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TITOLO DELLA TESI:

SYNTHESIS OF SMALL MOLECULES ACTIVE ON TUMOR RELEVANT CELLULAR SIGNALING PATHWAYS

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INTRODUCTION

1.1 SMALL MOLECULES AND TARGET THERAPY

The homeostatic balance of a complex organism is an extremely precise set of signalling mechanisms which all the physiologic activities depend on. A minimal abnormality or a wrong interaction among the components is enough to arise serious, degenerative and invalidating pathologies, birth defects, nervous affections. The pharmaceutical research is focused on the analysis and an increasingly more detailed comprehension of each step of these mechanisms to find the best target therapy and to attack the key deviations responsible of the cascade of the events which lead to diseases. The strategy is based on the identification and the druggability evaluation of driver genes. However, several elements have to be taken into account: the complexity of the cancer genome, a relevant heterogeneity within each single cancer type leading to drug resistance and the ability to demonstrate a therapeutically meaningful biological effect in important experimental models. It is remarkable that many of the key-cancer genes are not suitable to be used as clinical drug-targets and that the identification phase generally requires many years.[1]

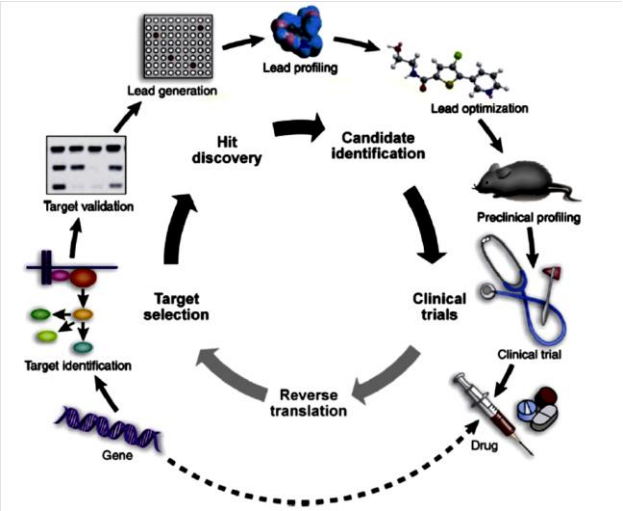


Figure 1.

In this variegated scenario, my PhD work is focused on the improvable potential of small molecules in interfering with occurring cells and tissues abnormalities and consequently reactivating normal functions. Furthermore, one of the greatest challenges is to find a way to avoid the adverse effects which have always represented a rate limiting feature, and ~~so~~ accordingly to increase the selectivity through a fine chemical manipulation of the well known and approved therapeutics.

1.2 HEDGEHOG SIGNALING PATHWAY

The importance of Hh signalling cascade was shown 30 years ago, by Nüsslein-Volhard and Wieschaus, by observing and studying the segmentation process during the embryogenesis of *Drosophila melanogaster*.

This study enabled to get insights in the regulating mechanisms of an embryo's development along the anteroposterior axis and to find out that the genes involved are also implicated in matching the structure of the adult cuticle. In fact, the induction of proper genetic mutations resulted in the development of 40% length of the wild-type larva with a ventral lawn of denticles, giving a typical phenotype looking like a hedgehog (Figure 2).[2]

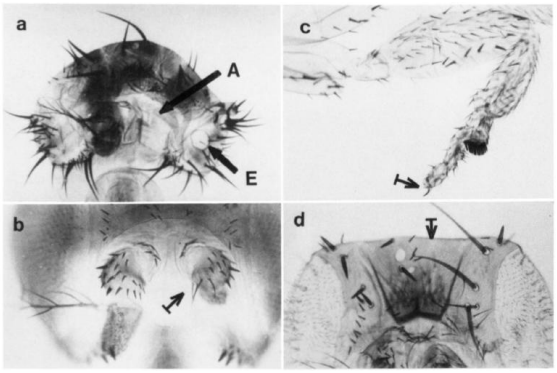


Figure 2.

The crucial role during the embryonic life and in adult tissues was then discovered in vertebrates. In mammals three Hh ligands, Sonic Hedgehog (Shh) Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) are responsible of signal transduction mediated by lipid-modifications and with the cooperation of several protein-based components. The development of kidney, lung, limb, nervous system depends on the physiological signalling of this pathway.

1.2.1 The Signal Transduction

The figure below compares the activity of the pathway in the presence of the Hh ligands with the one in the absence of them (Figure 3).

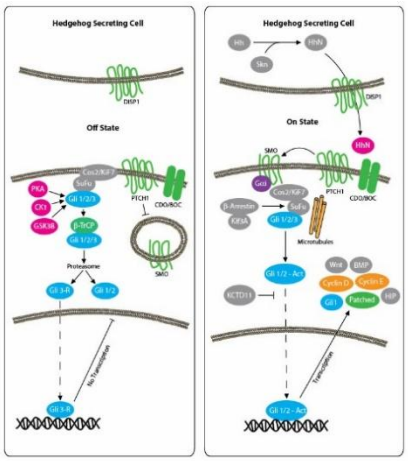


Figure 3.

In the OFF state these ligands are retained by the 12-transmembrane receptor *Dispatched* (Disp) in specific compartments inside the secreting cell, as inactive precursors. At the same time in the signal receiving cell, another 12-transmembrane receptor, *Patched* (Ptch) inhibits *Smoothened* (Smo) by a not yet clear mechanism in an intracellular vesicle, thus blocking all the cascade transcriptional events. However, it seems that Ptch prevents the interaction of Smo with some oxysterol moieties, and this explanation could be confirmed by the presence in Ptch structure of a Sterol Sensing Domain (SSD). Moreover, it's plausible that the Hh ligands themselves restrain the oxysterol release mediated by Ptch, acting independently from the cell inhibition mechanisms.

In this state Ptch inhibits the C-terminal phosphorylation and the Gαi protein binding on Smo, a 7-transmembrane receptor belonging to the G-Protein Coupled Receptor (GPCRs) family, mediated by GPCR kinase 2 (Grk2) and representing a functional modification to impede Smo actions. As a consequence, the protein kinase A (PKA) phosphorylates three zinc-finger transcription factors (Gli1, Gli2, Gli3) belonging to the Gli protein family and responsible to regulate the expression of Hh pathway target genes once entered into the nucleus. The phosphorylated forms of Gli2 and Gli3 are recognized by the F-box protein beta-transducin repeat containing protein (β-TrCP) and processed by proteolytic events to truncated inactive structures, unables to migrate into the nucleus. Moreover, the phosphorylation mediated by PKA inhibits the dissociation of o the Sufu-Gli3 complex. Sufu plays a relevant inhibiting role by binding the Gli factors but many aspects of its behaviour

are still unclear, like the Gli-Sufu complex localization in the cell and its dependence on PKA action. It seems that the phosphoinositide 3 kinase – Akt pathway (PI3K) controls the PKA action which directly depends on high cAMP intracellular levels.

This equilibrium is broken when the Hh ligands interact with Ptch in their active form and all the system pass in an ON state. Among the three isoforms, Shh is the most expressed one in vertebrates and from the quiescent state it undergoes modifications that allow its release in the extracellular environment. As a precursor it is a ~45KDa protein and it is at first auto-catalitically cleaved by the COOH terminal domain, resulting in two fragments which only the one containing the NH_2 terminal domain is involved in the following steps. These consist of the addition of a cholesterol moiety in the C-terminal side and a palmitate on the N-domain and result in the Cholesterol-modified Hh-Np active Shh. Disp mediates the release from the secreting cell and enables the interaction with Ptch1 (apparently the isoform Ptch2 has no relevant functions) and the subsequent activation of Smo. In fact, the Ptch-Hh complex migrates from the cell surface at the basis of the primary cilium into cytoplasmic lysosomes where it is degraded. At the same time the active Smo (with the phosphorylated C-terminal and the binding with the Gαi protein) enters and accumulates in the primary cilium, a particular structure only found in vertebrate cells –which is fundamental in displaying their own activity. Here it undergoes a second structure change enabling the reduction of the cAMP concentration and the inhibition of the PKA blockage on the Gli proteins, which enter the nucleus and promote the transcription of the Hh target genes. From different biomarker studies, it has emerged that the most expressed gene is *Gli1* and it enhances the activity of Gli2, Gli3 and Smo, whilst the expression of the *Ptch1* and the *HHIP* target genes induces a negative feedback on the pathway. In particular, the first acts on Smo, but the second does not seem to depend directly on Hh canonical way of action because it is able to bind extracellular Hh ligands. Actually, it needs to be considered the presence of three more mechanisms involved, which are supposed to exclude some of the classical components:[3]

1. Atypical interactions > the members involved in the signal transduction are the same and their activity depend on the Gli transcription factors.
2. Independent Gli-mediated transcription > proper of the axon guidance events, the Shh-induced cytoskeletal rearrangement and the Shh-dependent induction of endothelial cell angiogenesis.[4]
3. Interaction with other pathways > for instance the modulation of the Cyclin B1 action on the cell cycle progression. This protein is captured and blocked by Ptch1, but released by

Smo. In addition, it's known that Ptch1 shows apoptotic induction ability while Smo is an anti-apoptotic factor.[5]

1.3 PATHWAY'S ALTERATION AND TUMORIGENESIS

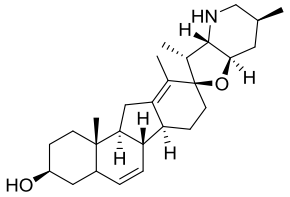
1.3.1 Negative Modulation

Each component of the pathway can develop mutating forms during the transcription process, and the cell control mechanisms have the role to limit the errors. Sometimes they are overpowered and the result is the development, proliferation and the metastatic expansion of different cancer disease such as medulloblastoma and basal cell carcinoma (BCC).

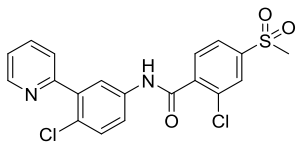
Different small molecules are currently in trials as Hh inhibitors and some of them approved by FDA are on the market. On the basis of the ability of tumour cells to become resistant, a more detailed analysis of the pathway's components and the enhancement of potency and the selectivity by discovering new small molecules would be a great challenge. The very first Hh inhibitor is Cyclopamine, a natural alkaloid which belongs to the jervine family, a metabolite of the plant *Veratrum californicum* responsible of congenital deformities observed in cyclopean lambs in the USA during the 1950s.[6]

This steroidal compound blocks Hh signalling (IC_{50}=300nM) through a direct binding on Smo which represents a key therapeutic target. [7]

Therefore, a high throughput screening was carried out on a library of commercial compounds, in order to avoid teratogenic effects and to improve the pharmacological profile. The synthetic small molecule GDC-0449 (Vismodegib) showed a strong interaction with Smo and an antitumor activity in clinical trials against locally advanced basal-cell carcinomas, which are mainly caused by genetic alteration of PTCH1 with a subsequent constitutively activation of Smo with uncontrolled basal-cell proliferation. Vismodegib was approved by FDA in 2012. It shows an IC_{50} of 16.1μM even though it causes different grades of side effects including dispnea, muscle spam, dehydration, pneumonia etc.



Cyclopamine

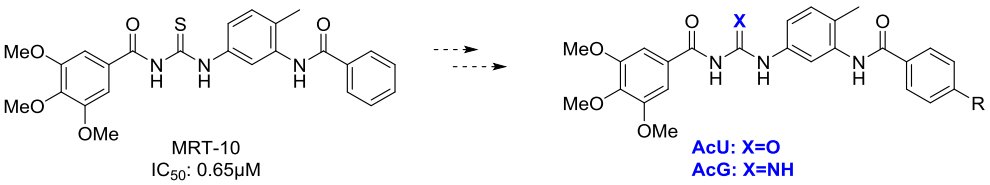


GDC-0449

Figure 5.

A phase1 trial showed that it inhibits Smo through a direct binding and it blocks accordingly all the downstream cascade effects.[8]

The complex structure of Smo has been better highlighted through X-ray methods, which allowed to elucidate in details that this G-Protein Coupled Receptor (GPCR) belongs to the class F (frizzled family) but also shows distinctive features. Three loops form the extracellular domain (ECL) and the intracellular amino-terminal part is a cysteine-rich domain. Other important evaluations are related to ~~on~~ how the mutating expression of some of Smo genes (D473H and LDE225 particularly) are responsible of the observed tumour cells resistance to the current treatments (and to focus on new classes of compounds in order to overcome this problem). The already known antagonists can be divided in two classes: type1 (binding the ECL) and type2 (binding the intracellular domain). A pharmacophoric model of Smo was used to identify by virtual screening a structure model able to bind all the receptor and so enhancing the inhibiting effects. The acylthiourea MRT-10 with an IC_{50} of 0.65±0.15 μM in the ability to reduce osteoblast differentiation of C3H10T1 multipotent mesenchymal cells encouraged further developments leading to optimized acylureas and acylguanidines. [9]



Scheme 1.

1.3.2 Positive Modulation

On the other hand there's evidence of a predominant responsibility of the pathway ipo-functionality in a conspicuous group of deficiency such as myocardial infarction, ischemic heart disease and diabetes disorders. From recent studies it's emerged the ability of the brain to generate new cells during the adult life because of the presence of stem cells, with high proliferating and plastic properties, in different regions. Their maintenance and differentiation into new neurons to replace the old ones is regulated by Hh pathway. Deviations can occur in each regulated step and lead to demyelinating events, or neurological, psychiatric, neurodegenerative disorders.[10,11]

Hh pathway is an integral component in the central nervous system (CNS) and in the peripheral (PNS), it controls the neuronal cell fate, and the positive modulation of Smo produces positive effects against Parkinson's disease and the general surviving and regeneration of the nervous tissues. Among the three ligands, IHh is involved in the

development of osteoprogenitor cells. Indeed, many degenerative bone disorders respond very well to pathway's reactivation therapies. The proof of the crucial role of this cascade can be found in the development of skin, hair cyclic growth, and fat formation. In this case a non-canonical Smo-Ca+ -Ampk axis is responsible for blocking only the white adipogenesis and for promoting the insulin dependent glucose uptake in muscle and brown fat tissues. This would explain the utility of the agonists in case of diabetes.[12]

Some endogenous molecules, such as oxysterols with osteoinductive and anti-adipogenic effects interact and modulate the pathway.[12] This is probably related to a similar structure observed in the Hh precursor proteins which are associated with cholesterol moieties. They all show inhibitor and agonist behaviours and synthetic analogues have been prepared in order to optimize the potency of activation and the related effects, and to elucidate the mechanism of action, which is related to a direct bound on Smo, upon the extracellular cysteine rich domain (CRD).

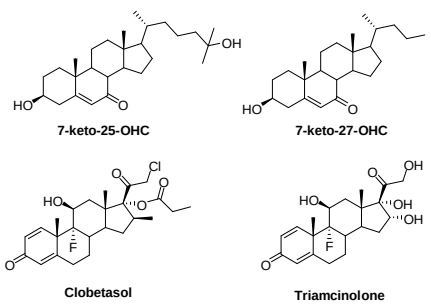


Figure 6

Similar results have been obtained with synthetic glucocorticoids (Figure 6) which can be included in the agonist class for the ability to induce proliferation of cerebellar granular cell precursors (GCPs). For example, there is some evidence of coronary vessel increase, insulin production, human embryonic stem cell differentiation, and facilitation in tissue and organ repair. Interesting results have been collected from experiments conducted in bone diseases such as osteopenia and osteoporosis related to downregulation of the pathway and treated with Hh agonist small molecules. Moreover, they promote the formation of new bone tissue after mouse amputation. Indeed, important results are emerging from experiments on obesity and diabetes cases, demonstrating that Smo agonists are capable to stimulate the adipocyte maturation and glucose utilization by acting on the AMPK (AMP-activated protein kinase) a key component in the glycogen synthesis and glycolysis.[13]

Purmorfamine (Pur) is the first natural compound with osteogenic properties observed both in mice and in human mesenchymal cells. In vitro experiments on the C3H10T1/2 lineage show that it is capable to stimulate cell proliferation, to promote the differentiation of mesenchymal progenitor cells into osteoblasts, and to block spontaneous adipogenesis. All these effects are related to the Pur ability to enhance the expression of genes involved in Hh pathway signalling such as Gli1, Gli2, Patched, Patched2, IGF-binding protein-3. Furthermore, other results show that it does not influence the Hh protein genes expression.

Hh signalling reporter assay (luciferase assay) also confirms the positive modulation of Pur on the pathway.[14]

SAG is a synthetic chloro-benzothiophene derivative, a Hh agonist, identified after a high-throughput screen of 140000 small molecules in a luciferase assay, able to interact with Smo.[12] Elucidation of its mode of action results from the observation on Shh-LIGHT2 cells in the photoaffinity labelling and the fluorescence binding assays which allowed to confirm an EC_{50} of ~ 3 nM for this molecule. Moreover, it was possible to demonstrate that the pathway's activation is independent from the Ptch proteins, and that it is directly associated with the interaction on the heptahelical bundle of Smo. However, Ptch may contributes to this activation through some endogenous cellular components. SAG binds and inhibits also some components involved in the downstream effects of the signal transduction when its concentration raises up over 1 µM and that its positive modulation on the pathway is maybe related to an enhanced interaction between Smo and a cellular effector physiologically involved in the process.[15] On the basis of its structure and behaviour a series of SAG derivatives have been obtained and tested, resulting in improved potency (EC_{50}: ~ 0.3 nM) and positive activity.[15]

Therefore, a pharmacophoric model was built based on their skeletal structure to verify the grade of interaction of a library of commercial compounds different from the series of SAG and a quinolinone derivative showed a promising profile. This can be included in the group of the positive modulators of Smo and represents the progenitor of a new class (Figure 7).

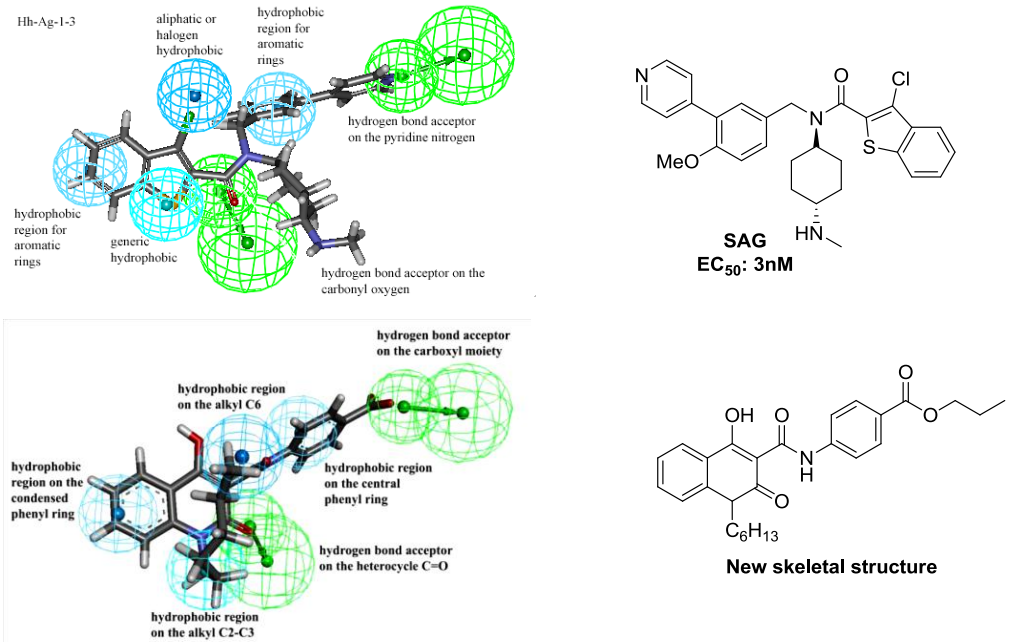


Figure 7.

