent (acetonitrile / ammonium formate 5mM, 95:5, v/v) with a Ultrasphere® C18 5µm column (2 mm i.d. x 100 mm length (Interchim, Montluçon, France) running at a flow-rate of 0.25 mL/min.

Supporting Information.

HPLC-MS/MS chromatograms and high resolution mass spectra (HRMS) of X-IFO compounds (**6, 9a-f**).

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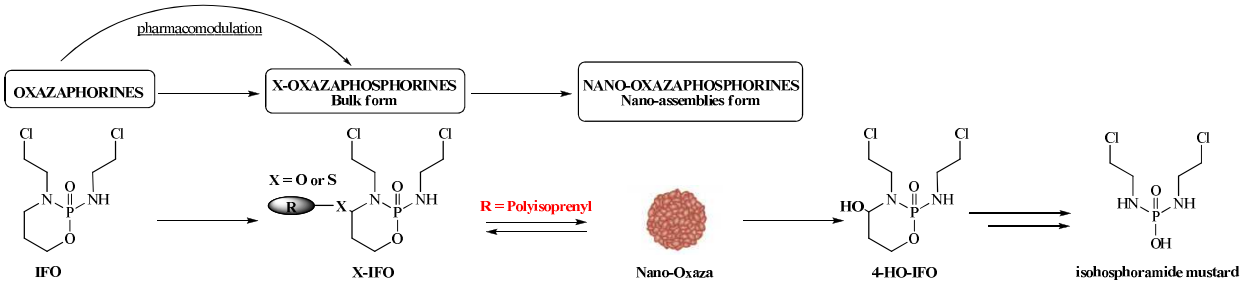
ABBREVIATIONS

IFO, ifosfamide; Mesna, 2-mercaptoethanesulfonate of sodium; NAs, nano-assemblies; NHS, N-hydroxysuccinimide; Et_3N, triethylamine; ACN, acetonitrile; AcOEt, ethyl acetate; PDI, polydispersity index; RPMI, Roswell Park memorial institute medium; DMEM, Dulbecco's

modified eagle medium; FCS, fetal calf serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; SCZ, semicarbazide; MRM, multiple reaction monitoring; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry.

Table of Contents graphic.



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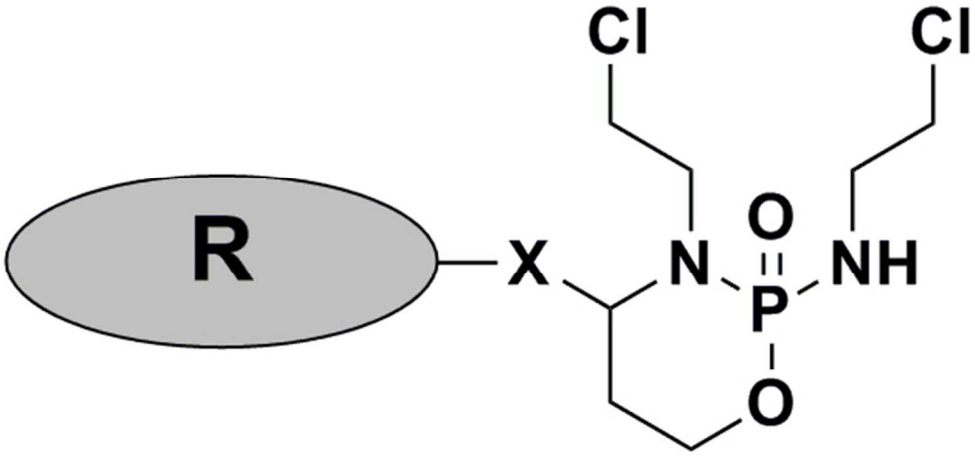

Figure in Abstract
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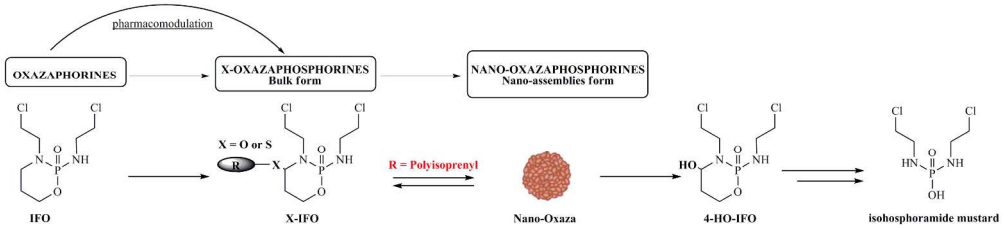

