A Codrug Approach for the Potential Treatment of EML4-ALK Positive Lung Cancer.

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ABSTRACT: We report on the synergistic effect of PI3K inhibition with ALK inhibition for the possible treatment of EML4-ALK positive lung cancer. We have brought together ceritinib (ALK inhibitor) and pictilisib (PI3K inhibitor) into a single bivalent molecule (a codrug) with the aim of designing a molecule for slow release drug delivery that targets EML4-ALK positive lung cancer. We have joined the 2 drugs through a new, pH-sensitive linker where the resulting codrugs are hydrolytically stable at lower pH (pH 6.4), but rapidly cleaved at higher pH (pH 7.4). Compound (**19**), which was designed for optimal lung retention, demonstrated clean liberation of the drug payloads *in vitro* and represents a novel approach to targeted lung delivery.

Of the non-small cell lung cancers (NSCLCs) 3-7% possess chromosomal rearrangements of anaplastic lymphoma kinase (ALK).[1,2] ALK gene translocations such as EML4-ALK, resulting from inversions in the p-arm of chromosome 2, lead to the fusion of the echinoderm microtubule-associated protein-like 4 (EML4) and ALK genes and produce constitutively active ALK fusion proteins.[3,4] The ALK-EML4 fusion gene[5] contains exons of ALK that code for the intracellular kinase domain of ALK and eight EML4 exons, all of which activate downstream signals of the Ras/Raf/MEK/ERK1/2, JAK/STAT and PI3K/AKT/mTOR pathways,[6] ultimately leading to uncontrolled cell growth and thus its oncogenic nature.

As ALK is required for oncogenic activity, ALK inhibitors, such as crizotinib,[7,8] have been developed as targeted anti-tumour therapies. Ceritinib **1** [9,10] developed by Novartis, has been approved by the FDA (April 2014) for patients that have relapsed on crizotinib.[11] Similarly to crizotinib, ceritinib initially shows promising results. However, patients relapse as resistance rapidly emerges. The situation is similar with alectinib[12] and lorlatinib,[13] where patients initially respond, however relapse is inevitable. Current treatments involve taking these ALK inhibitors in succession as resistance evolves. [14,15]

Another strategy for overcoming resistance to ALK inhibitors is to use upfront combination therapies,[16] targeting one or more signalling nodes that suppress the survival and emergence of resistance.[17,18] However, it is unclear which effector is most critical for EML4-ALK driven cell survival. Drug combinations have been shown to slow down the evolution of resistance, as drugs in combination simultaneously operate with different mechanisms of action, thus reducing the probability of mutations. As the overall goal is to identify a bivalent inhibitor where the two drugs are conjugated with a pH-dependent cleavable linker (Figure 1), the aim of this work is to identify a drug combination that exhibits synergy in a 1:1 molar ratio. Therefore, the drug combinations were tested in a 1:1 molar ratio.

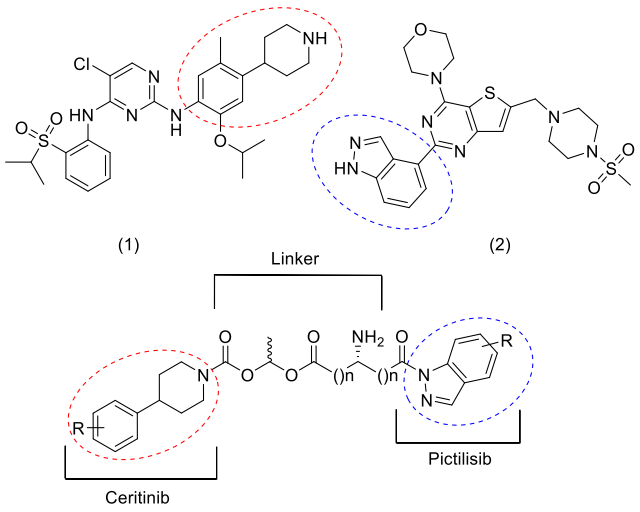


Figure 1. Ceritinib **1,** pictilisib **2**, and the proposed pH-sensitive codrug linking strategy.