1.4 THE MRN COMPLEX

1.4.1 DNA repair pathways

The dissociation of the two strands of the DNA helix (Double-Strand Breaks or DSBs) results from the breakage of the phospho-sugars on an identical position on both the filaments and causes the loss of genetic information and chromosome rearrangement. DSBs represent the cell response to the oxidizing events generated during metabolism by the reactive oxygen species (ROS), as well as to exogenous agents (i.e. ionizing irradiations) as well as to chemicals (mitomycin C, DNA topoisomerase inhibitors, camptothecins, and antitumoral drugs). The cell restores the physiological functions through the so called DSBs repair mechanisms. However, they are susceptible to defects, which trigger human neurological diseases and serious pathologies such as Ataxia telangiectasia, Nijmegen Breakage and the Severe Combined Immunodeficiencies (SCID).

In normal conditions DNA replication errors, such as DSBs, are repaired by Non-homologous end-joining (NHEJ) or homologous-directed repair (HDR) which differs from the need or the lack of need of homologous sequences:

NHEJ: is the main DSBs repair pathway in mammals and acts in all cell cycle maybe through the Ku70/80 heterodimer which first binds the two-ended DSBs and allows the NHEJ process to start. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) then activates a signalling cascade and the final restored conditions. The scaffold proteins XRCC4 and XLF contribute to help the process, as well as the DNA ligase 4 and the Artemis endonuclease belonging to the DNA-PKcs class.[16]

HDR: it's involved in meiotic recombination, replication fork stabilization and one-ended DSBs in late S/G2 phase and it's the only one able to act in both meiotic and mitotic phases.[17] It needs the presence of an undamaged single DNA strand at the DSB end. Double-stranded DNA filaments are taken together through Spo11, a topoisomerase II-like protein bridging the two Dna ends. A process called DNA end resection creates the new filament through the digestion of the DSB 5' strand, and requires the Mre11/Rad50/Nbs1 (MRN) complex to bind DSB ends. In fact, Mre11 removes Spo11 and degrades the filament through an endonuclease activity in 5' -3' direction while it is able to create the new one 3' -5' in an exonuclease process. Nbs1 activates the endonuclease CtIP protein which helps Mre11 in the resection and contemporarily the nucleases EXO1 and DNA2 together with the kinases ATM and ATR are implicated to regulate the activity of all the resection factors. During the new 3' -5' filament formation the RPA complex protects from degradation as long

as the RAD52, BCRA2 and PALB2 help the Rad51 binding and the final homology restoring.[16]

These evidences suggest that the MRN complex is essential for cell and tissue homeostasis and its activity is related to all the other cell regulators, included the Hedgehog Signalling Pathway.[18]

1.4.2 Architecture of the MRN Complex

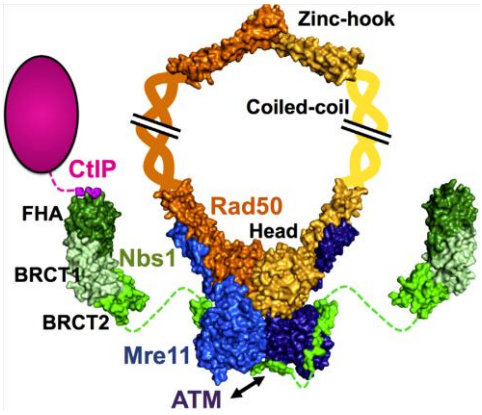


Figure 8. structural overview of the MRN Complex.[19]

Rad50: 1312 amino acids form this protein in humans, the biggest in the complex and belonging to the ABC ATPase superfamily. It shows two subunits and each one has a head formed by both the N and the C-terminal ends dimerizing with the head of the other subunit. Another dimerization occurs on the opposite site where there are two zinc-hook each one composed by two cysteine and coordinated together a Zn^{2+} atom.

Mre11 can undergo mutations which dump on its exonuclease activity, on the interaction with Rad50 and Nbs1 and probably on the endonuclease activity even if the mechanism is not yet clear. To better understand how Mre11 nuclease works it's necessary to know its structure in detail and then to alternatively block its activities. Hence, the known and characterized Mre11 inhibitor Mirin has been used to study in detail the Mre11 structure and to compare the activity of new inhibitors modifying this small molecule in different critical positions. Since the mechanism by which Mirin blocks Mre11 is unknown, it has been bounded to Mre11 in a co-crystallized complex which allowed to define more in detail some determinant Mre11 sites:

- Di-Mn active site in a capping domain and restricting ds-DNA access.
- Two β to α connecting loops interact with DNA and are regulated by Rad50
- One loop contains the exonuclease critical His61 and the other loop contains Asn93 which probably interacts with ssDNA

Keeping in mind the observation of the co-crystal structure it's plausible that Mirin is positioned close to the His61 and that one works by rotating the DNA phosphate backbone to open the double DNA filament from the end. Then, to underline the importance of the mirin structural features some modifications were applied synthesizing the derivative PFM39 which shows an amino group in place of the Mirin hydroxyl group and similarly binds Mre11 in the His61 region blocking the exonuclease activity.

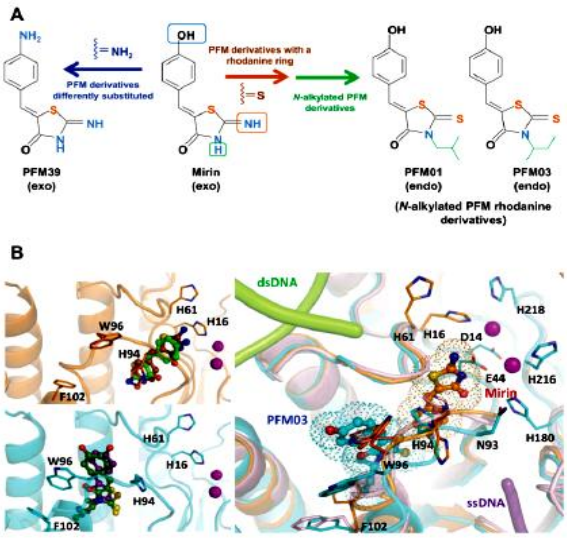


Figure 9.

At the same time, it is necessary to interfere also with the endonuclease activity to completely repair eventual complex modifications and, as told before, the Asn93 plays a critical role in the interaction with the ssDNA, which seems to be difficult within damages. The strategy adopted is the introduction of alkyl chains on the nitrogen group of a rhodanine-based structure resulting in PFM01with an isobutyl and in PFM03 with a sec-butyl. The two derivatives inhibit the Mre11 wrong endonuclease activity, facilitating the Asn93 to shift along the ssDNA-binding groove, and thank to the crystal structures observation it was possible to establish that the alkyl chains cause a shifted interaction respect to the exonuclease inhibitors Mirin and PFM39 and in particular it occurs upon the Mre11 dimer interface justifying the effects on Asn93 (Figure 9).

It was possible to confirm the impact on the Mre11 nuclease activity of all these inhibitors testing fibroblast of patients affected of Ataxia telangiectasia like disorder (ATLD) related with a significant lower level of the MRN complex respect to healthy cells.

Further studies allowed to determine that these two kinds of inhibitors are related with distinct DSB repair phenotypes and that the Mre11 endonuclease activity is carried out upstream of the exonuclease in HDR and, to confirm that a PFM01 and PFM03 treatment resulted in a complete repair, instead with Mirin or PFM39 a partial defect remains.[17]

1.5 MRN AND HH SIGNALING PATHWAY COOPERATION

The close interdependence existing between all the embryonic and postnatal development and the Hedgehog pathway is actually shared by the MRN complex and it's been demonstrated that the downstream events are mediated by the MYCN transcription factor in

both cases. Experiments on mice with the MYC gene depletion or deficiency result in early stage death or hypoplasia in several organs, including the nervous system because the neural cell proliferation is controlled by the MRN complex directly induced by the MYC factor.[18]

Several data confirm the strict cooperation of MRN and the MYC down-regulator, for example it's been observed the increased expression of the three MRN components and the mRNA after induced MYCN overexpression in neuron-like cells. On the same cell-line it's been verified the importance of the MRN complex in the MYCN-dependent proliferating events by knocking out the NBS1 gene or treating the cells culture with Mirin, resulting in deletion of the cell reproducing cycle. Results are in line with the hypothesis because the cell proliferation is compromised. At the same time the MYC component is associated with the replication stress and if it is overexpressed it is responsible of severe DNA damages, but this process is normally regulated by the MRN complex as reported after the observations on MYCN+ and MYCN- cells which allowed to confirm that. Moreover, studies were conducted on the proliferative expansion of the external granule cell layer (GPC) of the cerebellum which is finely regulated by Hh pathway and the MRN complex in sequence, in the early and last stage respectively, both acting through the MYC component. In fact, in this neonatal process it orchestrates the high proliferative events and consequently cause a stress condition assisted by different stress markers which represent at the same time the proliferation stopping signal once they're accumulated and reach a specific concentration able to induce the cell differentiation and the contemporary reduction of the MRN components. To better understand the cooperation between the Hh pathway and the MRN complex mediated by MYC, cultured cells were treated with SAG, one of the most known Hh agonists, inducing proliferation and observing the simultaneous MRN activation after the Gli factors activity inside the nucleus and the resulting expression of several proliferative factors including MYC. Furthermore, these correlation is confirmed by MYC knockdown experiments in SAG induced cells which results in a lower expression of Mre11, Rad50 and Nbs1.[18]

The MRN complex limits the DNA damage accumulation during the proliferative cell activity and its inhibition causes the DSBs progression and the final cell death. It can be observed after the Mirin treatment and the subsequent block upon Mre11 in its proper exonuclease activity. A different scenario is observed after treatment with a selective inhibitor of the Mre11 endonuclease activity which allows the DNA DSBs repair through alternative pathways.[18]

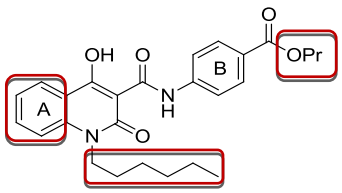
It’s been demonstrated that it’s able to block also single DNA stranded filaments prior to completion of second-strand DNA synthesis on the adeno-associated virus (AAV) and this inhibition is independent of downstream signalling and repair factors. Two kinases, ATM and ATR, are the first activated signalling elements after the complex transduction into the nucleus and it’s plausible that it requires many subunit proteins to express its activity. To demonstrate this hypothesis, it’s been blocked the expression of a Mre11 gene (H129N) involved in the DNA binding and nuclease activities and tested on recombinant AAV (rAAV) inhibition. As supposed, the lack of the H129N amino acid prevents Mre11 inhibiting functions and, to further investigate how the complex can be blocked it’s been considered the role of the two Ad proteins E1B55k/E4orf6 expressed by the rAAV that are effectively able to reduce Mre11 activity by degrading all the complex. Another possible mechanism of action is the binding of incoming genomes through the Mre11 phosphoesterase and DNA binding domains loaded by the MdcI and, in particular, it occurs on Mre11 and Nbs1 proteins, as shown from an *in vitro* binding. Other mode of action proposed include the sequester of the genome to a nucleus site inaccessible to gene expression factors and DNA synthesis, and a steric block to binding of transcription factors. Moreover, MRN is able to dimerize through a Rad50 terminal hook domain and this feature allows and facilitate the homologous recombination repair and stabilize replication fork progression, but the exact mechanism it is not elucidated yet.[20]

RESULTS AND DISCUSSION

2.1 HH AGONISTS.

2.1.1 New potential Smo Modulators

Based on the structure of the new quinolinone carboxamide GSA-10, identified by virtual screening as a new Smo positive modulator, structural modifications on its three principal sides were introduced with the aim to know more about where and how the interaction on this receptor exactly takes place:



GSA-10

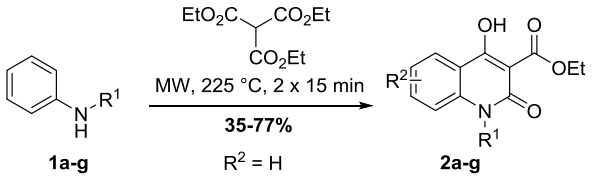
Figure 10.

- The N1 chain
- The ester moiety
- The condensed phenyl ring (A)

All the obtained derivatives were then tested to verify the activity on stem cell lines cultures.

2.1.2 Synthetic Procedure

The synthetic approach follows the one used to obtain GSA-10. The commercially available N-alkylated anilines **1a-g** were irradiated under microwaves at 225 °C with triethylmethane tricarboxylate to give the corresponding 3 carboxy-quinolone **2a-g**, differing for substituent on the N1, in almost good yields (35-77 %).



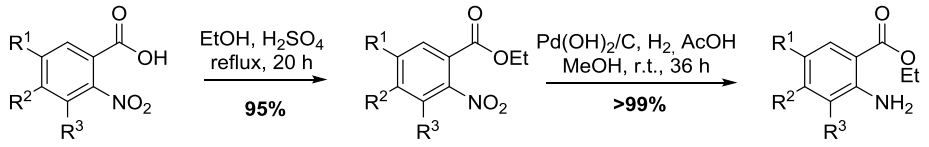
2a: R^1= C_6H_{13}; **2b**: R^1=C_5H_{11}; **2c**: R^1=C_4H_9; **2d**: R^1=C_3H_7;
2e: R^1=Me; **2f**: R^1=Allyl; **2g**: R^1= Bn;

Scheme 2.

The same procedure did not work when we tried to introduce substituents on the condensed aromatic ring, maybe because of the negative influence of the substituted anilines under the original reaction conditions.

The new adopted procedure yielded the formation of a series of conveniently functionalized nitrobenzoates **4a-d,** quantitatively obtained through a Fisher esterification in EtOH and in the presence of H_2SO_4 from the corresponding 2-nitrobenzoic acids **3a-d.** The desired

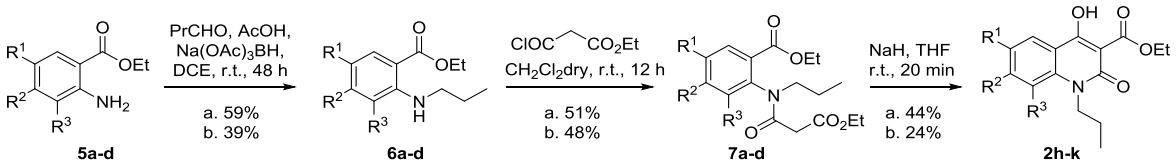
anthranilic derivatives **5a-d** were finally obtained by reduction under H_2 atmosphere with $Pd(OH)_2/C$ and AcOH (Scheme 3).



a.: R^1=F, R^2=R^3=H; **b.**: R^1=R^2=OMe, R^3=H; **c.**: R^1=Cl, R^2=R^3=H; **d.**: R^1=Me, R^2=R^3=H

Scheme 3.

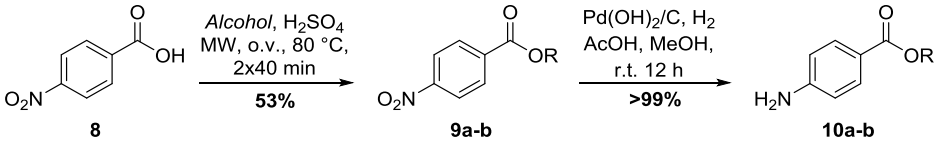
The reductive amination with propanal and $Na(AcO)_3BH$ in DCE furnished the N-propyl corresponding products **6a-d**, which underwent to subsequent acylation with ethyl malonyl chloride (**7a-d**) and cyclization in the presence of NaH allowing to obtain the 3 carboxy-quinolinones **2h-k** in good yields.



a.: R^1=F, R^2=R^3=H; **b.**: R^1=R^2=OMe, R^3=H; **c.**: R^1=Cl, R^2=R^3=H; **d.**: R^1=Me, R^2=R^3=H

Scheme 4.

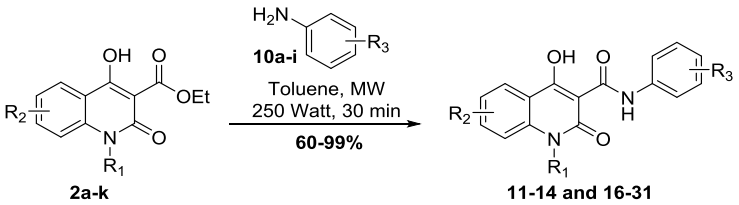
The last step is the coupling with the anilines **10a-i**. Two of them (**10a-b**) were prepared starting from the *p*-nitrobenzoic acid **8** converted into the corresponding esters **9a-b** with the appropriate alcohol (*n*-PrOH or *n*-BuOH) and H_2SO_4, under MW irradiation at 80 °C, two times for 40 minutes in open vessel, and finally reduced into the *p*-amminobenzoates **10a-b** (Scheme 5).



a.: R=propyl **b.**: R=butyl

Scheme 5.

The final step is the same as for GSA-10, heating **2a-k** and **10a-i** with microwaves at 220 °C in toluene for 30 min or 60 min depending on the substrates. The desired derivatives **11**-**14** and **16**-**31** were obtained in good yields (60-99%).



Scheme 6.

The *t*-butyl ester **14** was transformed into the corresponding acid **15** with TFA for 2 h.[11] the structure of **15** is reported in Figure 11 with the value of its EC_{50}.

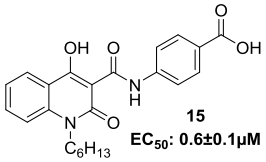


Figure 11.

All the other compounds synthesized and evaluated are reported in the figure below (Figure 12).

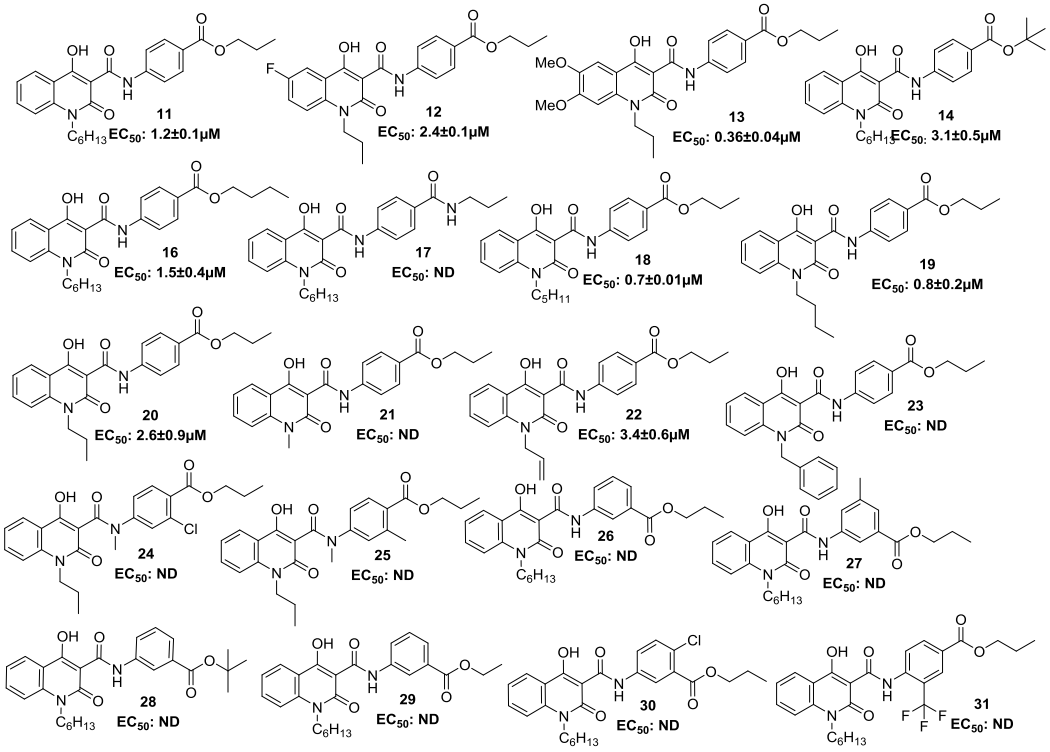


Figure 12.