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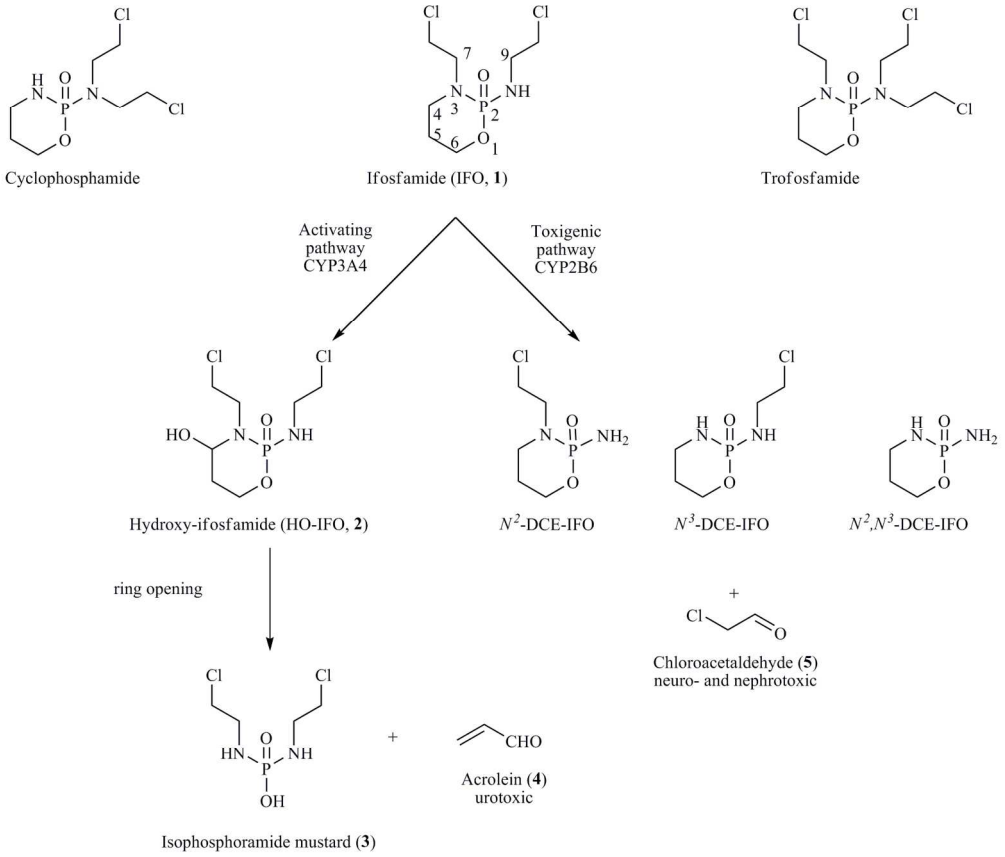

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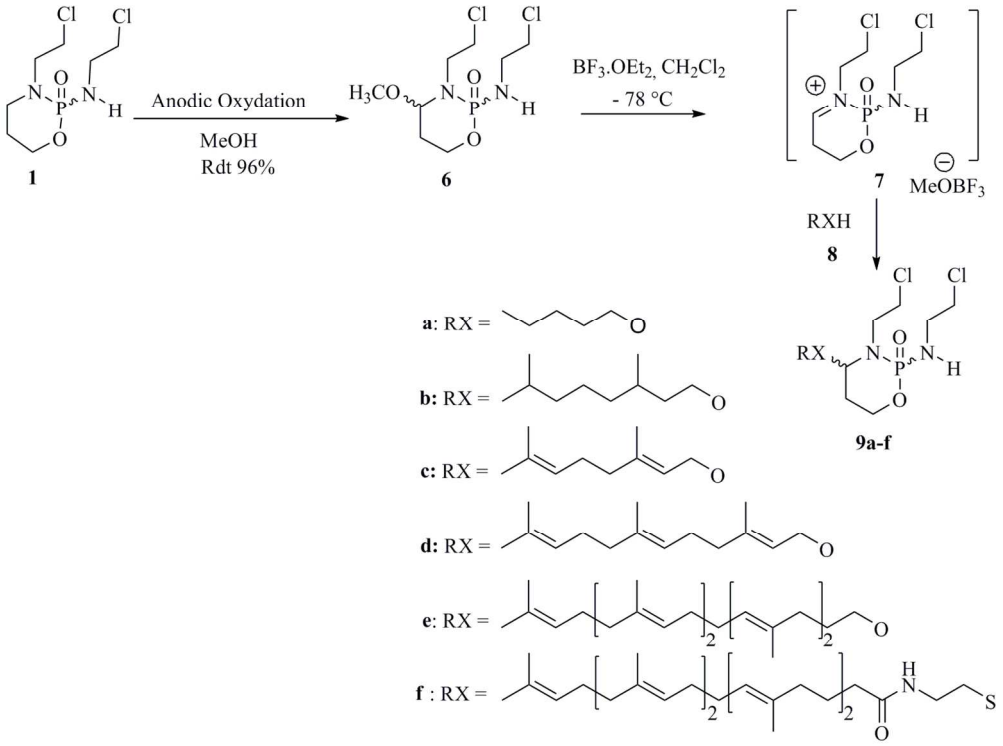