The combination of ceritinib (**1**) and the pan PI3K inhibitor pictilisib (**2**)[19] was investigated. We initially examined *in vitro* activity adopting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays against an isogenic pair of human lung carcinoma cell lines, A549 and A549 EML4-ALK, referred to as ALK^- and ALK^+ respectively. The A549 cell lines used, and their respective known mutations are listed in Table 1.

Table 1. Cell lines used in assays

Cell Line	EML4-ALK	PI3KCA	Other Mutations
A549	-	wt	CDKN2A, KRAS
CCL-185IG (A549 isogenic cell line)	+	wt	CDKN2A, KRAS

In vitro evaluation of pictilisib revealed GI_{50} values of 1217 nM ± 85 and 1175 nM ± 141 against ALK$^-$ and ALK$^+$ A549 cell lines respectively. Ceritinib, showed greater inhibitory activity against ALK$^+$ cells than ALK$^-$ cells (GI_{50} values 565 ± 102 and 845 ± 87 nM respectively; Figure 2).

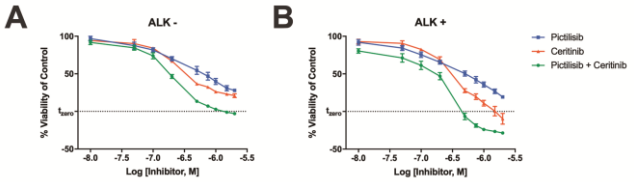


Figure 2. The concentration-response curves for ceritinib, pictilisib and their combination against the ALK- cell line (A) and ALK+ cell line (B). (*n*=3) The dotted line shows the T_{zero} value, and points below this line indicate that an agent is cytotoxic. Growth is represented as percentage viability of the control, which has been normalized to 100%, where 0% is the T_{zero}

Evaluating synergy using the Webb method[20] (Figure 3) showed no statistical significance between the observed and expected effects across all drug concentrations in ALK$^-$ cells. Conversely, against the ALK$^+$ cell line there was good statistical significance of synergy at concentrations >500 nM.

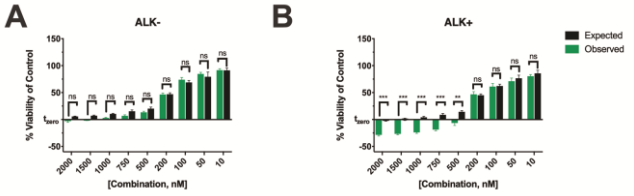


Figure 3. Combination study of ceritinib and pictilisib against the ALK$^-$ (A) and ALK$^+$ (B) cell lines, analyzed by the Webb fractional product method, comparing the expected (additive) effect against the observed effect. Where the observed % viability is less than the expected, synergy can be assumed. Statistical significance was determined by calculation of the p-value using t-tests where: *** P = 0.001, ** P = 0.01, * P = 0.05, ns P > 0.05. Error bars represent the SEM.

The Chou and Talalay method for drug combinations analyses, based on the median-effect equation [21, 22] is discussed in detail in SI. The resulting combination index (CI) theory offer quantitative definition of additive (CI=1), antagonistic (CI>1) or synergistic (CI<1) effects across a range of drug combinations (Table 2). The CIs at a fractional effect (Fa) of 0.5 (effectively at the IC_{50}) were found to be 0.72 and 0.37 for the ALK$^-$ and ALK$^+$ cell lines respectively. At higher concentration, at a fractional effect of 0.35, the CIs were 0.24 against both the ALK$^-$ and ALK$^+$ cell lines. The CI plots for both cell lines are shown in Figure 4. The synergy observed against the ALK$^+$ cell line is greater and observed across a broad range of drug concentrations. Isobolograms at IC_{50}, IC_{75} and IC_{90} clearly illustrate synergistic growth inhibition, with strongest synergy at doses achieving higher growth inhibitory effects (Figure 4A and 4B).[23,24] Clearly, synergy between pictilisib and ceritinib is observed against the ALK$^+$ cell line.