2.1.3 Biological Results

Compound **11**, named GSA-10, fits well in the pharmacophoric model similarly to the known Hh positive modulators Pur, SAG and all its derivatives (Figure 13). [21]

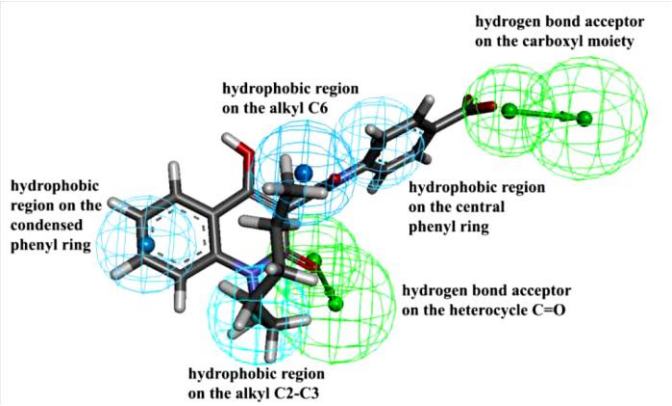


Figure 13.

From this study emerged that the interaction on the active site of Smo provides for two hydrogen bond acceptor group (HBA1-2) and four hydrophobic regions (HY1-4).

Below are reported the details of the fitting analysis:

- SAG: the chlorobenzothiophene superposes on the HY2-HY3-HY4 and the central phenyl ring on HY1; the amide carbonyl group on the HBA2, the pyridine nitrogen on HBA1.
- Pur: central phenyl corresponds to the HY1, the cyclohexyl to HY2, the naphthyl rings to HY3 and HY4; the O at the morpholine ring to HBA1 and the O at the naphthyl ring together with the purinyl heterocycle to HBA2.
- GSA-10: the carbonyl oxygen of the ester side with the HBA1 and the carbonyl oxygen on the quinolone moiety with HBA2; the central phenyl ring with HY1, the last methyl group on the N alkyl chain with HY2, the condensed phenyl ring is associated with HY3 placing its C2-C3 atoms on HY4.

GSA-10 shows an activity (EC_{50}: 1.2±0.1μM) comparable to that of Pur (EC_{50}: 0.8±0.1μM), but lower than SAG, promoting the differentiation into osteoblasts through the stimulation of the alkaline phosphatase (AP).

SAG, Pur and GSA-10 share this typical effect by binding on Smo, but going through a more detailed analysis, relevant differences were observed. For example, GSA-10 does not stimulate the transcription of the Hh proteins even at 10μM concentration compared to the SAG EC_{50} of ~0.15 μM; it doesn't cause a dose-dependent increase of the GCP proliferation

in rat like SAG does. Another important observation is that GSA-10 doesn't affect the typical mode of action of SAG consisting in the accumulation of Smo at the primary cilium. Indeed, we can conclude that it's not a SAG antagonist and that there is an allosteric active site on Smo mediating the GSA-10 agonist activity. This hypothesis is corroborated by further assays including the demonstration that GSA-10 and SAG have opposite effects on the Gli1 transcript levels with a decreasing effect of GSA-10. Moreover, in the presence of forskolin and CTX which are positive regulators of cAMP levels and inhibitors of the Hh pathway, SAG activity is blocked while the one of GSA-10 is potentiated of ~3.8-fold and increased in a dose-dependent manner. It's been demonstrated that these two structurally different Hh agonists act synergistically on Smo as shown by the dose-response curve of the AP activity measured in presence of both molecules, resulting in potentiating effects. Added evidences of a different mode of action occur when the ability to inhibit the binding of the inhibitor Cyclopamine on hSmo is tested: GSA-10 is not able to produce this effect, even at high concentration (~30μM). At the same time the analogous positive modulation on the pathway of the two compounds is confirmed through the competitive inhibition on the AP response mediated by MRT-83, one of the most potent Smo antagonists.
These results suggest the existence of distinct active sites and mode of action on Smo, all of them giving a positive modulation of the pathway. A group of known antagonists was used to test the competitive interaction in the presence of SAG and GSA-10 and the results confirmed this hypothesis.[21]

The same AP stimulation test in the differentiation into osteoblasts of pluripotent mesenchymal cells C3H10T1 was conducted for all the quinolinone derivatives and allowed to deduce how the activity is influenced by the generated structural modification.
The ester group and the aromatic ring on the right side play a key role, since the substitution with heterocycles, acidic or amide functions in place of the ester and even a shorter or longer alkyl chain are responsible of either decreasing or opposite activity. Once tested that the ether substitution has no activity as well, we deduced that the propyl ester chain on the C4 position of the aromatic ring is necessary for the interaction and the final activity.
Turning the attention to the heterocyclic N substituents, it has been shown that the activity is stabilized in the submicromolar range by reducing the alkyl length from a hexyl to a pentyl or butyl chain, while a shorter methyl group as well as a benzyl moiety resulted in inactive compounds. Interestingly, the N-propyl and the N-allyl derivatives showed a similar micromolar activity (~3μM).

Finally, testing the modifications introduced on the condensed aromatic portion, it was clear that the activity of F, Cl, Me groups on the 6 position was not so relevant. Instead, 6,7-dimethoxy derivative (EC_{50}: 0.36 μM) has an enhanced potency compared to GSA-10 and comparable with SAG.

All these results are in line with the fitting mode of the synthesized derivatives trough the pharmacophoric model for Smo agonists, confirming the importance of a suitable interaction with the 4 hydrophobic regions and the 2 hydrogen bond acceptors. In particular, the interaction depends on:

I. Conformational freedom of the C_{phenyl}-$C_{carbonyl}$ bond, which allows the two O of the ester group to be good acceptor for the hydrogen bond region. The same is for the acidic derivative, but not for the amide one due to the stiffening caused by the N hydrogen bond, which limits the carbonyl oxygen to act as acceptor. Finally, the O of the ether derivative relies too far from the HBA region

II. The quinolone N-alkyl substitution is tolerated and efficient at different lengths with correspondences on two HY regions covered by C2-C3 and C6 in the hexyl chain, and well reproduced by butyl and propyl derivatives.

III. Tests performed on the quinolone derivatives, carrying a 6-F, 6-Cl, 6-Me and 6,7-diOMe on the aromatic condensed ring, revealed a good fitting profile in the HY regions. Interestingly, dimethoxy substitution only, gave positive influence on activity.[11,3]

In conclusion, the acid and the dimethoxy derivatives **15** and **13** gave results almost similar to their progenitor GSA-10 and confirmed that they both can be considered as novel osteogenic agents.

2.2 HH ANTAGONISTS.

The same approach was adopted for the synthesis and activity evaluation of new potential inhibitors, since GDC-0449 (Vismodegib) is unable to suppress some Smo mutated forms and so causes the development of resistance.

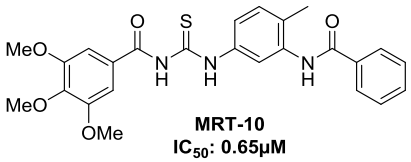
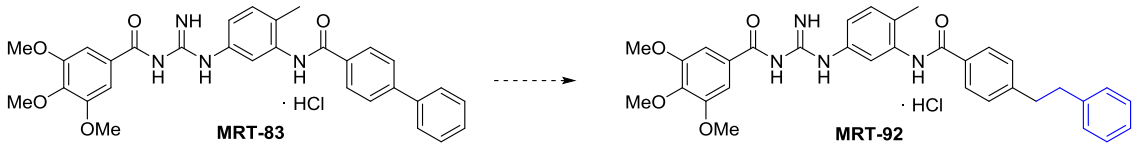


Figure 11.

The acylthiourea (AcTU) MRT-10 shows a promising profile with an IC_{50}: 0.65µM (Figure 2) and it was possible to transform it easily into the corresponding acylureas (AcU) and acylguanidines (AcG) with the aim to obtain a much more good profile of activity. [7]

The AcG are the most interesting class of compounds based on the exhibited antagonist behaviour of MRT-83, which shows an IC_{50} comparable to that of GDC-0449 and better than Cyclopamine. These results emerged from the exploration of different substituents on the right side of the molecule including pyridinyl, morpholino-phenyl, phenylamino, napthyl moieties and many others. Thus, MRT-83 development represents today the most important challenge.

Indeed, keeping in mind the observation on the pharmacological evaluation of MRT-83, the acylguanidine MRT-92, which has an even more elongated right side of the structure, is considered the best inhibitor of Smo, since it is able to circumvent the resistance developed by some Smo mutants towards the commercially available GDC-0449 (Scheme 7).[9]



Scheme 7.

To obtain the acylthioureas, ammonium thiocyanate is treated with 3,5-dimethoxybenzoyl chloride and the appropriate benzamide for 1 h, giving the desired products in almost good yields (40 to 80 %). Simply suspending these compounds in CH_3CN, in the presence of CuCl and NaOH 2.5 M and undergoing to microwave irradiation, the conversion into the acylureas occurs. Finally, the purification by flash chromatography furnishes the derivatives in discrete to good yields.[7]

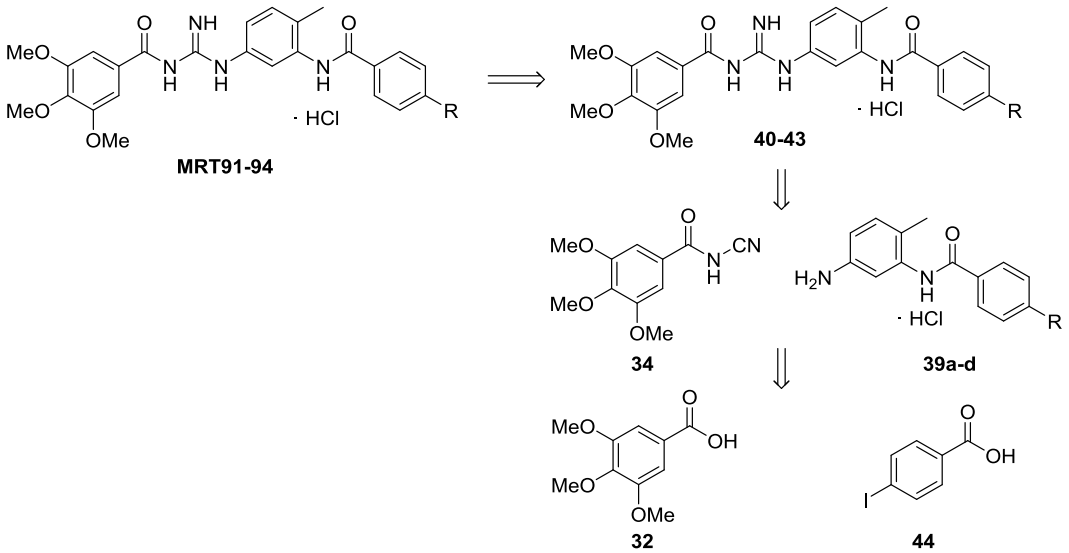
However, my work focused on the synthesis of the AcG, which can be obtained from the corresponding AcTUs, but also through a different synthetic procedure, developed in this

work. This class shows the best pharmacological profile, in agreement with the one shown by MRT-83, the acylguanidine carrying a biphenyl moiety on the right side of the molecule.

2.2.1 Synthetic procedure

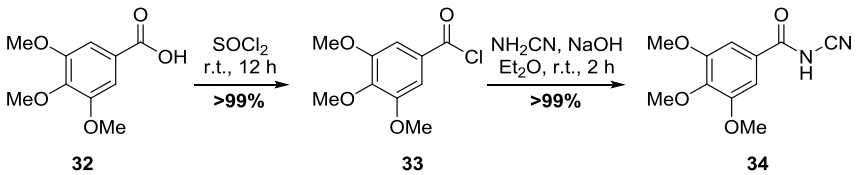
The retrosynthetic strategy used to obtain this class of compounds gives the hydrochloride final products **MRT91-94,** presenting a better solubility profile than their corresponding non-salted forms **40-43**, obtained through a coupling reaction between the cyanamide **34** and different hydrochloride aminoanilides **39a-d** (Scheme 8).

These two moieties are in turn furnished from a multiple steps synthetic procedure involving opportunely substituted benzoic acids.



Scheme 8.

The 3,4,5-trimethoxy benzoic acid **32** is transformed into the corresponding chloride **33** in quantitative yield in the presence of $SOCl_2$ for 12 h at room temperature. Then, reacting with NH_2CN and NaOH in Et_2O it furnishes the desired cyanamide **34** in quantitative yield (Scheme 9).

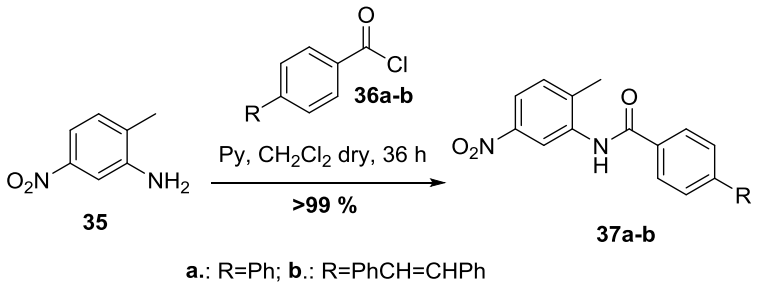


Scheme 9.

At the same time, the synthesis of the different aminoanilides foresees the procedure described in a previous article .[7] The aminoanilides are obtained from the corresponding nitroanilides with reduction of the nitro group mediated by $SnCl_2$ with 70% of yield, or

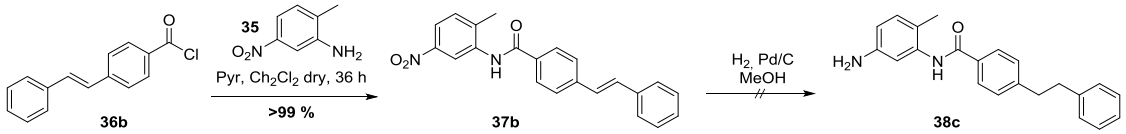
under microwaves in the presence of NH_4CO_3-Pd/C with a 75% yield. Some of the nitro-adducts (**37a-c**) have been prepared.

For the synthesis of **37a-b**, the 2-methyl-5-nitroaniline **35** reacts with the chlorides **36a-b** in CH_2Cl_2 dry and in the presence of Py to give the desired compounds in quantitative yields (Scheme 10).



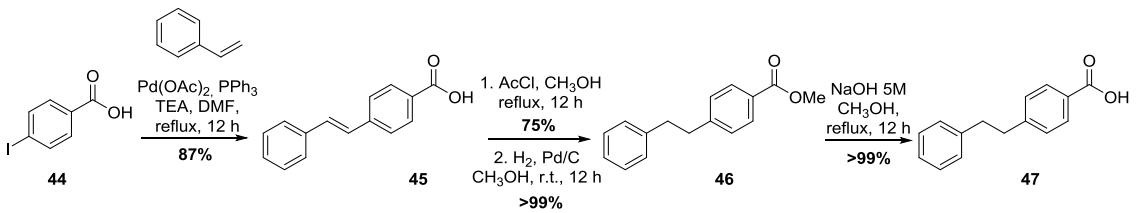
Scheme 10.

It was not easy to reduce the double bond through the hydrogenation of **36b** or **37b** trying to reduce at the same time the nitro group and obtain the aminoanilide **38c** (Scheme 11).



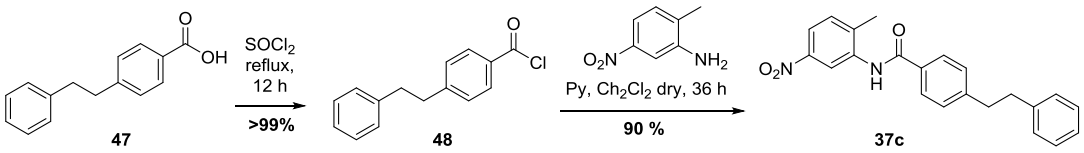
Scheme 11.

After many attempts, we found out that the best strategy to prepare **37c** was to synthesize first the biphenyl allyl acid **45** from p-iodobenzoic acid **44** which undergoes a Heck reaction with the styrene in DMF dry catalysed by $Pd(OAc)_2$ with PPh_3 and TEA as base, refluxing for 12 h and results in 87% of conversion. The second step is the esterification of the carboxylic group (yield: 75%) followed by the double bond reduction under H_2 atmosphere, to isolate the compound **46** in quantitative yield. Finally, the acid **47** is obtained after the hydroxylation with NaOH 5M in CH_3OH (Scheme12).



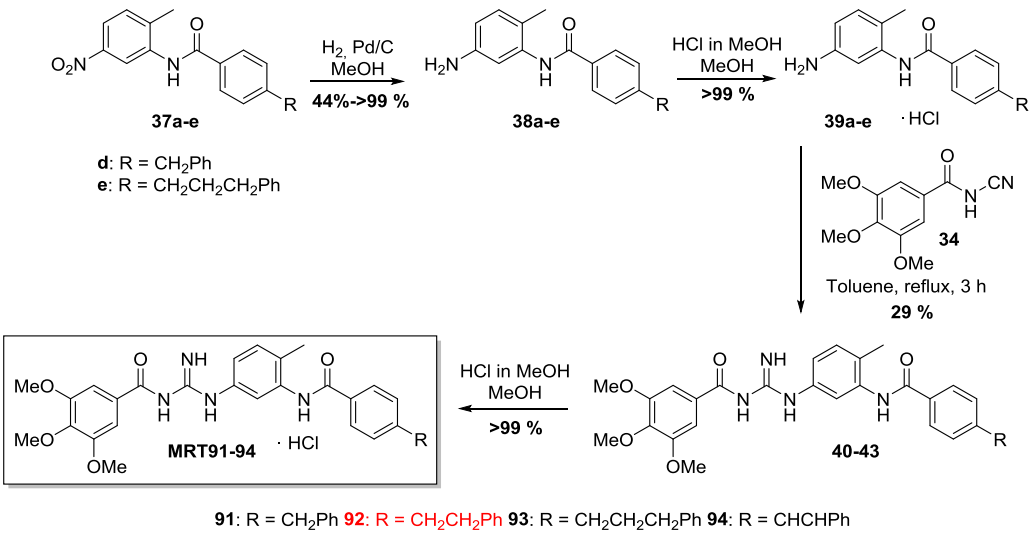
Scheme 12.

The acid **47** is transformed into the corresponding chloride **48** by treatment with $SOCl_2$ under N_2 atmosphere for 12 h monitored by mass spectrometry and treated with **35** as described in Scheme 11, to give the nitro-derivative **37c** in very good yield (90%, Scheme 13).



Scheme 13.

A general procedure for the synthesis of MRT-83 derivatives is the following:



Scheme 14.

This scheme includes also the commercially available nitroanilides **37d** and **37e**. The aminoanilides **38a-e** are obtained in 44% to quantitative yields by reduction under H_2 atmosphere. Then, they are transformed into the corresponding hydrochloride salts **39a-d** with HCl in CH_3OH. This last step was crucial for the following coupling with the cyanamide **34** in toluene heating to reflux and stirring for 3 or 12 h depending on the substrates. Here are reported the acylguanidines **40-43** obtained with 29% of yield, which are finally treated with HCl in MeOH to obtain **MRT91-94** in quantitative yield (Scheme 14).

2.2.2 Biological results

The figure below shows the virtual interaction between MRT-83 and the pharmacophoric model hypothesised for Smo.[7]

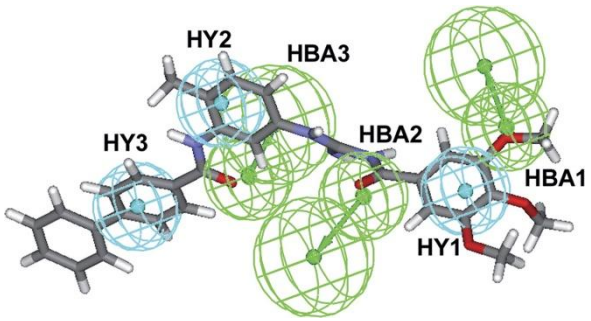


Figure 14.

Each compound underwent inhibitory tests following the use of Shh as ligand and Shh-light2 cells that are NIH3T3 cells stably transfected with a Gli-dependent firefly luciferase reporter, to establish the most important features of these Hh antagonists.