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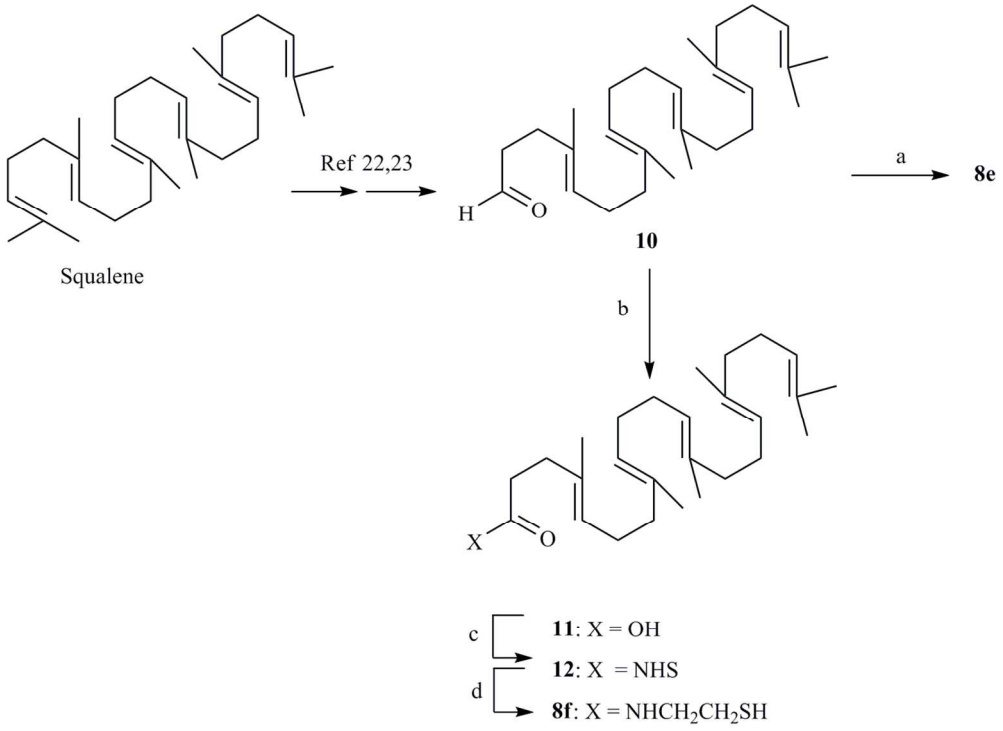