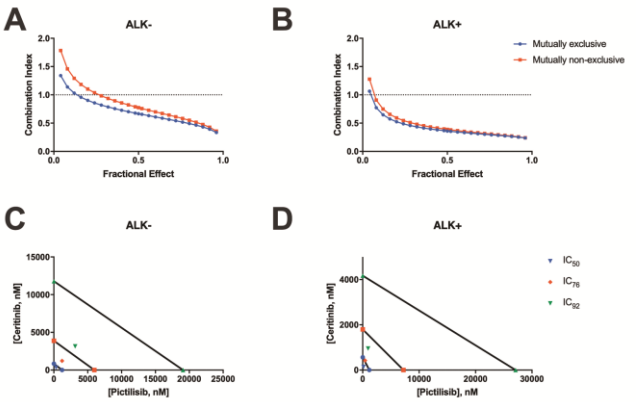


Figure 4. CI plots generated using the Chou and Talalay method for a 1:1 molar ratio of pictilisib and ceritinib against the ALK$^-$ cell line (A) and the ALK$^+$ cell line (B). A CI at a given fractional effect of 1 is an additive effect, >1 is antagonistic and <1 is synergistic. (B) and (C) isobolograms generated from the Chou and Talalay method IC_{50}, IC_{75} and IC_{90}.

Table 2. The parameters used to calculate the combination index of combinations against the ALK$^-$ (grey) and ALK$^+$ cell lines.

Drug	m-value[#]	D_m Value[#]	Combination Index (Fa) 25	(Fa) 50	(Fa) 76	(Fa) 96
Ceritinib	0.76 (0.07)	0.85 (0.09)				
Pictilisib	0.72 (0.09)	1.22 (0.09)	0.95	0.72	0.55	0.35
Combination	0.88 (0.07)	0.67 (0.05)				
Ceritinib	0.76 (0.07)	0.57 (0.1)				
Pictilisib	0.64 (0.08)	1.12 (0.1)	0.52	0.37	0.30	0.24
Combination	0.28 (0.05)	0.60 (0.22)				

The CI is given as an average of the mutually exclusive and mutually non-exclusive combination indices. [#] (± SEM) μM.

After observing substantial synergy specifically in ALK$^+$ cells, western blot analyses were adopted to explore signal transduction activation following treatment of cells with pictilisib (500 nM and ceritinib (500 nM). Figure 5 demonstrates the presence of the EML4-ALK fusion protein in lysates prepared from ALK$^+$ A549 cells. The presence of STAT3 protein is evident in both A549 populations irrespective of EML4-ALK expression (Figure 6A). In contrast, downstream of ALK, activation of the JAK-STAT pathway (P-STAT3) is detected exclusively in ALK$^+$ cells. Also evident is inhibition of STAT3 signalling following treatment of cells with ceritinib alone, and almost complete abolition of P-STAT3 after treatment of cells with the drug combination (Figure 6B).

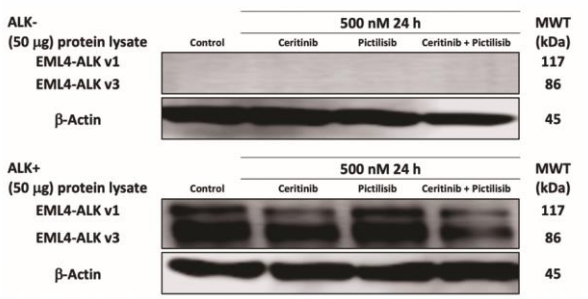


Figure 5. (A) Immunoblot analysis confirming lack of EML4-ALK in the ALK- cell line. (B) Immunoblot analysis confirming the presence of EML4-ALK in the ALK+ cell line. The Concentration-response curves for **23**, ceritinib, pictilisib and their combination against the ALK- cell line