Dividing the general structure in three zones, it can be evaluated that:

- In the left (L) zone the presence of alcoxyl moieties is crucial for the inhibiting effects which depend on their position on the aromatic ring. The best results emerged with a trimethoxy aromatic frame;
- In the right (R) zone the presence of an aromatic portion is also required, as suggested by the observation of detrimental effects when the aromatic ring is substituted by cyclohexyl, isopropyl, thiophene or furan ones. The same results occur when methoxy or chlorine groups are introduced on the aromatic portion, while they are enhanced adding a second phenyl ring or an indole fragment;
- Fixing the biphenyl moiety, the amide bond anchoring it to the central core revealed to be fundamental since its transformation into a urea failed;
- To study the effects of different substituents on the central aromatic ring, a chlorine, fluorine and methyl residue were introduced with retain of the activity while a methoxyl group provoked the disappearance of the activity. It has been deduced that especially a methyl and a chlorine chain gives the advantage to separate the central phenyl from the amide bond.

MRT-92 is the outcome of the hypothesis that the elongation of the MRT-83 biaryl moiety might be able to interact totally with the active site of the receptor covering the part, which remains free when MRT-83 interacts. The importance of an ethyl linker between the two aromatic rings is then confirmed by measuring the ability to block the differentiation of the mesenchymal progenitor cells into osteoblasts induced by the agonists SAG and GSA-10. In fact, the presence of a longer or unsaturated linker (MRT-93, MRT-94) results in reduced activity. Shh-light2 cells are a NIH3T3 cell line used to define and characterize MRT-92 functional properties through comparison with MRT-83, GDC0449, LDE225.

The recovered data indicate that MRT-92:

- Inhibits Smo translocation on the primary cilium, contrasting SAG activity, which induces it;
- Does not execute Wnt signalling pathway inhibition, even if Smo structure is very similar to those frizzled receptors involved in this pathway;
- Blocks Granule Cell Precursors (GCPs) proliferation both in genetic manipulation and induced by Hh pathway activation;

Important deductions about MRT-92 mode of action emerged from results of experiments on a tritiated analog [^{3}H]MRT-92 bound to HEK-hSmo membranes and then dissociated in the presence of GDC-0449, and other known inhibitors such as itraconazole and the agonist SAG in order to better understand how the interaction with Smo occur. The inhibitors tested interfere with the [^{3}H]MRT-92 binding to HEK-hSmo membranes, validating the hypothesis that MRT-92 interacts with almost the whole Smo structure. Furthermore, it has been demonstrated the main role of the following structure areas:

1. The trimethoxy phenyl moiety for aromatic and hydrophobic interactions (in the ECD);
2. The central acylguanidine for 4 hydrogen bonds (in the ECD);
3. The right side aromatic and hydrophobic region penetrating inside and binding the intracellular domain.

Finally, by mutating specific Smo genes it was possible to circumscribe the aminoacids responsible of the interactions and to evaluate that this new antagonist overcomes the resistance occurring to GDC-0449 from the D473H Smo mutant.[9]

2.3 EPMF COMPOUNDS

Considering the growing interest for the Hh Signalling Pathway modulators, we explored the possibility to manipulate MRT derivatives belonging to the class of Smo inhibitors.

Based on MRT83 and MRT92 structures, the new proposed potential inhibitors differ for the presence, on the left aromatic ring, of one or two OMe groups and an OH variably positioned on C5, C6 or C7.

They are reported in Figure 13:

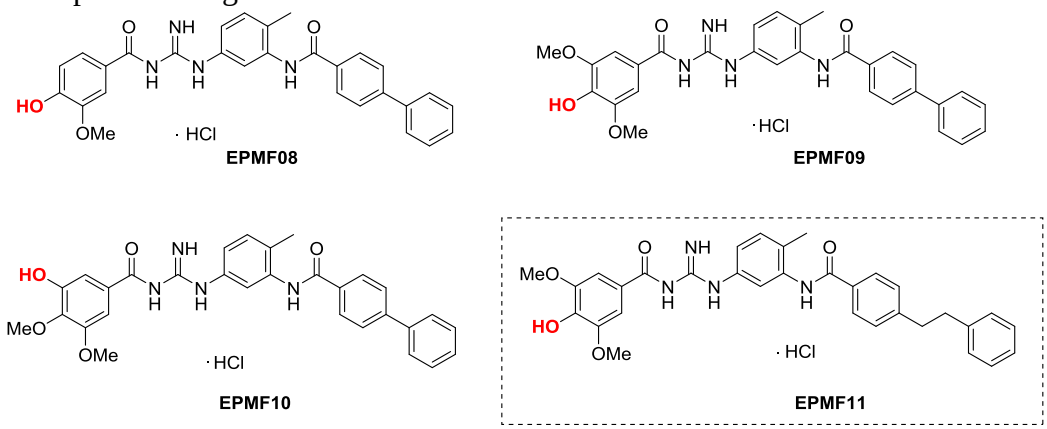
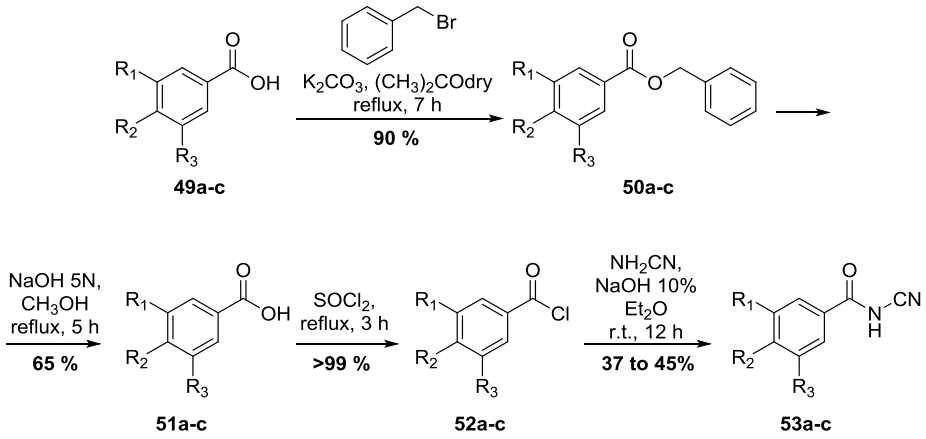


Figure 13.

2.3.1 Synthetic procedure

The synthetic procedure is in line with the one used for the corresponding MRT83 and MRT92, but starting from the suitable OH aryl substituted acids **49a-c**.



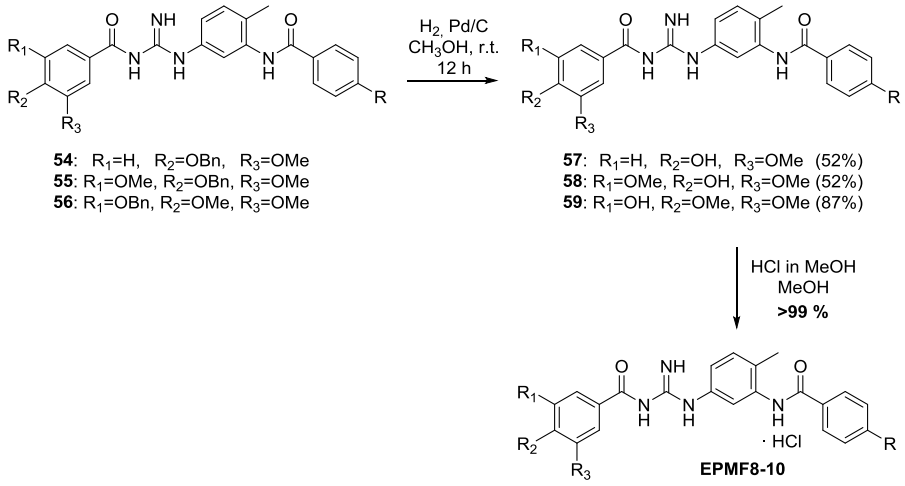
49 a: R_1=H, R_2=OH, R_3=OMe **b.**: R_1=OMe, R_2=OH,R_3=OMe **c.**: R_1=OH, R_2=OMe, R_3=OMe

50 a.: R_1=H, R_2=OBn, R_3=OMe **b.**: R_1=OMe, R_2=OBn, R_3=OMe **c.**: R_1=OBn, R_2=OMe, R_3=OMe

Scheme 15.

Starting with a double benzyl protection on the aryl and the carboxylic OH functions with benzyl bromide and K_2CO_3 in dry Acetone heating to reflux for 7 h, the desired products **50a-c** are obtained with very good yields. After the saponification in CH_3OH with NaOH 5N refluxing for 4 h the resulting acids **51a-c** are transformed into the corresponding chloride **52a-c** which were further transformed in cyanamides **53a-c** in NH_2CN, NaOH 10% aqueous solution in Et_2O at r.t. for 12 h in discrete yields (Scheme 15).

The acylguanidines **54-56** are obtained by coupling between **53a-c** and the **39a-c** following the procedure described in Scheme 12. Unfortunately, we found some problems starting from **53b** and **39d** and so the synthesis of **EPMF11** is still in progress. The final compounds are obtained through the debenzylation of **54-56** under H_2 atmosphere in the presence of Pd/C in CH_3OH for 12 h at room temperature with yields of 52% (**57**, **58**) and 87% (**59**) and the transformation into the hydrochloride salts in CH_3OH with HCl in CH_3OH with quantitative yields (Scheme 16).



Scheme 16.

2.3.2 Biological results

EPMF08-10 have been sent to the biological assays to test their inhibitory activity on the standard fibroblast cell line NIH3T3. In particular, the figure below (Figure 14) shows the ability of these compounds to modulate the Gli1 protein level in the presence of the agonist SAG (100nM) and the antagonist GANT61 (5μM). At the same time, it has been evaluated their densiometric quantification using DMSO as standard. Moreover, it has to be specified that **EPMF03-04** compounds correspond to the antagonists MRT92 and MRT83 respectively, and that the acronym EPMF is used to specify that the tests here reported have been conducted by another research group. **EPMF09** shows a relevant ability to inhibit the Gli1 proteins expression, comparable to that of MRT83 (**EPMF04**). A similar result emerged from the densiometric quantification test, in which the DMSO value is taken as standard and the values related to EPMF04 and EPMF09 are comparable again (Figure 14).

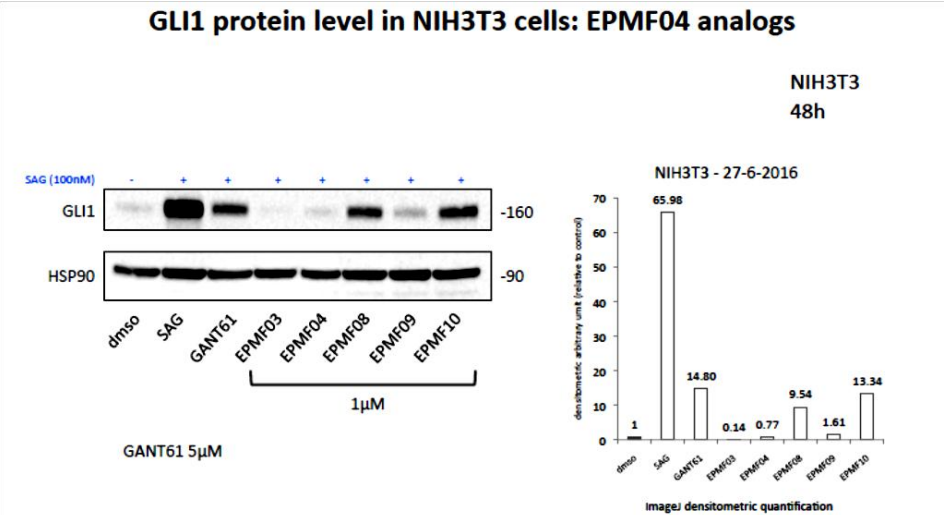
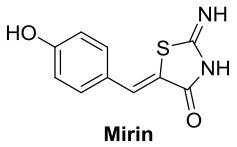


Figure 14.

With these data in hands, the greatest challenge is now the application of these compounds in the field of the bioconjugation strategy for cancer therapies.[22]

2.4 Mre11 INHIBITORS.

As told before, the MRN complex is essential for cell life and this is the reason why it is always difficult to understand its mechanisms. Anyway, it is possible to reproduce exactly the signalling pathway through some cell-free systems and to obtain chemical genetic screening to find potential inhibitors. Using this strategy, it was possible to isolate Mirin (Figure 15), a thiazolidinone compound able to block the MRN activation of the ATM (ataxia-telangiectasia mutated) pathway since it is an antagonist of the exonuclease activity of Mre11.[23]



Mirin

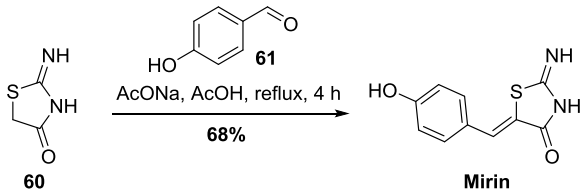
Figure 15.

Moreover, it represents the solution to study Mre11, without disrupting the MRN complex, in its nuclease dependent and independent functions. In particular, Mirin:

- Interferes with MRN inhibition and its lethal genetic inactivation;
- Through its exonuclease activity is centrally involved in the HDR (homology-directed repair) in mammals;

It has been established that Mirin is a weak inhibitor and so it limits the possibility to acquire a total overview on its behaviour, including the hypothesis of other targets except Mre11. For these reasons, Mirin derivatives have been prepared, belonging to the PFM class and in which the pseudothioidantoine nucleus is maintained or is substituted by a rhodanine one. They are all almost easily prepared and after pharmacological assays three of them emerged for their enhanced activity in respect to their progenitor. In this work, it's been developed the synthesis of these compounds with the aim to improve reaction yields, and to find out an even more detailed profile of their interaction with the complex and their activities.

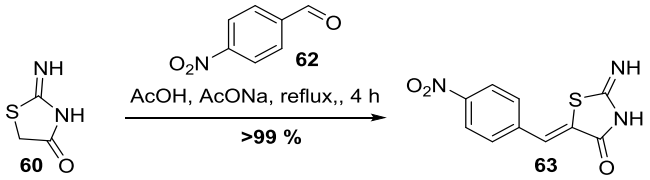
First of all, Mirin has been synthesized through the following procedure:



Scheme 17.

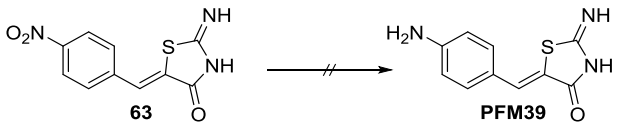
2.4.1 Synthetic procedure

The 2-iminothiazolidin-4-one (pseudothiohydantoin) **60** is treated with *p*-OH-benzaldehyde **61** in the presence of AcONa in AcOH and refluxed for 4 h, to give the desired product in a good yield (68%, Scheme 17). Then, we focused on the synthesis of PFM39, which differs from Mirin only for the presence of a primary amino group on the para aryl position in place of the OH function.



Scheme 18.

As shown in Scheme 18, with the same conditions it was easy to prepare the nitro derivative **63** in quantitative yield, but the reduction of the nitro group with $SnCl_2.H_2O$ in EtOH refluxing for 2 h didn't work because of the difficulty to remove $SnCl_2$ from the final product (Table 1, Exp.1).



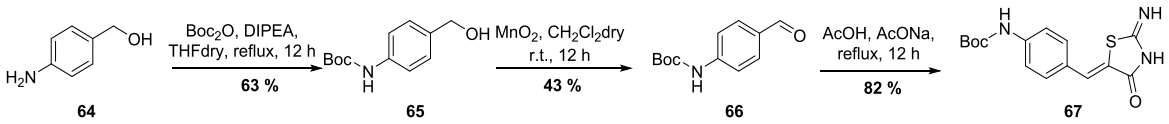
Exp. No	Conditions	Yield %
1	$SnCl_2.2H_2O$, EtOH, reflux, 2 h	-
2	In (powder), NH_4Cl, H_2O/EtOH, reflux, 12 h	-

Table 1.

When we used In (powder) with NH_4Cl in H_2O/EtOH at the reflux temperature for 12 h we recovered only the starting material (Table 1, Exp.2).

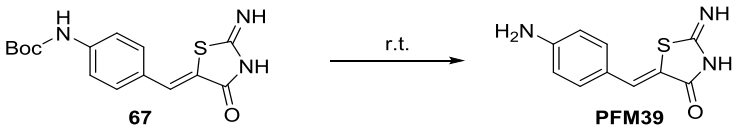
Therefore, we changed the strategy starting from the *p*-aminobenzyl alcohol (PABA) **64** which first undergoes the Boc protection on the primary amino group giving **65** with 63% of yield. The resulting product is oxidized into the corresponding *p*-Boc-amino aldehyde **66**.

Next, the Knoevenagel reaction with the pseudothiohydantoine follows the same conditions as described above, in AcOH with AcONa at reflux for 12 h and furnished the compound **67** (82%, Scheme 19).



Scheme 19.

The final Boc removal from the amine group was first experimented with HCl 4M in CH_3OH for 2 h but it didn't work and only **67** was recovered (Table 2, Exp.1). The same happens when we tried with trifluoroacetic acid (TFA) in CH_2Cl_2 at room temperature for 12 h (Table 2, Exp.2). The best result is obtained raising the amount of TFA up to 27eq, or in other words in a 1:1 ratio for 2 h, furnishing the final desired product in quantitative yield (Table 2, Exp.3).

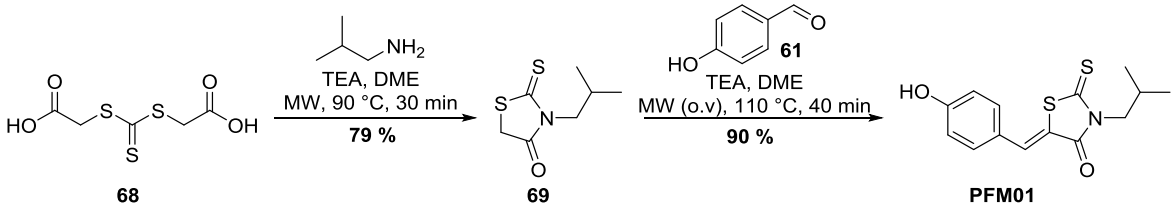


Exp. No	***Conditions***	***Yield %***
1	HCl 4M, CH_3OH, 2 h	s.m.
2	TFA (2eq), CH_2Cl_2, 12 h	s.m.
3	TFA (27eq), CH_2Cl_2, 2 h	**>99**

Table 2.

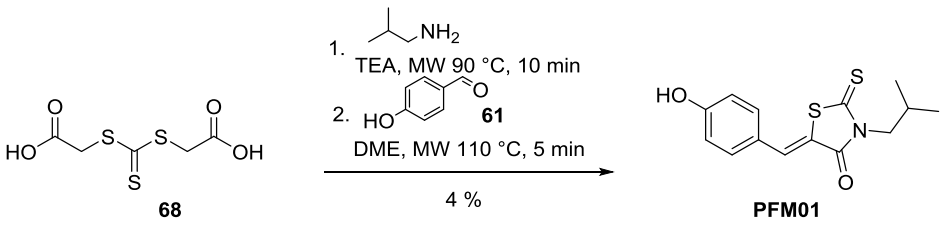
Then we turned our attention on the rhodanine derivatives PFM01 and PFM03 and even if the only difference in their structure is the alkyl chain on the N atom, a unique synthetic strategy did not work for both.

PFM01 is prepared starting from the reaction between the biscarboxymethyltrithyo-carbonate **68** and the isobutylamine in the presence of TEA and dimethoxyethane (DME) as solvents under MW irradiation at 90 °C for 30 min, to obtain the alkylated rhodanine **69** with 79% of yield. **69** was finally treated with p-OH benzaldehyde **61** and TEA in DME again under MW heating at 110 °C for 40 min in open vessel and the final desired product was obtained with 90 % yield (Scheme 20).



Scheme 20.