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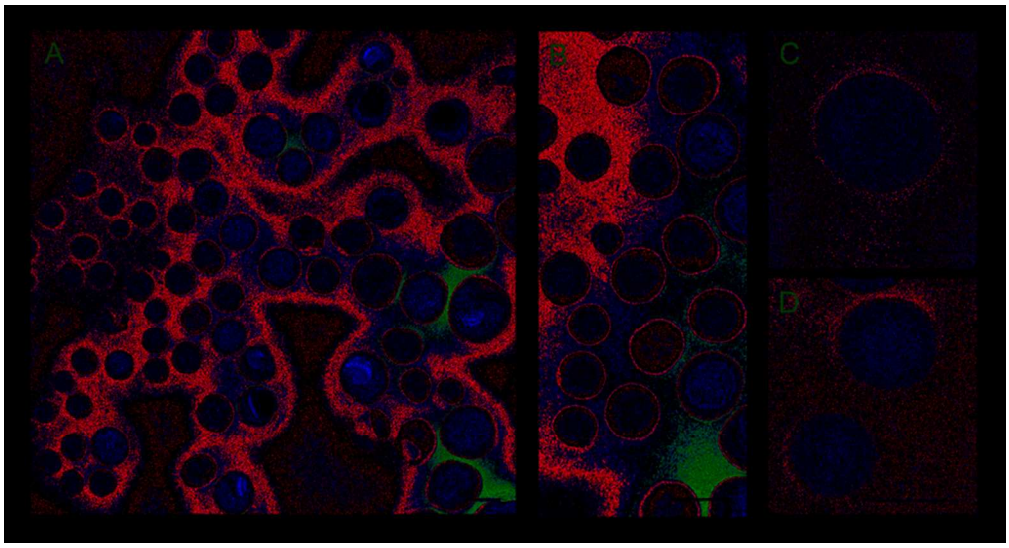

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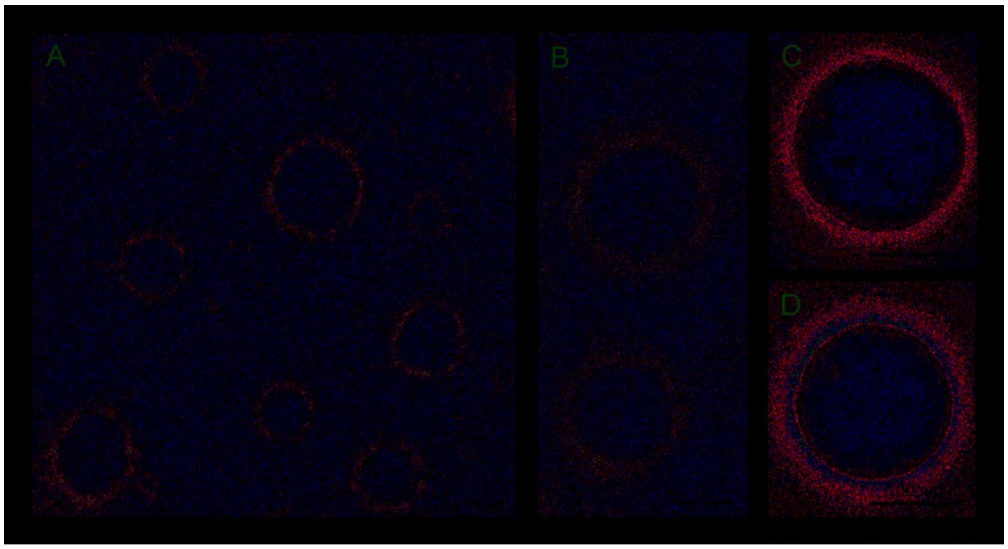

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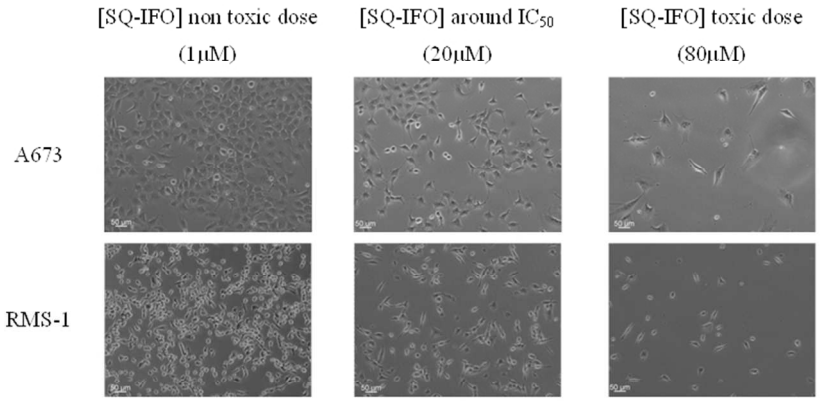