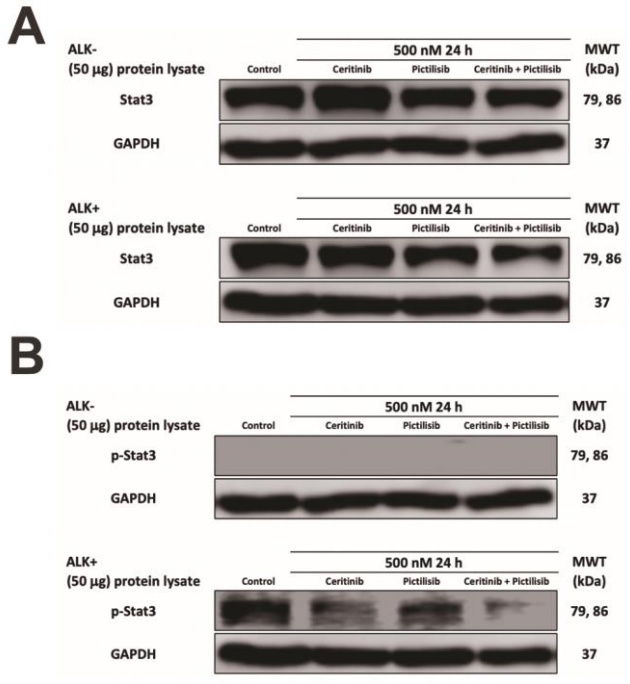


Figure 6. Immunoblot analysis of total STAT3 (A) and P-STAT3 (B) against the ALK+ and ALK- cell lines after 24 h treatment of pictilisib, ceritinib and their combination.

A further issue associated with ceritinib treatment is adverse toxicity where >50% patients in clinical trials experienced adverse events that necessitated a reduction in dose. There are also serious side effects observed with pictilisib (diarrhoea, nausea, taste alteration, rash, fatigue, itchiness, vomiting and decreased appetite) where a suggested daily oral dose is 340 mg.[25] With these adverse effects, either dose-reduction or localized, topical application is desirable. One strategy to achieve such outcomes would be delivery *via* controlled release i.e. in the form of a prodrug. Inhalation is an increasingly important delivery approach for respiratory therapeutics and yet there still remains an unmet clinical, commercial and practical need for a 'once-a-day' treatment. There has been much debate about the various strategies for the rationalization of agents that exhibit a sustained duration of action when applied topically to the lung. [26] Examples of such approaches are compounds that display a reduction in solubility and permeability, where slow dissolution into the airways smooth muscle affords the potential for extended lung retention.[27] In addition, increasing lipophilicity has been shown to be an important parameter in delivering compounds with an extended duration of action.[28, 29] Along with modulation in lipophilicity, it has been shown that the incorporation of a dibasic pharmacophore within the compounds, leads to an increase in the duration of action of inhaled compounds.[30]

With these concepts in mind, we have brought together ceritinib and pictilisib into a single bivalent molecule (a codrug)[31,32] with the aim of designing a molecule for slow release drug delivery that could target EML4-ALK positive lung cancer. The codrug is designed as a bivalent ligand, sporting a pH-dependent cleavable linker which is more hydrolytically stable at lower pH (pH 6.5 *vs* pH 7.4). This approach is interesting in that the pH of the lung environment is slightly acidic (pH ~6.5 to 6.8) enabling a potential lung tissue retention for the biologically inactive codrug.

The piperidine ring on ceritinib and the indazole of pictilisib were identified as appropriate synthetic handles for exploration. In a codrug model system (**5**), 4-phenyl piperidine (**3**) and 5-bromo indazole (**4**) were used as drug mimetics. With the indazole as a superior leaving group than the piperidine, this was used to form an *α*-amino amide. The α-amino amide was chosen with the hypothesis that the amino group will either aid hydrolysis through anchimeric assistance *via* hydrogen bonding of the non-protected amine to water (pKa ~ 7.08).[33] This hypothesis forms the basis of the pH-dependent linker (Figure 7). [34] An aspartic acid analogue was chosen to form part of the linker due to the well-known cyclization mechanism of amino acid cleavage, which would present itself following hydrolysis of the alpha amino amide, releasing pictilisib.