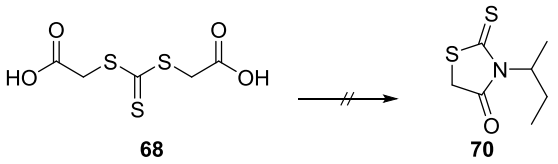
We tried also a one-pot reaction following the procedure described in a previous work [17], adding the aldehyde **61** to a mixture of **68** and isobutylamine with TEA in DME after 10 min of microwaves irradiation at 90 °C and raising up the temperature to 110 °C for other 5 min, but only a mix of the product and the aldehyde was recovered and it was difficult to purify it (Scheme 21).



Scheme 21.

However, applying the procedure described in the Scheme 20 in a gram scale reaction, the yield were highly reduced and new experiments are still in progress to find the best work conditions.

When the biscarboxymethyltrithyo-carbonate **68** was treated with the *sec*-butylamine to obtain the suitable rhodanine for PFM03, we tried to carry a solvent-less reaction again under MW irradiation at 350 °C for 3 min, since the procedure used for PFM01 didn't work. However, only traces and a mix of the starting material and the product **70** was recovered (Table 3, Exp.1). Thus, we adopted two different methods described in literature on similar substrates. The first consist in a preliminary treatment of **68** with carbonyldiimidazole (CDI) in THF at room temperature for 1.5 h followed by the addition of the *sec*-butylamine and refluxing for 4 h, but we didn't obtain the rhodanine **70** (Table 3, Exp.2).[24]

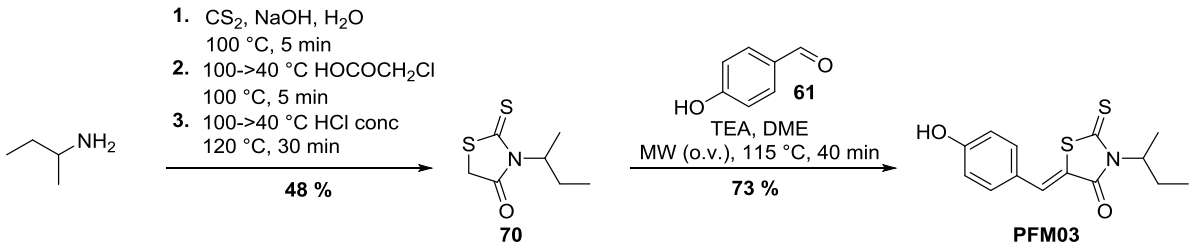


Exp. No.	Conditions	Yield %
1	*sec*-butylamine, MW, 350 °C, 3 min	Traces+s.m.
2	1.CDI, THF, 25 °C, 1.5 h 2.*sec*-butylamine, reflux, 4 h	byproducts

Table 3.

The second contains three steps. The sec-butylamine reacts with carbon dioxide CS_2 and NaOH in H_2O in a MW tube at 100 °C for 5 min, then the chloroacetyl chloride is added in a

second cycle of 5 min at 100 °C, and the resulting mixture is treated with concentrated HCl running the last MW cycle at 120 °C for 30 min. The desired product **70** is obtained with 60 % yield and it was used for the knoevenagel reaction to give the final compound PFM03 with 73 % yield (Scheme 22).[25]



Scheme 22.

2.4.2 Biological results

It is necessary to know the structure of Mre11 in all its details to better understand how this nuclease works and, then, to alternatively block its activities. Hence, the known and characterized Mre11 inhibitor Mirin has been used to study in detail the Mre11 structure and to compare the activity of the new PFM inhibitors. Since the mechanism by which Mirin blocks Mre11 is unknown, it has been bounded to Mre11 in a co-crystallized complex to define more in detail some determinant Mre11 sites. The main emerged features are:

- Di-Mn active site is in a capping domain and it restricts ds-DNA access;
- Two β to α connecting loops interact with DNA and are regulated by Rad50;
- One loop contains His61, while the other loop contains Asn93 which probably interacts with ssDNA.

Basing on the observations of the co-crystal structure it's plausible that Mirin is positioned close to His61 and it works by rotating DNA phosphate backbone opening the double DNA filament from the end. Then, to underline the importance of the Mirin structural features we include some modifications to synthesize the derivative PFM39. This compound shows an amino group in place of the Mirin hydroxyl group and it similarly binds Mre11 in the His61 region blocking the exonuclease activity.

At the same time, it is also necessary to guarantee a normal endonuclease activity which, together with the exonuclease activity, allows the restore of MRN complex. As already told Asn93 plays a critical role in the interaction with the ssDNA, however this association is perturbed when the complex is not working properly. In order to obtain such properties, we

synthesized two compounds having a rhodanine core and carrying an isobutyl (PFM01) and a sec-butyl (PFM03) on the nitrogen group. These two derivatives modulate endonuclease activity of Mre11, facilitating Asn93 shift along the ssDNA-binding groove. From the crystal structures, it was possible to establish that the alkyl chains cause a shifted interaction respect to the exonuclease inhibitors Mirin and PFM39 and in particular it occurs upon the Mre11 dimer interface justifying the effects on Asn93.

It was possible to confirm the impact on the Mre11 nuclease activity of all these inhibitors testing fibroblast of patients affected of Ataxia telangiectasia like disorder (ATLD) related with a significant lower level of the MRN complex respect to healthy cells.

Further studies allowed to determine that these two inhibitors are related with distinct DSB repair phenotypes and that the Mre11 endonuclease activity is carried out upstream of the exonuclease in HRconfirming that a PFM01 and PFM03 treatment resulted in a complete repair, while with Mirin or PFM39 a partial defect remains.[17]

Interestingly, the close interdependence between all the embryonic and postnatal development and the Hedgehog pathway is actually shared by the MRN complex and it's been demonstrated that the downstream events are mediated by the MYCN transcription factor in both cases. Experiments on mice with the MYC gene depletion or deficiency result in early stage death or hypoplasia in several organs, including the nervous system because the neural cell proliferation is controlled by the MRN complex directly induced by the MYC factor.[18]

Several data confirm the strict cooperation of MRN and the MYC down-regulator. For example it's been observed an increased expression of the three MRN components and the mRNA after inducing MYCN overexpression in neuron-like cells. On the same cell-line it's been verified the importance of the MRN complex in the MYCN-dependent proliferating events by knocking out the NBS1 gene or treating the cells culture with Mirin, resulting in deletion of the cell reproducing cycle blocking Mre11 in its proper exonuclease activity. Results are in line with the hypothesis because the cell proliferation is compromised. At the same time the MYC component is associated with the replication stress and if it is overexpressed it is responsible of severe DNA damages, but this process is normally regulated by the MRN complex as reported after the observations on MYCN+ and MYCN- cells which allowed to confirm that. Moreover, studies were conducted on the proliferative expansion of the external granule cell layer mediated by the granular progenitor cells (GPC) of the cerebellum which is finely regulated by Hh pathway and the MRN complex in sequence, in the early and last stage respectively, both acting through the MYC component.

In fact, in this neonatal process it orchestrates the high proliferative events and consequently cause a stress condition assisted by different stress markers which represent at the same time the proliferation stopping signal once they're accumulated and reach a specific concentration able to induce the cell differentiation and the contemporary reduction of the MRN components. To better understand the cooperation between the Hh pathway and the MRN complex mediated by MYC, cultured cells were treated with the Hh-agonist SAG inducing proliferation and observing the simultaneous MRN activation after the Gli factors activity inside the nucleus and the resulting expression of several proliferative factors including MYC. Furthermore, theis correlation is confirmed by MYC knockdown experiments in SAG-induced cells which results in a lower expression of Mre11, Rad50 and Nbs1. It has to be noticed that while the treatment with a Mre11 exonuclease inhibitor acts on the DSBs directly leading to their accumulation, a selective endonuclease inhibition gets involved alternative pathways.[18]

PFM39, PFM01 and PFM03 are the first and unique inhibitors of Mre11. Anyway, these compounds can interact also with other kind of receptor and it would be very important to know their behaviour through clinical trials in order to make their structure more drug-like. Moreover, these small molecules allow to know the exonuclease and the endonuclease activities of Mre11 and, for this reason, they can be considered excellent biological tools.

2.5 BIOCONJUGATION

2.5.1 Antibody Drug Conjugates (ADC)

For reasons due to the co-financing of my fellowship, I also worked in collaboration with Sigma-tau in the frame of the project for the preparation of bioconjugates. The bioconjugation represents a new strategy for antitumoral therapy and its main purpose is to obtain more selectivity and to overcome the various side effects caused by the nonspecific toxicity of most anticancer drugs. The low selectivity is mostly related to the ability of the antitumoral drugs to inhibit the uncontrolled cell proliferation and differentiation, but these processes are in common with some physiological tissues behaviour in continuous regeneration such as cutaneous annexed ones.

Antibody-Drug Conjugates (ADCs) combine the specificity of monoclonal antibodies (mAb) and the potency of cytotoxic molecules (Figure 16). These complexes allow the delivery of different anticancer agents as prodrugs directly to the target cancer cells, avoiding the

healthy tissues. Then, chemical or enzymatic transformations lead to the final cytotoxic activity.

Figure 16.

At the same time, it has to be considered the difficulty to raise the molecular target, with five fundamental steps to overcome:

- Circulation: the linker between mAb and the drug must ensure a stable association in the bloodstream to avoid any damage to the normal tissues
- Antigen Binding: an high immunoaffinity is necessary, in order to avoid its alteration after the drug conjugation
- Internalization: the drug must reach a sufficient level of concentration inside the cell
- Drug Release: the drug is active only after the complete release in its original active form
- Drug Action: high cytotoxic agents (subnanomolar IC_{50}) are the most suitable to conjugate considering the difficulty to kill the tumour cell with a relatively low cell-concentration of the drug

The linker plays a crucial role: it mediates the interaction between mAb and drug, it is responsible of the drug release and obviously of its structure-dependent activity. Therefore, the linker's main features are:

- Low MW
- Stability in the bloodstream
- Favourable pharmacokinetic
- Ability to release the drug in the tumour site

The linkers are classified in two typologies: cleavable and non-cleavable, hereafter described.

2.5.2 Cleavable Linkers

Chemically labile

These linkers are sensible to pH-difference between the extracellular and intracellular environment: the acid cleavable hydrazone linkers stably circulate in the 7.3-7.5 bloodstream pH, while they undergo to cleavage once internalized in endosomes (pH: 5.0-6.5) and lysosomes (pH: 4.5-5.0). Disulfide bond linkers also belong to this category and in this case they are cleaved by glutathione, present at high concentration inside the cell and almost absent in the blood.

Enzymatically labile

In this case the linker is a peptide and the drug release inside the tumour cell is mediated by some lysosomal proteases, and they are stable in the bloodstream. One of the most promising one has a valine-citrulline dipeptide.

The enzymatic cleavable linkers show many advantages compared to the acid and glutathione labile ones. In particular, the half-life of the latter linkers in mice is of about 6 days while it's just 2 days for the former typology. Some doxorubicin ADCs derivatives show this behaviour as well.

2.5.3 Non-cleavable Linkers

These linkers are characterized by the presence of thioether bonds, they are internalized with all the ADC complex inside the cancer cell where the lysosomal activity causes mAb degradation thus releasing the linker-drug moiety with high specificity and avoiding any damage to nearby tissues. They can be active even in the extracellular site since they're easily cleaved, thus allowing the interaction between the free drug and different cells.[26]

Our investigations are based on the knowledge available on *Kadcyla* (Trastuzumab Emtansine), a FDA approved non-cleavable ADC for the treatment of advanced breast cancer, and on the enzymatically cleavable *Adcetris* (Brentuximab Vedotin) for the *Hodgkin lymphoma* (HL) and *systemic anaplastic large cell lymphoma* (sALCL). In this work we developed new ADCs by with the aim to conjugate our cytotoxic compounds.

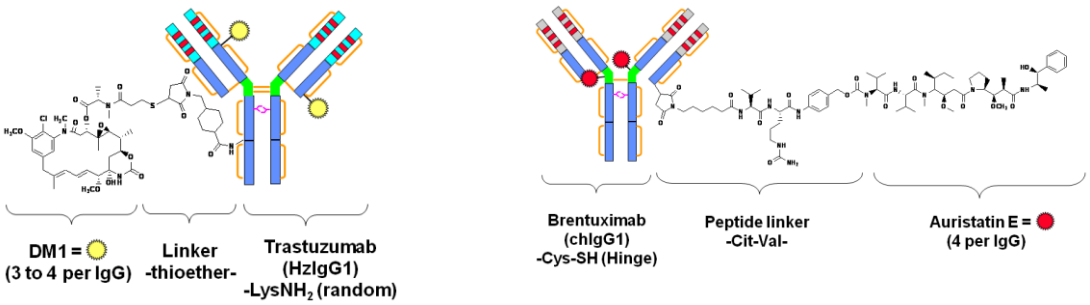
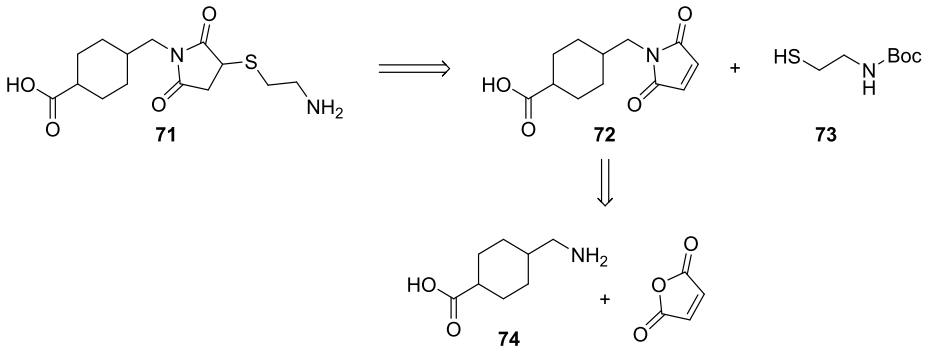


Figure 17. Kadcyla [27]and Adcetris[28]

2.6 NON-CLEAVABLE KAD LINKERS

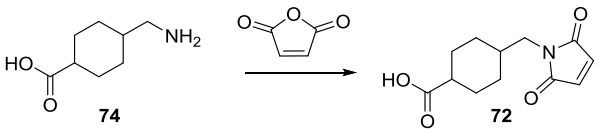
2.6.1 Synthetic procedure

The first experiments is focused on the synthesis of the non-cleavable linker **71** which was supposed to be obtained from the Michael reaction between the maleimide **72** and the boc-2-amino ethanethiol **73** (Scheme 23).



Scheme 23.

To prepare **72** the trans-4 (aminomethyl) cyclohexanecarboxilic acid **74** was used to react with the maleic anhydride in CH_3CN at room temperature varying the reaction time from 2 h to 48 h since the main product was the acid **75**. We tried to carry the reaction in DMA for 24 h and then to add AcONa and Ac_2O heating the mixture at 65 °C, but we isolated the acid **75** again. In the last two attempts **74** was heated with maleic anhydride without solvent under MW irradiation at 100 °C for 10 min, or at 120 °C for 40 min in the presence of Ac_2O without good results (Table 4).



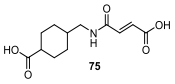
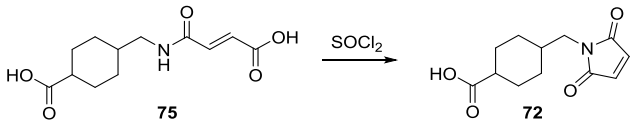
Exp. No	Conditions	Yield%	Intermediate 65% yield 
1	CH_3CN, 25 °C, 2 h	-	**75**
2	CH_3CN, 25 °C, 48 h	-	**75**
3	**1.**DMA, 25 °C, 24 h **2.**Ac_2O, AcONa, 65 °C, 5 h	-	**75**
4	MW, 100 °C, 10 min	-	**75**
5	Ac_2O, MW 120 °C, 40 min	-	**75**

Table 4.

Finally, we considered to treat **75** with $SOCl_2$ at room temperature for 2 h and 12 h to promote the intramolecular cyclization, but only the corresponding chloride **76** was obtained as emerged from mass spectrometry (Table 5).



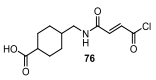
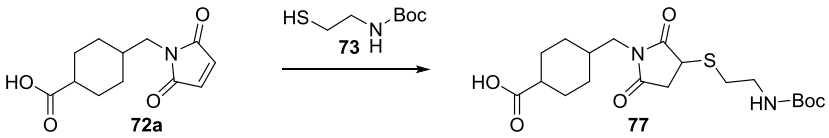
Exp. No	Conditions	Yield%	Intermediate 65% yield 
1	2 h	-	**76**
2	24 h	-	**76**

Table 5.

We also observed the formation of a white solid **72a** with a NMR profile comparable to that of our desired product even if its low solubility in $CHCl_3$ and CH_2Cl_2 looked unusual. In fact, the following experiments to prepare the Boc protected derivative **77** failed when we tried to reflux the mixture of **73** and **72a** in EtOH for 12 h, in CH_3OH and in the presence of isopropylamine at room temperature for 12 h, and using $SOCl_2$ at room temperature for 12 h recovering only **73** and **72a** (Table 6).



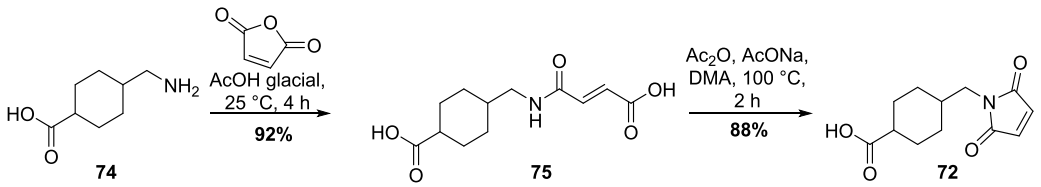
Exp.	Conditions	Yield%
1	EtOH, reflux, 12 h	**72a**+**73**
2	CH_3OH, isopropylamine, 25 °C, 12 h	**72a**+**73**
3	$SOCl_2$, 25 °C, 12 h	**72a**+**73**

Table 6.

Indeed, by analysing the COSY 2D NMR experiment we found out that **72a** was a salt of the desired product **72** and it didn't react in the following steps.

The literature suggested a 2-step procedure to synthesize the maleimide: a first reaction between the aminoacid **74** and the maleic anhydride in AcOH glacial at room temperature for 4 h to give **75** pure as crude product. This acid underwent the cyclization by treatment with Ac_2O and AcONa without the solvent at 100 °C for 2 h and giving **72** and the starting material **75** with a 30 % yield.[29]

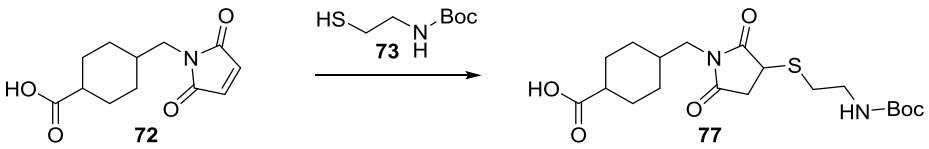
Carrying the reaction in DMA, we obtained a bigger amount of **72**, the yield was improved from 38% to 88% (Scheme 24).



Scheme 24.

The maleimide **72** differs from **72a** for the solubility in CH_2Cl_2 and $CHCl_3$ and for the NMR chemical shifts (Figure 18).

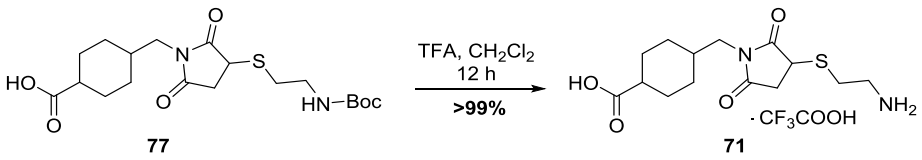
Changing solvents and temperature, we obtained the desired compound **77** with a 63% yield after treatment with **73** in CH_3CN at room temperature for 12 h, as shown in entry 6 in Table 7.