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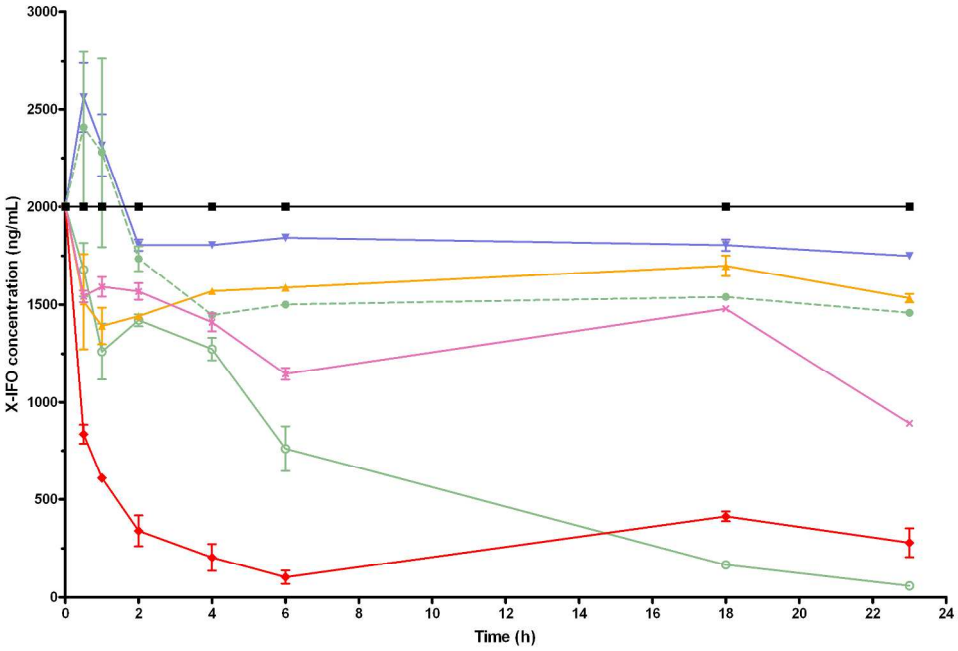

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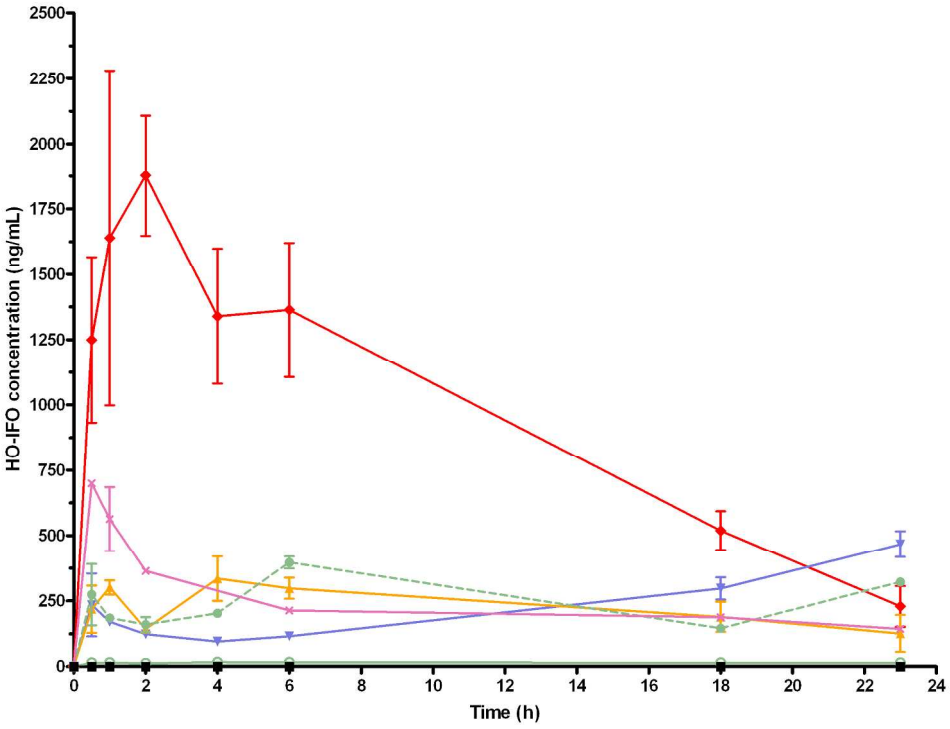