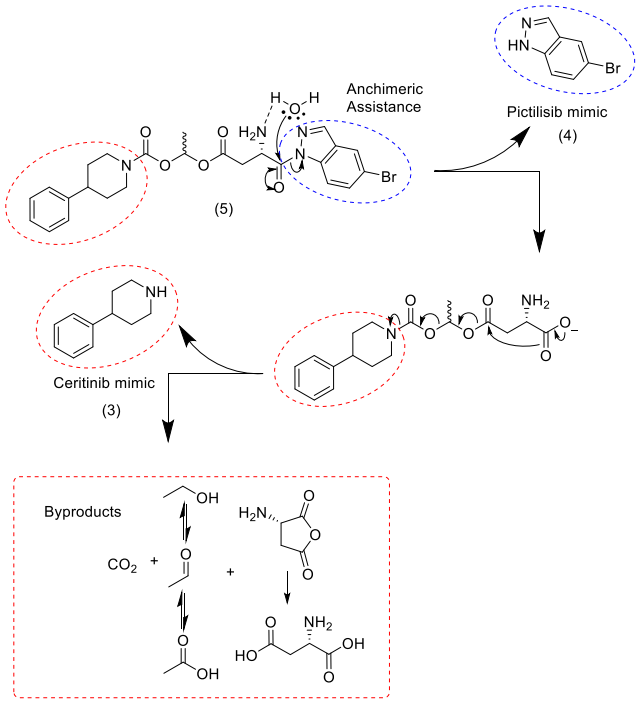


Figure 7. The hypothesized cleavage mechanism of the codrug. The α-amino group delivers water to the site of hydrolysis by anchimeric assistance. This anchimeric assistance can either be hindered or assisted, depending on the pH, as the amino group will have a different percentage ionization at differing pH. Following the delivery of water and release of pictilisib, the codrug has been designed to degrade *via* a well-known cyclization mechanism,

yielding the anhydride (which is subsequently hydrolyzed to the amino acid). This is followed by collapse, eliminating acetaldehyde (which disproportionates to acetic acid and ethanol - as seen by NMR), carbon dioxide, and ultimately ceritinib. It is known that release of acetaldehyde could have inflammatory effects in the lung tissue, however this could be alleviated in future work through modification of the acetal linker group.

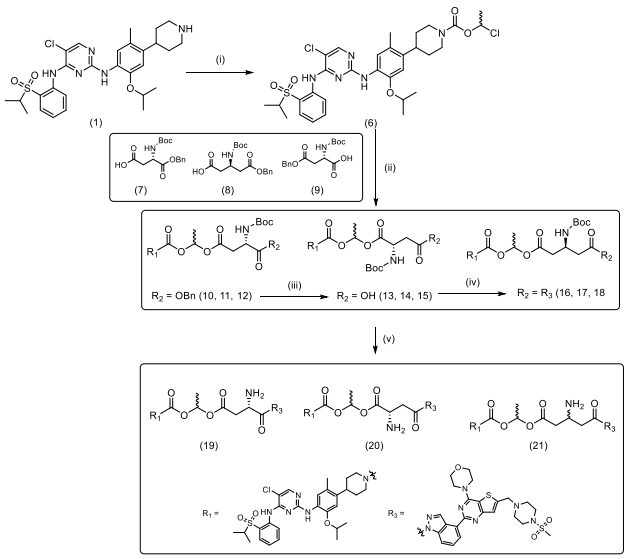
The concept was investigated through the synthesis of a codrug model **5**. A kinetic study was performed using a time-course NMR method where the time course experiment was run at pH 6.4, 6.8 and 7.4 and the corresponding half-lives and rate constants calculated (Table 3 and SI).

Table 3. Half-lives and rate constants for the release of drug mimetics from the codrug model system 5 at a range of pH.

pH	$t_{1/2}$ (hr)	k_1 (s^{-1})	percentage unionized
6.4	4.64 ± 0.5	0.22 ± 0.003	17.3%
6.8	0.70 ± 0.11	0.84 ± 0.06	34.4%
7.4	0.39 ± 0.7	1.37 ± 0.17	67.6%

Immediately it could be seen that as the pH increases the model codrug cleaves more rapidly (Table 3). The fact that the rate of reaction increases with the percentage unionized is evidence in favour of the hypothesis that the *α*-amino group delivers water to the amide carbonyl through anchimeric assistance. After proof of concept with the model system, cleavable codrugs (**19-21**) were synthesized starting from ceritinib and pictilisib (Scheme 1).