Exp.	Conditions	Yield%
1	EtOH, reflux, 12 h	-
2	CH_2Cl_2, 25 °C, 3 h	-
3	CH_2Cl_2, 25 °C, 12 h	-
4	CH_2Cl_2, 25 °C, 72 h	-
5	Isopropylamine, CH_2Cl_2, 25 °C, 3 h	-
6	CH_3CN, 25 °C, 12 h	63

Table 7.

The final linker resulted from treatment of **77** with TFA in CH_2Cl_2 for 12 h to remove the Boc protection and to obtain the desired compound as the triflate **71** in a quantitative yield (Scheme25).



Scheme 25.

The linker **71** is designed for small molecules with free OH groups because it can interact with the primary NH_2, while the carboxylic OH conjugates with the lysine of the mAb (Figure 18).

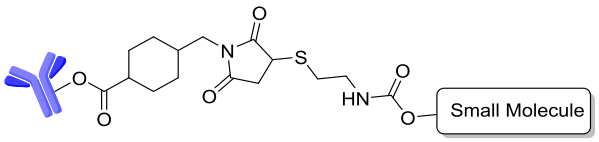
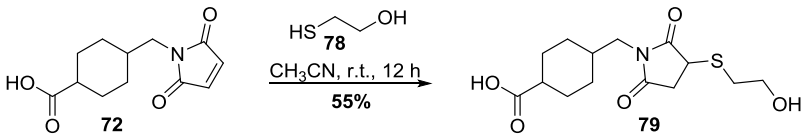


Figure 18.

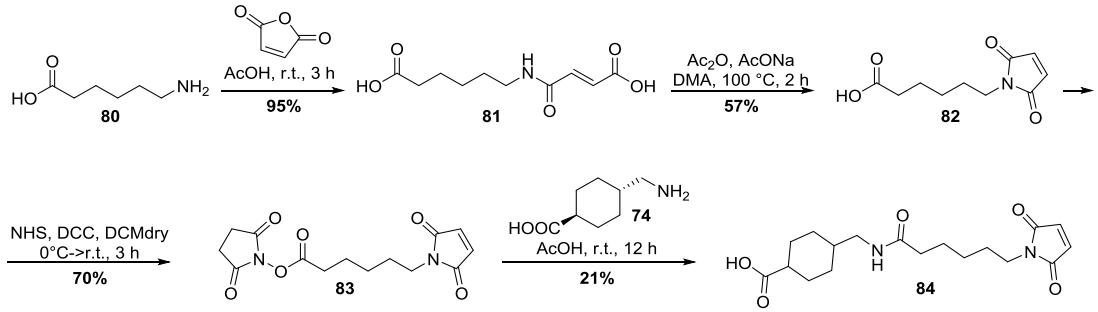
The aim of the Sigma-tau project was to prepare payloads of different drugs and starting from the structure of the linker **71** we introduced suitable modifications in the linker to allow the conjugation with the functional groups characteristic of each drug.

The maleimide **72** was used to react with the 2-mercaptothiol under the same conditions of the Table 7, entry 6 to give the linker **79**.



Scheme 26.

Finally, the linker **84** was synthesized for drugs with free SH groups. The caproic acid **80** reacted with maleic anhydride in AcOH (glacial) under N_2 atmosphere stirring at room temperature for 4 h and gave the desired product **81** with 95% yield. Treating **81** with Ac_2O and AcONa in DMA at 100 °C for 2 h we obtained the maleimide derivative **82.** After the activation of the carboxylic OH with NHS in the presence of DCC in CH_2Cl_2 dry we obtained the compound **83** (70% yield), which was used for the coupling with the trans-4-(aminomethyl) cyclohexanecarboxylic acid in glacial AcOH stirring at room temperature for 12 h. The final linker **84** was isolated with 21% yield only (Scheme 27).

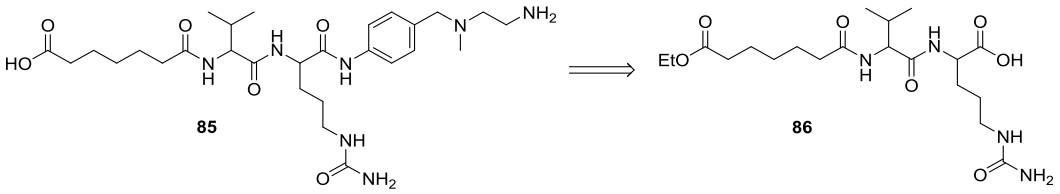


Scheme 27.

2.7 CLEAVABLE Ad LINKERS

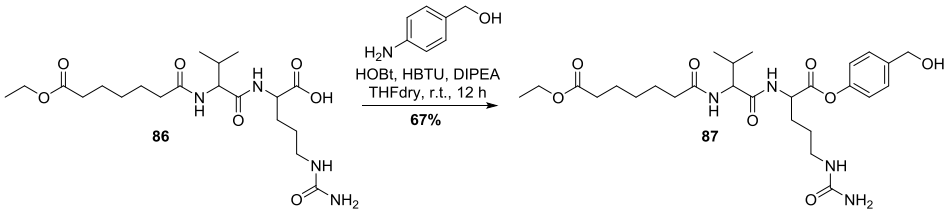
2.7.1 Synthetic procedure

The main feature of the enzyme-labile linkers is the presence of amino acids. Our purpose was to prepare the linker **85** starting from the Valine-Citrulline derivative **86** already available and prepared by another research group (Scheme 28).



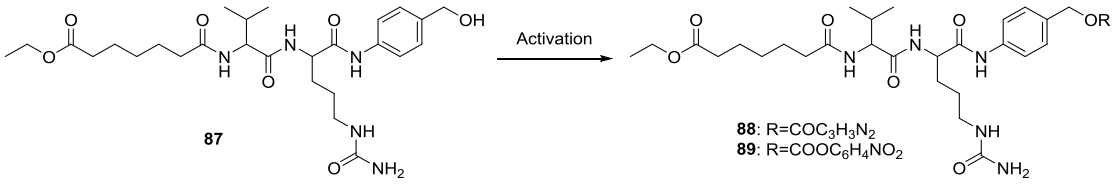
Scheme 28.

Therefore, the first step was the peptide coupling with the p-aminobenzyl alcohol (PABA) in the presence of HOBt and HBTU. After 12 h, the desired product **87** was easily isolated with a discrete yield (67%, Scheme 29).



Scheme 29.

In order to facilitate the reaction with the secondary amine of the N'-methyl-N-boc etylendiamine **90**, the benzylic OH of **87** was activated by treatment with CDI in THF dry for 72 h. The obtained product **89** (36% yield) was not pure because of the presence of residual CDI (Table 8, Exp.1).



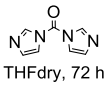
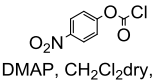
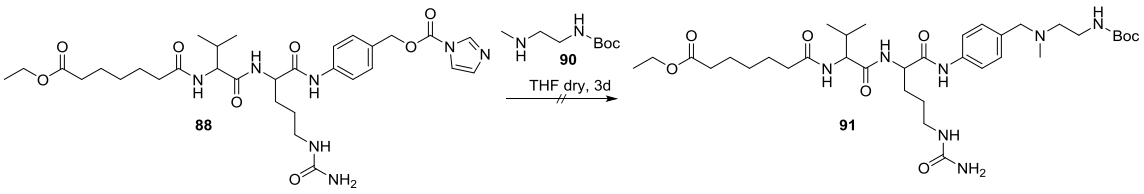
Exp. No	Conditions	Compd. Yield %
1	 THFdry, 72 h	**88** 36(CDI)
2	 DMAP, CH_2Cl_2dry, 1 h	**89** Traces(DMAP)

Table 8.

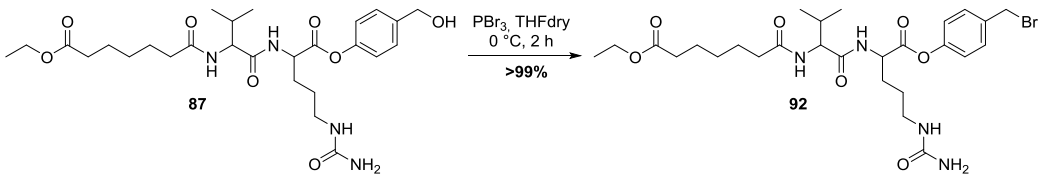
A similar problem emerged when we tried to activate the OH group with PNP-chloroformate, because in this case the activated linker **89** was obtained in traces and contaminated with DMAP (Table 8, Exp.2).

Anyway, **88** was used for the following step with **90** in THF dry. Even after 72 h there were only traces of the product **91** (Scheme 30).



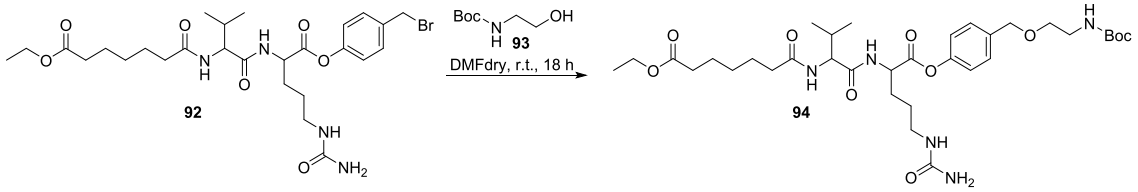
Scheme 30.

Finally, we tried to use PBr_3 to transform the benzylic OH into a good leaving group and the desired product **92** was isolated in quantitative yield stirring the mixture at 0 °C for 2 h in THF dry (Scheme 31). Anyway, it shows some stability problems and for these reasons it has to be used immediately for the following step.



Scheme 31.

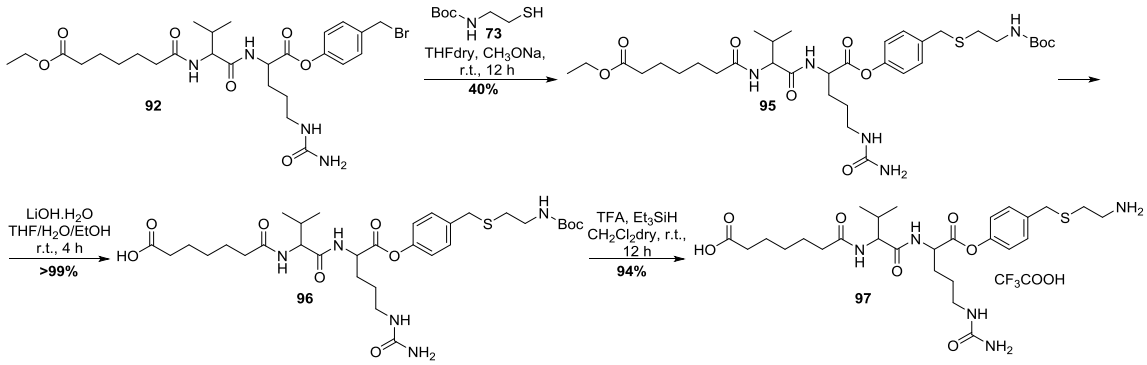
Poor results were obtained by trying again the reaction with the diamine **90** as well with the boc-etanolamine **93** either using NaH or KOH (Table 9).



Exp. No	Conditions	Yield %
1	NaH	-
2	KOH	-

Table 9.

In the last experiment, we used the boc-amino-ethanethiol **73** in the presence of CH_3ONa in THF dry at room temperature for 12 h. This time we obtained the desired product **95** with 40% yield. After the hydrolysis of the OEt group with $LiOHxH_2O$ in $THF/H_2O/EtOH$ (1:1:1 ratio), the resulting acid **96** was deprotected with TFA and Et_3SiH in CH_2Cl_2 dry for 12 h furnishing the final linker **97** with 94% yield (Scheme 32).



Scheme 32.

2.8 CONJUGATION OF KNOWN CYTOTOXIC MOLECULES

At that point, we had available different kind of in-house prepared linkers belonging to both cleavable and non-cleavable type and a series of small molecules already known for their cytotoxic properties and suitable for the conjugation. The drugs of our interest are shown in the figure below and the conjugating functional groups evidenced.

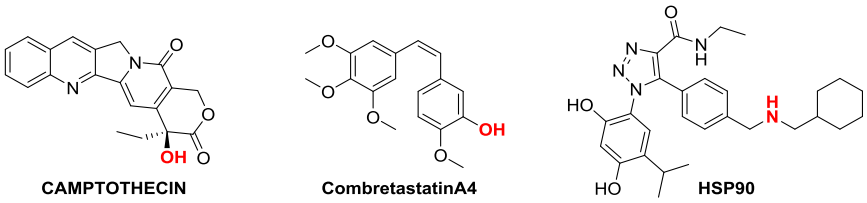
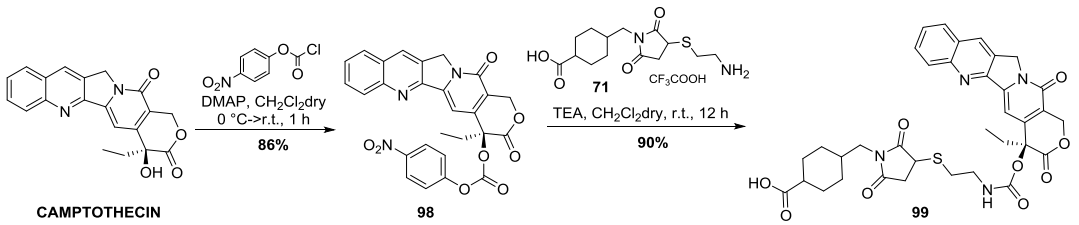


Figure 19.

2.8.1 CAMPTOTHECIN Conjugates - Synthetic procedure

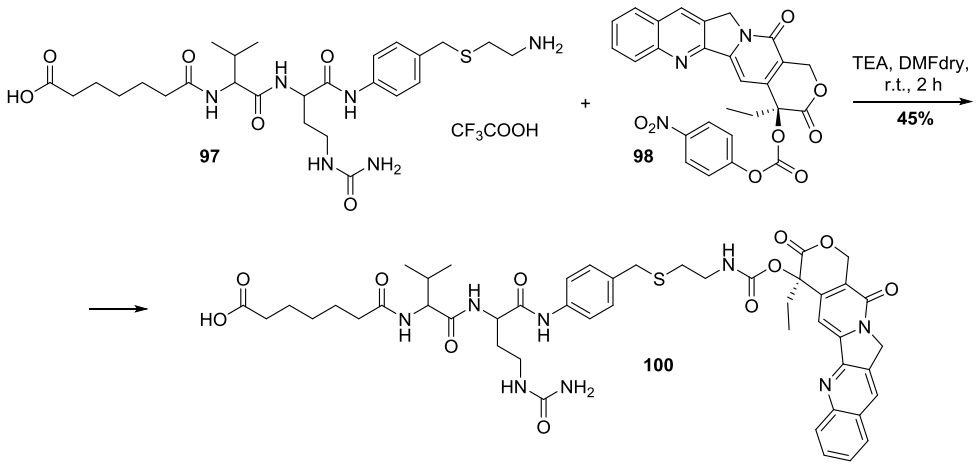
We had available an extended literature on the Camptothecin analogues bioconjugates which are easily formed taking advantage of the versatile OH group opportunely activated and loaded on linker chains.[31]

Kad-Camptothecin is obtained with a very good yield after the activation of the toxin using PNP-chloroformate and DMAP as base in CH_2Cl_2 dry for 1 h according to an already described procedure, and with a yield of 86%.[32] Then, the activated compound **98** and the triflate linker **71** reacted in the presence of TEA for 12 h at room temperature to give the expected product **99** in a good yield (90%, Scheme 33).



Scheme 33.

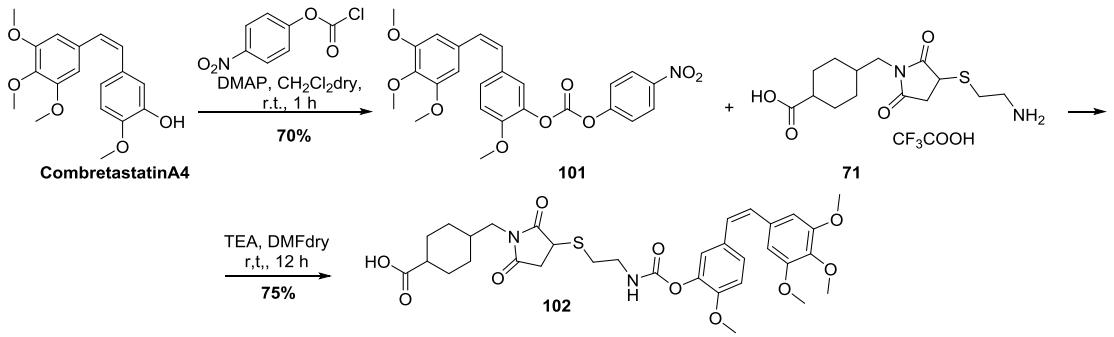
Ad-Camptothecin was prepared with a similar approach. In detail, the coupling with the linker **97** was conducted in DMF dry with TEA at room temperature for 2 h and the final compound **100** was isolated with a lower yield than the Kad derivative **99** (45%, Scheme 34).



Scheme 34.

2.8.2 Combretastatin Conjugate - Synthetic procedure

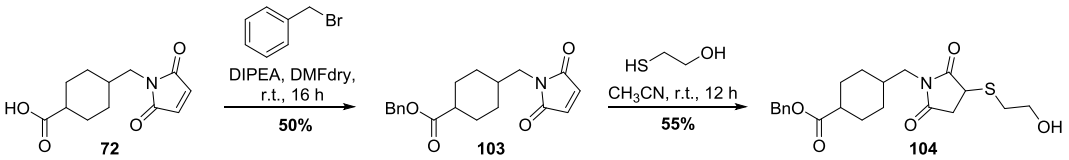
The strategy adopted to conjugate this drug is similar to the one developed for the camptothecin derivatives since they both have an OH group. This is activated with PNP-chloroformate and DMAP in CH_2Cl_2 dry (**101**, 70% yield) and it then undergoes the coupling reaction with the Kad linker **71** in DMF dry with TEA. The desired product **102** is obtained with a discrete yields (75%, Scheme 35).



Scheme 35.

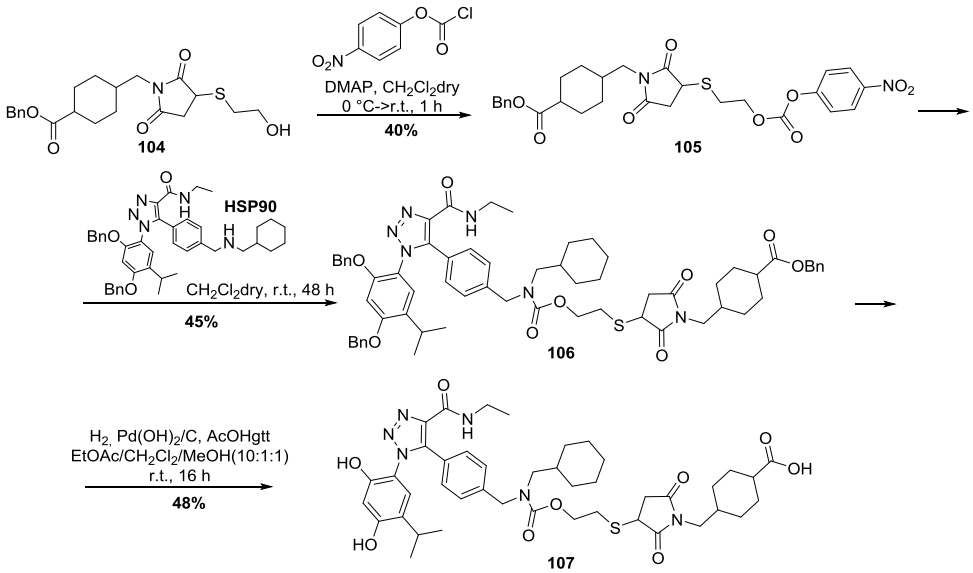
2.8.3 HSP90 Conjugate - Synthetic procedure

The synthesis of this small molecule's been developed in our group and it's used as a pro-drug to be conjugated, in a di-benzylated form.[33]In this case, we focused on the secondary amine moiety for the conjugation with the linker **79** previously prepared. We thought to protect the carboxylic OH of the linker **79** with a benzyl group and to remove it in the last step of the synthesis together with the ones on the phenolic OH of HSP90. Therefore, the maleimide **72** was treated with benzyl bromide and DIPEA in DMF dry at room temperature for 16 h and the benzylated derivative **103** was then submitted to the Michael reaction with the 2-mercaptoethanol under the conditions already described in Scheme 25 to give the compound **104** with 55% yield (Scheme 36).