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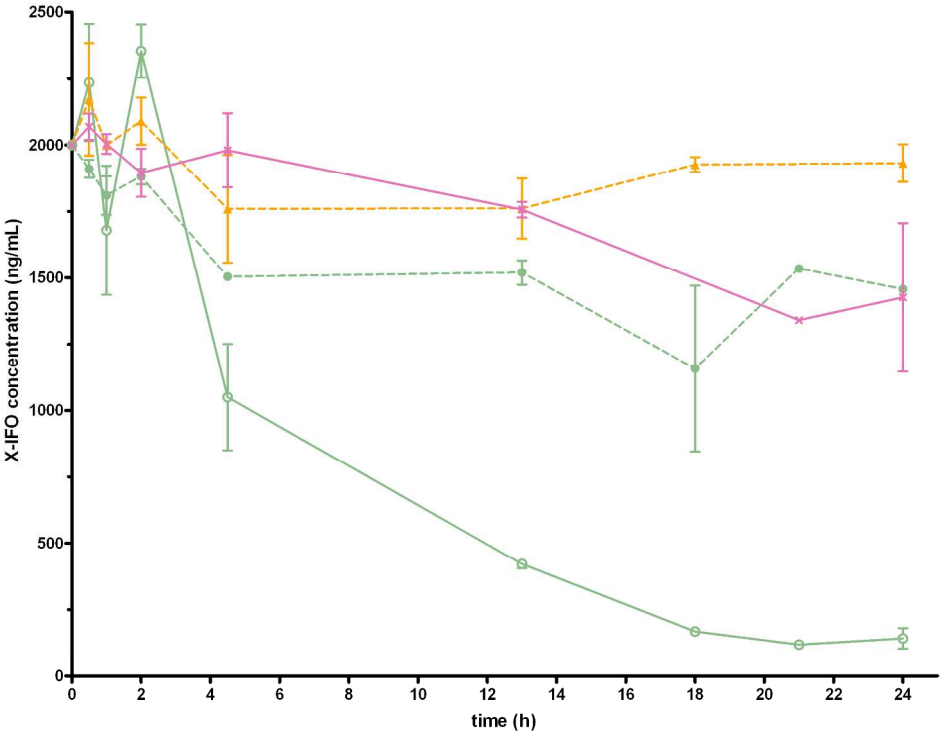

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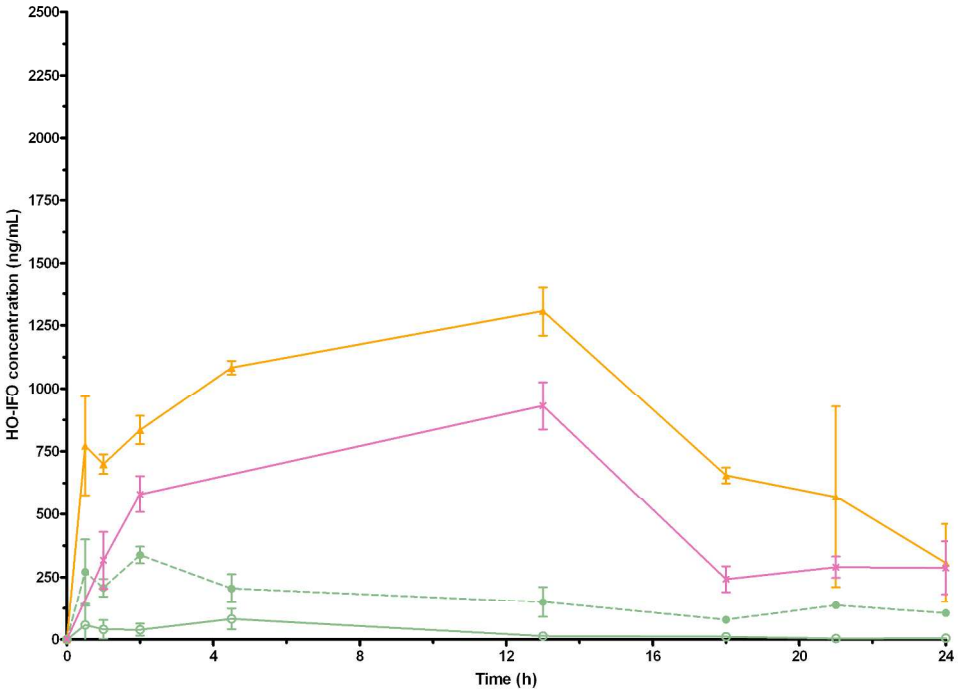

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