Scheme 1. Synthesis of the codrugs (19-21)



Scheme 1. Reagents and conditions: (i) **1** (1.0 eq.), chloroethyl chloroformate (1.1 eq.), NMM (2.0 eq.), CH_2Cl_2, -10 °C, 45 min; (ii) *N*-boc-Cbz-protected amino acid **7-9** (0.8 eq.), Ag_2O, NBu_4Br (0.16 eq.) , Toluene, 60 °C, overnight, Yield 34-77%; (iii) Pd/C, H_2, EtOAc, rt, overnight, Yield 76-99%; (iv), **2** (1.0 eq.), HATU (1.1 eq.), DIPEA (3.0 eq.), CH_2Cl_2, rt, 6 hr, Yield 3-11%; (iv) TFA, CH_2Cl_2, rt, 1 hr, 100%.

The synthesis of codrugs (**19-21**) started with the acylation of ceritinib (**1**) with 1-chloroethyl chloroformate (ACE-Cl) in the presence of *N*-methyl morpholine to give (**6**). Coupling of the amino acid partners (**7-9**) was achieved using silver oxide containing a catalytic amount of *tert*-butyl ammonium bromide in toluene at 60 °C. Following deprotection, the corresponding carboxylic acids (**13-15**) were reacted with **2** to give the Boc-protected codrugs (**16-19**). Once required, the protecting group was removed with TFA to give the codrugs (**19-21**). The protected codrugs were stable in air at room temperature >1 year after synthesis

The hydrolytic stability of (**19-21**) was investigated in buffered solutions supplemented with 4% bovine serum albumin (BSA) to aid solubility (Table 4). In addition, rat plasma stability was assessed (Table 5). The stability results for (**19**) at both pH 6.5 and pH 7.4 (Figure 8) show clean conversion to pictilisib and ceritinib.

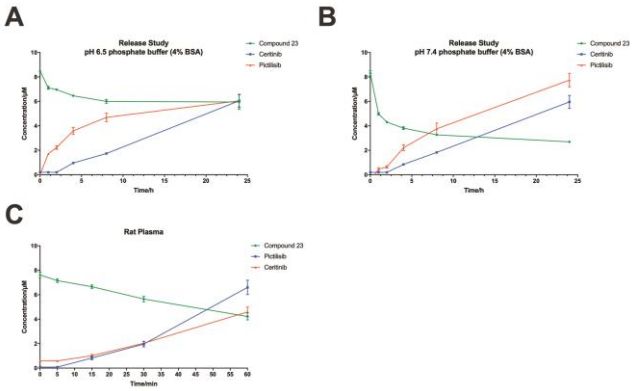


Figure 8. Release of **19** at (A) pH 6.5 and (B) in pH 7.4 phosphate buffer containing 4% BSA showing degradation of the codrug with release of pictilisib followed by the subsequent release of ceritinib. (C) Stability study of **19** in rat plasma.

Table 4. Summary of half-lives and rate constants for the codrug systems in buffer + 4 % BSA.

Example	pH	k/ s^{-1}	$t_{1/2}$/ h	r^2
19	6.5	0.036 ± 0.01	19.3	0.79
	7.4	0.094 ± 0.04	7.4	0.70
20	6.5	0.02 ± 0.03	34.7	0.93
	7.4	0.036 ± 0.01	19.3	0.74
21	6.5	0.058 ± 0.03	12.0	0.99
	7.4	0.091 ± 0.02	7.6	0.87

The compounds were assayed as a mixture of 2 diastereomers. However, **19** was purified by HPLC and it was observed that there was no difference in the rates of release of the single enantiomers.

From Table 4 it can be seen that the half-lives of the codrugs at pH 6.5 are longer than the half-lives at pH 7.4..

In rat plasma, the degradation of **20** is different to that of **19**, which showed a zero-order degradation (a linear plot of [codrug] *vs* time). Compound **20** shows a first order degradation profile.