Scheme 36.

The OH group of the linker **104** is activated using again the PNP-chloroformate at conditions reported in Scheme 32 to give **105** with 40% yield. After stirring **105** with the pro-drug HSP90 for 48 h in CH_2Cl_2 dry, the tri-benzylated conjugate **106** was obtained with 45% yield and deprotected under H_2 atmosphere with catalytic amount of $Pd(OH)_2/C$ for 16 h to furnish the desired product **107** with a 48% yield (Scheme 37).



Scheme 37.

2.9 PAYLOADS ACTIVATION

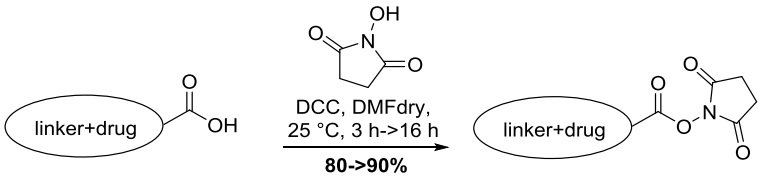
To link a payload (linker-drug) on a mAb, the cysteine and lysine moieties are very useful for:

1. Abundancy
2. Advantage of the application of basic reactions

Therefore, the last step for each payload prepared is the activation of the COOH function, shared by all the compounds, in order to react with the NH_2 of the lysine and the SH of the cysteine.

2.9.1 NHS Activation for lysine

The opportune payload is treated with NHS in the presence of DCC and in DMF dry, and stirred for 3 h or 16 h at room temperature depending on the substrates. The desired products are obtained with good yields (Scheme 38).



Scheme 38.

Conjugates for lysine:

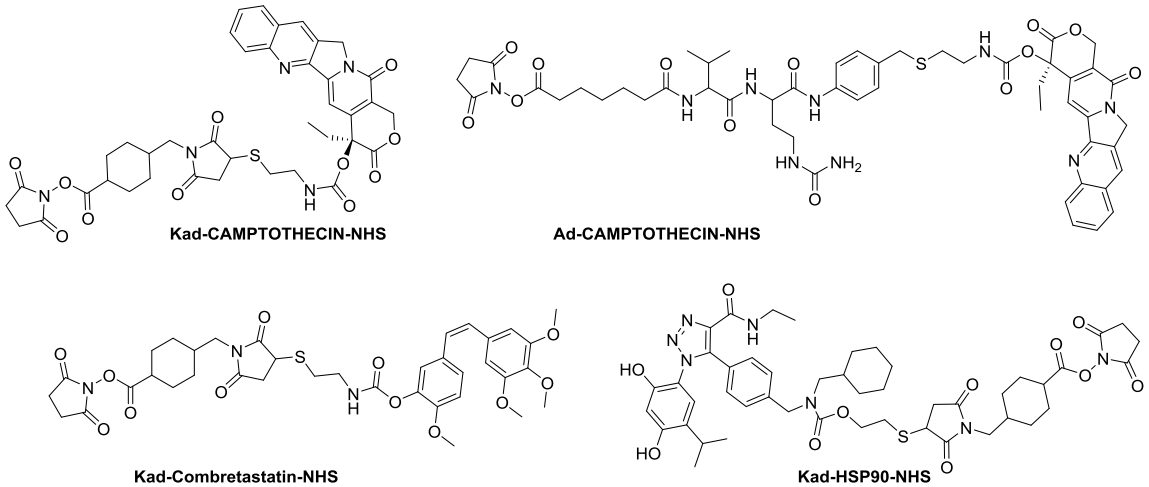
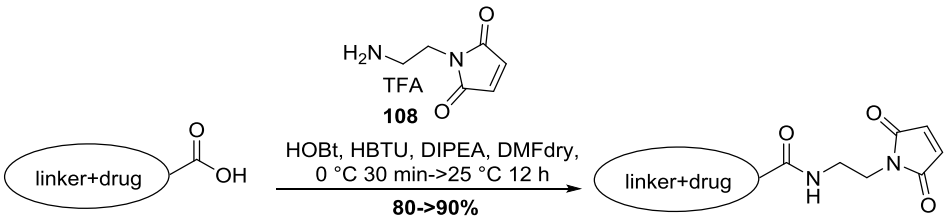


Figure 20.

2.9.2 Maleimide Activation for cysteine

The same payloads are activated with a maleimide to interact with the SH group of cysteine through a Michael type reaction. Each compound is treated with the N(2-aminoethyl)maleimido trifluoroacetic salt **108**, HOBt, HBTU and DIPEA in DMF dry to give the final products with good yields (Scheme 39).[30,34]



Scheme 39.

Conjugates for cysteine:

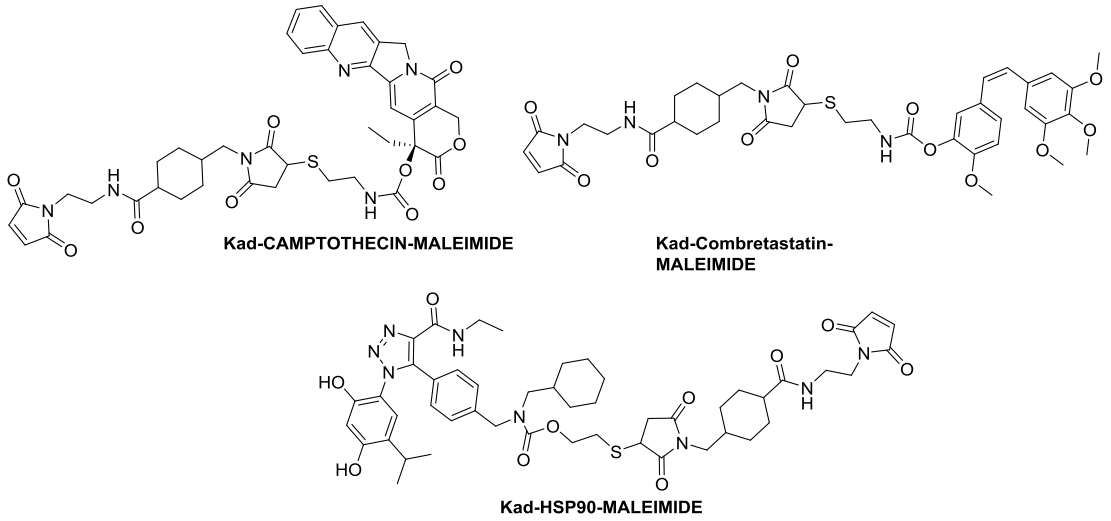


Figure 21.

All of the payloads have been sent to the company group to be conjugated with specific mAbs, but we cannot report the results of their activity in this work for reasons of intellectual property.

2.10 CONCLUSIONS

In this work, the hit optimization of the Hh pathway's agonists and antagonists is reported. In particular, it is developed on the base of the structure of the quinolone GSA10 (agonist) and the acylguanidine MRT83 (antagonist). All the compounds synthesized have been tested in collaboration with other research groups and some of them show a very good and promising profile in potency, selectivity and efficacy.

At the same time, a series of Mre11 inhibitors, the PFM compounds, have been prepared. From the biological assays emerged that these compounds are able to restore the activity of the DNA repair functions with both endonuclease and exonuclease mechanisms. Moreover, they are very useful biological tools and are currently used to know how the MRN Complex works. Finally they are the only Mre11 inhibitors known today.

An important part of this PhD work has been developed in the frame of a Sigma-Tau project, concerning the Antiboby Drug Conjugates (ADC) which combine the selectivity of the antibody and the potency of the cytotoxic drug, taken together through a molecular linker. Keeping in mind the structure of the commercially available *Kadcyla* and *Adcetris* conjugates, the aim was to synthesize new molecular linkers and new payloads with the already known cytotoxic drugs Campthotecin, CombretastatinA4 and HSP90. All of them have been sent to the research group which provided for the conjugation with the mAbs (monoclonal Antibodies). The results of the biological tests can't be reported for reasons of intellectual properties.

EXPERIMENTAL SECTION

3.1 HH AGONISTS

*General procedure for the synthesis of 4-hydroxy-quinolones **2a-g**. Ethyl 1-hexyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2a**).*

A mixture of N-hexylaniline (5 mmol) and triethyl methanetricarboxylate 3 (15 mmol) was irradiated with microwaves at the fixed power of 250 Watt (max temp 250 °C) for 20 minutes in open vessel conditions. The crude mixture was directly purified by flash chromatography (PE/AcOEt 4:1) giving the desired compounds **x** in moderate to good yields.

Ethyl 1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxylate **Yield**: 54%
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 – 8.02 (m, 1H), 7.63 – 7.50 (m, 1H), 7.23 – 7.12 (m, 2H), 4.43 (dd, *J* = 14.2, 7.1 Hz, 2H), 4.17 – 4.07 (m, 3H), 1.68 – 1.54 (m, 2H), 1.22 (ddt, *J* = 33.0, 25.9, 7.3 Hz, 8H), 0.88 – 0.71 (m, 4H).
^{1}H NMR (200 MHz, CDCl3): δ 14.13 (bs, 1H), 8.10 (d, 1H, J = 7.8 Hz), 7.58 (t, 1H, J = 8.12 Hz), 7.28-7.09 (m, 2H), 4.49 (q, 2H, J = 7.2 Hz), 4.12 (t, 4H, J = 7.6), 1.42-0.98 (m, 8H), 0.63 (t, 3H, J = 7.2 Hz).
ES-MS: 318 [M+H]+, 340 [M+Na]+, 356 [M+K]+, 657 [2M+Na]+.

Ethyl 1-pentyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2b**). **Yield**: 60%.
^{1}H NMR (200 MHz, $CDCl_3$): δ 13.88 (bs, 1H), 7.79 (d, 1H, J = 7.8 Hz), 7.33 (t, 1H, J = 7.3 Hz), 6.96-6.84 (m, 2H), 4.20 (q, 2H, J = 7.2 Hz), 3.87 (t, 4H, J = 7.6 Hz), 1.41-0.99 (m, 6H), 0.63 (t, 3H, J = 7.2 Hz). ES-MS: 304 [M+H]+, 326 [M+Na]+, 342 [M+K]+.

Ethyl 1-butyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2c**). **Yield**: 55%.
^{1}H NMR (200 MHz, $CDCl_3$): δ 13.91 (bs, 1H), 7.79 (d, 1H, J = 7.8 Hz), 7.33 (t, 1H, J = 7.3 Hz), 6.96-6.84 (m, 2H), 4.22 (q, 2H, J = 7.2 Hz), 3.87 (t, 4H, J = 7.6 Hz), 1.40-1.21 (m, 4H), 0.63 (t, 3H, J = 7.2 Hz).
ES-MS: 290 [M+H]+, 312 [M+Na]+, 328 [M+K]+.

Ethyl 1-propyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2d**). **Yield**: 53%.
^{1}H NMR (200 MHz, $CDCl_3$): δ 12.54 (bs, 1H), 8.24 (d, 1H, J = 7.92 Hz), 7.71-7.65 (m, 1H), 7.40-7.14 (m, 2H), 4.31-4.08 (m, 4H), 1.83-1.72 (m, 2H), 1.27-1.20 (m, 3H), 1.14-0.96 (m, 3H).

ES-MS: 297 [M+Na]+.

Ethyl 1-methyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2e**). **Yield**: 35%.
^{1}H NMR (200 MHz, $CDCl_3$): δ 12.64 (bs,1H), 7.30-7.26 (m, 4H), 4.35 (q, 2H, J = 7.3 Hz), 3.62 (s, 3H), 1.19 (t, 3H, J = 7.3 Hz).
ES-MS: 270 [M+Na]+.

Ethyl 1-allyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2f**). **Yield**: 77%.
^{1}H NMR (400 MHz, $CDCl_3$): δ 14.25 (bs, 1H), 8.15 (d, 2H, J = 8.4 Hz), 7.60 (t, 1H, J = 7.2 Hz), 7.24-7.19 (m, 2H), 5.95-5.85 (m, 1H), 5.18-5.06 (m, 2H), 4.94-4.96 (m, 2H), 4.47 (q, 2H, J = 6.4 Hz), 1.37 (t, 3H, J = 6.4 Hz).
^{13}C NMR (100 MHz, $CDCl_3$): δ 172.27, 171.47, 140.3, 133.84, 133.74, 131.49 , 125.29, 121.42, 116.58, 114.27, 61.91, 43.99, 13.74.
ES-MS: 296 [M+Na]+.
Ethyl 1-benzyl-1,2-dihydro-4-hydroxy-2-oxo-quinoline-3-carboxylate (**2g**). **Yield**: 49%.
^{1}H NMR (400 MHz, CDCl3): δ 14.32 (bs, 1H), 8.17 (d, 2H, J = 7.6 Hz), 7.50 (t, 2H, J = 7.6 Hz), 7.16-7.27 (m, 5H), 5.48 (s, 2H), 4.51 (q, 2H, J = 7.6 Hz), 1.46 (t, 3H, J = 7.6 Hz).
ES-MS: 262 [M+Na]+.

*General procedure for the synthesis of the nitrobenzoates **4a-d**.*
To a solution of the opportune 2-nitrobenzoic acid **3a-d** (8.81mmol) in EtOH (20ml) H_2SO_4 (53mmol) was added refluxed and stirred for 20 h. The residual solvent was evaporated under reduced pressure, NaHCO3ss was added fino a pH 8 and the mixture is extracted with CH_2Cl_2 (3x15ml). The organic layers were dried over Na_2SO_4, filtered and after the evaporation of the solvent, the desired compounds were obtained in good to excellent yields.
Ethyl 4,5-dimethoxy-2-nitrobenzoate (**4a**) **Yield:** >99%
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.00 (s, 1H), 4.30 (q, *J* = 7.1 Hz, 2H), 3.90 (d, *J* = 4.3 Hz, 6H), 1.28 (t, *J* = 7.1 Hz, 3H).
Ethyl 5-fluoro-2-nitrobenzoate. (**4b**) **Yield:** 95%
^{1}H NMR: (400 MHz, $CDCl_3$) 7.92-7.95 (t, J=8.8 Hz, 1H); 7.29-7.32 (m, 1H); 7.21-7.26 (m, 1H); 4.32-4.35 (t, J=6.4 Hz, 2H); δ 1.01 (t, J=1.2, 3H)

*General procedure for the reduction of the anthranilates **5a-d**.*

To a solution of the opportune nitrobenzoate **4a-d** (10.7mmol) in CH_3OH (40ml) acetic acid (glacial) was added in drops under N_2 atmosphere. After sonication and the addition of $Pd(OH)_2/C$ (1.1mmol), the solution was stirred at room temperature for 36 h under H_2 atmosphere. The resulting reaction mixture was filtered on celite washing with CH_3OH and CH_2Cl_2, evaporated under reduced pressure and purified on silica gel (Büchi) with PE/EtOAc 1:1 to give the desired compounds in quantitative yield.

Ethyl 2-amino-4,5-dimethoxybenzoate (**5a**) **Yield:** >99%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, *J* = 2.9 Hz, 1H), 6.08 (d, *J* = 4.3 Hz, 1H), 4.31 – 4.20 (m, 2H), 3.77 (dd, *J* = 11.9, 3.5 Hz, 6H), 1.37 – 1.27 (m, 3H).

*General procedure for the reductive amination of the anilines **6a-d***

To a solution of the anthranilates **5a-d** (12.1mmol) in DCE (50ml) under N_2 atmosphere. Propionaldehyde (24.2mmol) and glacial acetic acid (18.15mmol) were added in portions, and the resulting mixture is stirred for 30 min at room temperature. After cooling down to 0 °C and the addition of $Na(OAc)_3BH$ (60.5mmol), the solution was stirred for 48 h at room temperature and monitored by TLC. The reaction mixture was cooled down to 0 °C and extracted with CH_2Cl_2. The collected organic layer were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified on silica gel with PE/EtOAc 2:1 to give the desired products with 30% to 60% yield.

Ethyl 4,5-dimethoxy-2-(propylamino)benzoate (**6a**) **Yield:** 39%

^{1}H NMR: (400 MHz, $CDCl_3$) 7.59 (bs, 1H); 7.34 (s, 1H); 6.09 (s, 1H); 4.21-4.27 (q, *J*=6.8 Hz, 2H); 3.85 (s, 1H); 3.77 (s, 1H); 3.07-3.11 (t, *J*=6.8, 2H); 1.64-1.69 (q, *J*=7.2 Hz, 2H); 1.29-1.33 (t, *J*=6.8 Hz, 3H); 0.97-1.01 (t, *J*=7.6 Hz, 3H)

Ethyl 5-fluoro-2-(propylamino)benzoate (**6b**) **Yield:** 59%

^{1}H NMR: (400 MHz, $CDCl_3$) 7.55-7.58 (dd, J_1=2.8 Hz, J_2= 9.6 Hz, 1H); 7.49 (bs, 1H); δ 7.04 (d, *J*=8.4 Hz, 1H); 6.54-6-57 (q, *J*=4 Hz, 1H); 4.25-4.30 (q, *J*=6.8 Hz, 2H); 3.06-3.11 (q, *J*=6.8, 2H); 1.64-1.69 (q, *J*=7.2 Hz, 2H); 1.32-1.36 (t, *J*=7.2 Hz, 3H); 0.97-1.01 (t, *J*=7.6, 3H)

^{13}C NMR: (400 MHz, $CDCl_3$) δ 167.84, 151.6, 148.215, 122.172, 121.946, 116.782, 116.551, 112.17, 109.672, 77.372, 77.053, 76.735, 60.446, 45.01, 22.38, 14.25, 11.66

General procedure for the acylation with ethyl malonyl chloride.

To a solution of the compounds **6a-d** (1.76mmol) in CH_2Cl_2 dry (10ml) under N_2 atmosphere, ethyl malonyl chloride (1.76mmol) was added. The solution was stirred at room temperature for 12 h and the resulting mixture was diluted with CH_2Cl_2 (10ml), then washed with $NaHCO_{3ss}$ (10ml). The organic layer was collected and dried over Na_2SO_4, filtered and evaporated under reduced pressure. After purification by flash chromatography on silica gel (Büchi) with PE/EtOAc 2:1 the desired products **7a-d** were obtained with 50% to 70% yield.

Ethyl 2-(3-ethoxy-3-oxo-N-propylpropanamido)-4,5-dimethoxybenzoate (**7a**) **Yield:** 69%

^{1}H NMR: (400 MHz, $CDCl_3$) 7.46 (s, 1H); 6.71 (s, 1H); 4.25-4.31 (m, 2H); 4.06-4.11 (m, 2H); 3.91 (s, 6H); 3.87 (s, 2H); δ3.08 (d, J=1.6 Hz, 2H); 1.45-1.55 (m, 2H); 1.29-1.33 (t, J=6.8 Hz, 3H); 1.16-1.23 (m, 3H); 0.83-0.87 (t, J=7.6 Hz, 3H)

Ethyl 2-(3-ethoxy-3-oxo-N-propylpropanamido)-5-fluorobenzoate (**7b**) **Yield:** 51%

^{1}H NMR: (400 MHz, $CDCl_3$) 7.49-7.51 (dd, J_1=8.4 Hz, J_2= 6.8 Hz, 1H); 7.12-7.17 (m, 2H); 3.90-3.95 (q, *J*=7.2 Hz, 4H); 3.78-3.89 (m, 2H); 2.92 (s, 2H); 1.27-1.40 (m, 2H); δ 1.04 (t, *J*=1.2 Hz, 3H); δ 0.70 (t, *J*=8 Hz, 3H)

*General procedure for the synthesis of quinolones **8a-d***

NaH (1.22mmol) was washed 3 times under N_2 atmosphere with PE, which was then removed and replaced with THF dry (10ml). Finally, the compounds **7a-d** (1.22mmol) were added and the resulting mixture was stirred at room temperature for 20 min, then cooled down at 0 °C and diluted with CH_3OH. After the evaporation of the solvent, the crude products were purified on silica gel (Büchi) with PE/EtOAc 1:1 to furnish the desired quinolones **8a-d** with 40% to 90% yield.