Table 5. Summary of rate constants and half-lives for the codrug systems in rat plasma

Example	k	$t_{1/2}$/min	r^2	Order
19	0.069 ± 0.02 μM s^{-1}	58	0.85	zero
20	0.018 ± 0.001 s^{-1}	38	0.99	first
21	0.01 ± 0.001 s^{-1}	69	0.97	first

The biological evaluation of **19** was studied by MTT assay, and compared to the ceritinib/pictilisib combination (Figure 9).

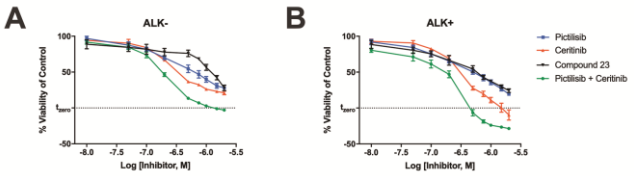


Figure 9. The concentration-response curves for (**19**), ceritinib, pictilisib and their combination against the ALK$^-$ cell line (A) and ALK$^+$ cell line (B). Points represent the means ± SEM (minimum 3 internal replicates). The dotted line shows the T_{zero} value, and points below this line indicate net cytotoxicity. Growth is represented as percentage viability of the control, which has been normalized to 100%, where 0% is the T_{zero}.

The concentration-response curve in the ALK$^+$ cell line appears to mirror the concentration-response of the combination but is right shifted. This suggests that the codrug had not fully cleaved to provide the drugs in the desired concentration to have the required biological effect. There most probable reason for this effect is that the codrug is highly protein bound. As accumulating evidence suggested that FBS negatively impacted biological activity, **19** was incubated in RPMI medium containing 10% FBS and the drugs' release was followed by HPLC. Even after 72 h the codrug had not released its active payloads and this is highly likely a consequence of very high protein binding (Figure 10).

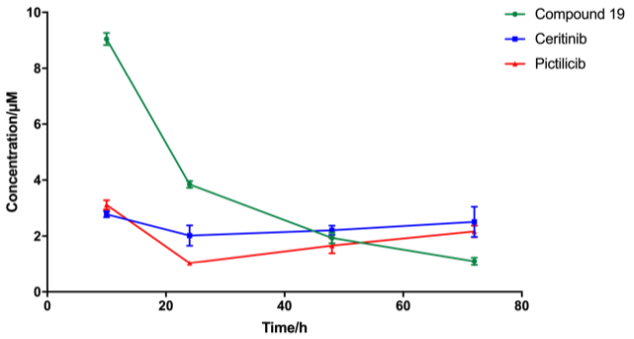


Figure 10. Release study of **23** in RPMI cell culture medium containing 10% FBS at 37 ºC. Minimal release of the drugs after 72 h indicates binding of the protected codrug to proteins.

To conclude, we have demonstrated the synergistic biological activity between pictilisib and ceritinib and have used this finding to design, synthesize and test a novel codrug system where the two drugs are joined by a new pH-cleavable linker. Whilst the resulting codrug did not display the anticipated enhanced biological response, we have suggested that this is likely because of the inherent high albumin binding exhibited by the codrug due to its high lipophilicity (cLogP 6.9).[33] However, we postulate that this could be a possible benefit for inhaled sustained delivery applications. Further work is on-going to explore the albumin binding dependence and cleavage of the codrugs and will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Representative synthetic procedures and analytical data for **5, 19-21**. Description of biological and stability assays. Immunoblot analysis of PI3K/AKT/mTOR pathway activation through phosphorylation of AKT (b) Ras/Raf/MEK/ERK activity through phosphorylation of ERK1/2.

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors.

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ABBREVIATIONS

AKT, Protein Kinase B; ALK, Anaplastic Lymphoma Kinase; CI, Combination Index; EML4, D_m, median effect dose; Echinoderm Microtubule-Associated Protein-like 4; ERK, Extracellular Signal–Regulated Kinases; Fa, Fractional Effect; FBS, Foetal Bovine Serum; GI_{50}, Conc. required to inhibit 50% of maximal cell proliferation; MEK, Mitogen-activated Protein Kinase; mTOR, Mammalian Target of Rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; RAF, Rapidly Accelerated Fibrosarcoma; STAT, Signal Transducer and Activator of Transcription.

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