Ethyl 4-hydroxy-6,7-dimethoxy-2-oxo-1-propyl-1,2-dihydroquinoline-3-carboxylate (**8a**) **Yield:** 91%

^{1}H NMR: (400 MHz, $CDCl_3$): δ16.38 (bs, 1H), 14.18 (bs, 1H), 7.45 (s, 1H), 6.62 (s, 1H), 4.43 (q, 2H, *J* = 7.2 Hz), 4.11 (t, 2H, *J* = 7.6 Hz), 3.94 (s, 3H), 3.91 (s, 3H), 1.71 (q, 2H, *J* = 7.6 Hz), 1.43 (t, 3H, *J* = 7.2 Hz), 0.99 (t, 3H, *J* = 7.2 Hz).

^{13}C NMR: (400 MHz, $CDCl_3$): δ 173.08, 170.8, 159.53, 155.25, 145.07, 137.29, 107.71, 105.61, 96.74, 96.36, 62.10, 56.23, 56.14, 44.05, 20.84, 14.28, 11.46.

ES/MS: 358 $[M+Na]^+$

R_f: 0.51 (CH_2Cl_2/MeOH 9.5:0.5)

Ethyl 6-fluoro-4-hydroxy-2-oxo-1-propyl-1,2-dihydroquinoline-3-carboxylate (**8b**) **Yield:** 44%

^{1}H NMR: (400 MHz, $CDCl_3$) 7.79-7.81 (t, *J*=7.2 Hz, 1H); 7.29-7.33 (t, *J*= 4.4 Hz, 2H); δ4.31 (t, *J*=7.2 Hz, 2H); δ4.08 (t, *J*=7.6 Hz, 2H); 3.28 (bs, 1H); 1.51-1.69 (t, *J*=7.2 Hz, 2H); 1.19-1.37 (t, *J*=7.2 Hz, 3H); 0.79-1.00 (t, *J*=7.2 Hz, 3H)

R_f: 0.23 (eluente PE/EtOAc: 3:1)

*General procedure for the synthesis of p-nitrobenzoates **9a-b**.*

P-nitrobenzoic acid **8** (6mmol) was solubilized in the appropriate alcohol (0.13mmol) and irradiated with microwaves at 80 °C for 2 cycles of 40 min in open vessel, monitoring by TLC until the complete conversion. The resulting mixture was diluted with EtOAc, washed with $NaHCO_{3ss}$ (3x10ml) and the organic layers were dried over Na_2SO_4 and filtered. After the evaporation of the solvent, the desired compounds were obtained in moderate yields as crude products.

Propyl 4-nitrobenzoate (**9a**) **Yield:** 40%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 – 7.74 (m, 1H), 3.99 – 3.86 (m, 1H), 1.48 – 1.33 (m, 1H), 0.61 (td, *J* = 7.2, 2.5 Hz, 1H).

Reduction of the p-nitrobenzoates.

To a solution of the *p*-nitrobenzoates **9a-b** (2.53mmol) in CH_3OH (15ml) under N_2 atmosphere, 2-3 drops of AcOH were added. The mixture was sonicated and $Pd(OH)_2/C$ was added in catalytic amount (0.25mmol). The reaction mixture was stirred under H_2 atmosphere at room temperature for 24 h and monitored by TLC. After filtration on celite washing with CH_3OH, the solvent was evaporated, and the crude product was purified on silica gel (Büchi) with PE/EtOAc 2:1 to give the desired anilines **10a-b** in good yields.

Propyl 4-aminobenzoate (**10a**) **Yield:** 60%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.6 Hz, 2H), 6.59 (d, *J* = 8.6 Hz, 2H), 4.15 (t, *J* = 6.7 Hz, 2H), 1.69 (dd, *J* = 14.2, 7.1 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H).

ES-MS: 202 $[M+Na]^+$

*General procedure for the synthesis of 4-hydroxy quinolones **11-31***

A mixture of **2a-k** (0.63 g 2 mmol) and **10a-i** (0.394 g, 2.2 mmol) in toluene (4 mL) was irradiated with microwaves at 250 Watt (max temperature set 250 °C) for 1 h in open vessel conditions. After cooling down to room temperature, the crude mixture was purified by flash

chromatography on silica gel (Büchi) with PE/AcOEt: 6/1 to give the desired final compounds **11-14** and **16-31** with moderate to good yields.

*Propyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamido)benzoate.**(11)*** **Yield:** 70%

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.30 (bs, 1H), 15.71 (bs, 1H), 8.24 (d, 1H, *J* = 8.0 Hz), 7.85 (d, 2H, *J* = 8.4 Hz), 7.77 (t, 1H, *J* = 8.4 Hz), 7.44-7.24 (m, 2H), 6.52 (d, 2H, *J* = 8.8 Hz), 4.27 (t, 2H, *J* = 7.6 Hz), 4.19 (t, 2H, *J* = 6.4 Hz), 2.85-2.80 (m, 2H), 1.76- 1.71 (m, 2H), 1.45-1.23 (m, 6H), 0.98 (t, 3H, *J* = 7.6 Hz), 0.89 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 197.23, 173.05, 170.67, 164.25, 139.13, 134.52, 130.97, 125.94, 123.06, 72.54, 65.95, 42.32, 29.71, 26.1, 21.95, 13.53, 10.11.

ES-MS: 473 [M+Na]+. **LC/MS:** RT 5.29 min; 473 [M+Na]+.

Purity: 98%.

Anal calcd for C26H30N2O5 C 69.31, H 6.71, N 6.22, O 17.76; found C 69.29, H 6.68, N 6.25. %.

*Propyl 4-(1-propyl-4-hydroxy-N-methyl-2-oxo-1,2-dihydroquinolin-3-carboxamido)-6-fluorobenzoate.**(12)*** **Yield:** 40%.

^{1}H NMR: (200 MHz, $CDCl_3$): δ 8.04 (d, 2H, *J*=8.6 Hz), 7.89 (dd, 1H, *J1* = 2.8 Hz, *J2* = 8.6 Hz, 1H), 7.76 (d, 2H, *J* = 8.6 Hz), 7.42 (m, 1H), 4.23 (q, 2H, *J* = 6.6 Hz), 1.78 (q, 4H, *J* = 7.6 Hz), 1.04 (t, 6H, *J* = 7.6 Hz).

LC/MS: RT 5.23 min; 427 [M+H]+. **Purity:** 98%. **Anal calcd for** C23H23FN2O5 C, H, N: calc. C 64.78, H 5.44, F 4.46, N 6.57, O 18.76; found C 64.80, H 5.41, N 6.53.

*Propyl 4-(1-propyl-4-hydroxy-N-methyl-2-oxo-1,2-dihydroquinolin-3-carboxamido)-6,7-dimethoxylbenzoate.**(13)*** **Yield:** 48%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.28 (bs, 1H), 12.87 (bs, 1H), 8.02 (d, 2H, *J* = 8.4 Hz), 7.76 (d, 2H, *J* = 8.8 Hz), 7.55 (s, 1H), 6.72 (s, 1H), 4.24 (q, 4H, *J* = 6.4 Hz), 4.00 (s, 3H), 3.96 (s, 3H), 1.82-1.75 (m, 4 H), 1.5-0.92 (m, 6H).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 171.19, 170.00, 166.25, 162.49, 155,14, 145.74, 141,64, 135,66, 130,68, 126.19, 125.70, 120.30, 109.07, 105.33, 96.92, 95.82, 77.33, 77.01, 76.69, 66.44, 56.25, 44.18, 20.17, 22.70, 21.01, 11.44, 10.54.

LC/MS: RT 5.95 min; 469 [M+H]+, 491 [M+Na]+, 959 [2M+Na]+. **Purity:** 98%. Anal calcd for

C25H28N2O7 C, H, N: C 64.09, H 6.02, N 5.98, O 23.91; found C 64.12, H 6.00, N 5.95.

*Tert-Butyl 3(1.hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate.**(14)***

Yield: 32%.

^{1}H NMR ($CDCl_3$, 400 MHz): d 16.45 (bs, 1H), 12.39 (bs, 1H), 8.80 (d, 1H, *J* = 6.8 Hz), 7.91 (d,

1H, *J* = 7.6 Hz), 7.75 *(*d, 1H *J* = 7.6 Hz), 7.66 (t, 1H, *J* = 7.6 Hz), 7.39 (t, 1H, *J* = 8 Hz), 7.30 (d, 1H, *J* =1.2 Hz), 7.26 (dd, 2H, *J1* = 8 Hz, *J2* = 7.6 Hz,), 4.24 (t, 2H, *J* = 6.4 Hz), 1.58-1.33 (m, 6H), 1.23 (s, 9H), 0.89 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR ($CDCl_3$, 100 MHz): d 172.04, 169.63, 165.33, 165.33, 162.51, 138.20, 137.31, 133.98, 132.94, 128.82, 125.76, 125.67, 125.17, 122.41,122.14, 116.30, 114.35, 97.03, 81.24, 42.52, 31.54, 30.89, 29.70, 28.22, 27.66, 26.69, 22.62, 14.02.

ES-MS: 465 [M+H]+, 487 [M+Na]+. **M.p.**: 120-125 °C. LC/MS: RT 5.72 min; 465 [M+H]+, 487 [M+Na]+. **Purity**: 97%. **Anal calcd for** C27H32N2O5 C, H, N: C 69.81, H 6.94, N 6.03, O 17.22; found C 69.83, H 6.97, N 6.00.

Butyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate (**16**). **Yield:** 75%. **^{1}H NMR**: (400 MHz, $CDCl_3$): δ 16.30 (bs, 1H), 15.71 (bs, 1H), 8.24 (d, 1H, *J* = 8.0 Hz), 7.85 (d, 2H, *J* = 8.4 Hz), 7.77 (t, 1H, *J* = 8.4 Hz), 7.44-7.24 (m, 2H), 6.52 (d, 2H, *J* = 8.8 Hz), 4.27 (t, 2H, *J* = 7.6 Hz), 4.19 (t, 2H, *J* = 6.4 Hz), 2.85-2.80 (m, 2H), 1.79-1.71 (m, 2H), 1.49-1.23 (m, 8H), 0.98 (t, 3H, *J* = 7.6 Hz), 0.89 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 197.23, 173.05, 170.67, 164.25, 139.13, 134.52, 130.97, 125.94, 123.06, 72.54, 65.95, 42.32, 30.82, 29.71, 26.1, 21.95, 19.12, 13.53, 10.11.

ES-MS: 487 [M+Na]+. LC/MS: RT 5.32 min; 487 [M+Na]+.

Purity: 97%. **Anal calcd for** C27H30N32O5 C, 69.81; H, 6.94; N, 6.03; O, 17.22; found C 69.79, H 6.95, N 6.05.

*1-Hexyl-4-hydroxy-2-oxo-N-(4-(propylcarbamoyl)phenyl)-1,2-dihydroquinoline-3-carboxamide (**17**)* **Yield:** 53%.

^{1}H NMR ($CDCl_3$, 400 MHz) d (ppm): 8.25 (d, *J* = 8 Hz, 2H), 7.77 (s, *J* = 8, 1H), 7.70 *(*t, *J* = 7.6 2H), 7.36 (d, *J* = 8 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 2H), 4.26 (t, *J* = 8 Hz, 3H), 3.41 (q, *J1* = 7.6 Hz, *J*2 = 6.4 Hz 4H), 1.53 (s, 2H), 1.33 (d, *J* = 3.6 Hz, 6H*)*, 1.00 (t, *J* = 6.8 Hz 3H), 0.89 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz): d (ppm): 172.11, 19.67, 127.83 125.83, 122.52, 120.73, 114.40, 77.63, 77.20, 77.00, 76.36, 73.05, 42.57, 41.76, 31.53, 29.69, 27.65, 26.68, 22.97, 22.61, 14.01.

ES-MS: 450 [M+H]+, 472 [M+Na]+.

M.p.: 205-210 °C.

LC/MS: RT 4.98 min; 472 [M+Na]+.

Purity: 98%.

Anal calcd for C26H31N3O4 C, 69.47; H, 6.95; N, 9.35; O, 14.24; found C 69.44, H 6.98, N 9.30.

Propyl 4-(1-pentyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate ***(18)***.

Yield: 67%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.30 (bs, 1H), 15.71 (bs, 1H), 8.24 (d, 1H, *J* = 8.0 Hz), 7.85 (d, 2H, *J* = 8.4 Hz), 7.77 (t, 1H, *J* = 8.4 Hz), 7.44-7.24 (m, 2H), 6.52 (d, 2H, *J* = 8.8 Hz), 4.27 (t, 2H, *J* = 7.6 Hz), 4.19 (t, 2H, *J* = 6.4 Hz), 2.83-2.79 (m, 2H), 1.76-1.71 (m, 2H), 1.45-1.27 (m, 4H), 1.01 (t, 3H, *J* = 7.6 Hz), 0.91 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 197.23, 173.05, 170.67, 164.25, 139.13, 134.52, 130.97, 125.94, 123.06, 72.54, 65.95, 42.32, 29.71, 26.1, 21.95, 13.53, 10.11.

ES-MS: 459 [M+Na]+.

LC/MS: RT 5.75 min; 459 [M+Na]+.

Purity: 98%.

Anal calcd for C25H28N2O5 C, H, N: calc. C68.79, H 6.47, N 6.42, O 18.33; found C 68.82, H 6.45, N 6.45.

Propyl 4-(1-butyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate ***(19)***.

Yield: 52%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.22 (bs, 1H), 15.38 (bs, 1H), 8.27 (d, 1H, *J* = 8.0 Hz), 7.85 (d, 2H, *J* = 8.4 Hz), 7.72 (t, 1H, *J* = 8.4 Hz), 7.44-7.19 (m, 2H), 6.48 (d, 2H, *J* = 8.8 Hz), 4.27 (t, 2H, *J* = 7.6 Hz), 4.19 (t, 2H, *J* = 6.4 Hz), 2.83-2.61 (m, 2H), 1.70-1.45 (m, 4H), 1.01 (t, 3H, *J* = 7.6 Hz), 0.91 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 197.23, 173.02, 170.67, 164.27, 139.13, 134.52, 130.97, 125.94, 123.06, 72.54, 65.90, 42.37, 29.31, 21.80, 13.13, 10.11.

ES-MS: 445 [M+Na]+.

LC/MS: RT 5.68 min; 445 [M+Na]+.

Purity: 97%.

Anal calcd for C24H26N2O5 C, H, N: calc. C 68.23, H 6.20, N 6.63, O 18.94; found C 68.19, H 6.18, N 6.60.

Propyl 4-(1-propyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate ***(20)***.
Yield: 42%.
^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.36 (bs, 1H), 12.83 (bs, 1H), 8.25 (d, 1H, *J* = 8.4 Hz), 8.04 (d, 2H. *J* = 8.4 Hz), 7.77 (d, 2H, *J* = 8.4 Hz), 7.76-7.67 (m, 1H), 7.37-7.24 (m, 2H), 4.25 (q, 4H, *J* = 6.4 Hz), 1.78 (q, 4H, *J* = 7.2 Hz), 1.05 (q, 6H, *J* = 7.2 Hz).
^{13}C NMR: (100 MHz, CDCl3): δ 133.71, 130.27, 125.42, 119.94, 114, 76.57, 66.02, 43.55, 21.10, 10.86.
LC/MS: RT 5.71 min; 409 [M+H]+; 431 [M+Na]+.
Purity: 96%.
Anal calcd for C23H24N2O5 C, H, N: C 67.63, H 5.92, N 6.86, O 19.59; found C 67.60, H 5.94, N 6.84.

Propyl 4-(1-methyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate ***(21)***
Yield: 42%.
^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.52 (bs, 1H), 12.81 (bs, 1H), 8.24 (d, 1H, *J* = 8 Hz), 7.85 (d, 2H, *J* = 8.4 Hz), 7.76 (d, 2H, *J* = 8.4 Hz), 7.71 (t, 1H, *J* = 8 Hz), 7.38 (d, 1H, *J* = 7.6 Hz), 7.32 (t, 1H, *J* = 8 Hz), 4.26 (t, 2H, *J* = 6.4 Hz), 3.71 (s, 3H), 1.78 (q, 2H, *J* = 7.2 Hz), 1.02 (t, 3H, *J* = 7.2 Hz).
^{13}C NMR: (100 MHz, $CDCl_3$): δ 173.23, 164.25, 141.32, 133.81, 130.30, 125.28, 123.06, 119.89, 113.94, 66.03, 31.51, 22.26, 13.67.
ES-MS: 403 [M+Na]+.
LC/MS: RT 5.62 min; 481 [M+H]+.
Purity: 98%.
Anal calcd for C21H20N2O5 C, H, N: C 66.31, H 5.30, N 7.36, O 21.03; found C 66.29, H 5.31, N 7.32.

Propyl 4-(1-allyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate ***(22)***.
Yield: 46% **^{1}H NMR**: (400 MHz, $CDCl_3$): δ 16.45 (bs, 1H), 12.72 (bs, 1H), 8.25 (d, 1H, *J* = 8 Hz), 8.03 (d, 2H, *J* = 8.4Hz), 7,75 (d, 2H, *J* = 7.6 Hz), 7.66 (t, 1H, *J* = 8), 7.32-7,24 (m, 2H), 6.00-5.94 (m, 1H), 5.24 (d, 1H, *J* = 8 Hz), 5.08 (d, 1H, *J* = 10.8 Hz), 4.94 (d, 2H, *J* = 2.8 Hz), 4.25 (t, 2H, *J* = 6.8 Hz), 1.80-1.75 (m, 2H), 1.01 (t, 3H, *J* = 8.4 Hz).
^{13}C NMR: (100 MHz, $CDCl_3$): δ 172.11, 161.18, 140.96, 133.74, 130.92, 130.27, 125.98, 125.28, 122.38, 119.91, 116.73, 114.60, 66.03, 44.19, 21.71, 10.1.
ES-MS: 407 [M+H]+, 429 [M+Na]+.

Mp : 132-135 °C.

LC/MS: RT 5.88 min; 457 [M+H]+, 479 [M+Na]+.

Purity: 97%.

Anal calcd for C23H22N2O5 C, H, N: C 67.97, H 5.46, N 6.89, O 19.68; found C 67.99, H 5.44, N 6.86.

*Propyl 4-(1-benzyl-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-carboxamido)benzoate (**23**).*

Yield: 40%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.51 (bs, 1H), 12.74 (bs,1H), 8.23 (d, 1H, *J* = 8 Hz), 8.03 (d, 2H, *J* = 8.4 Hz), 7.75 (d, 2H, *J* = 8.4 Hz), 7.52 (t, 1H, *J* = 8 Hz), 7.32-7.17 (m, 7H), 5.54 (s, 2H), 4.25 (t, 2H, *J* = 6.8 Hz), 1.80-1.77 (m, 2H), 1.02 (t, 3H, *J* = 8 Hz).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 172.26, 169.23, 162.55, 140.93, 139.07, 135.37, 133.81, 130.26, 128.58, 125.97, 125.23, 119.91, 114.76, 96.57, 66.03, 45.50, 21.74, 10.02.

LC/MS: RT 5.87 min; 457 [M+H]+, 479 [M+Na]+.

Purity: 99%.

Anal calcd for C27H24N2O5 C, H, N: C 71.04, H 5.30, N 6.14, O 17.52; found C 71.05, H 5.34, N 6.11.

*Propyl 4-(1-propyl-4-hydroxy-N-methyl-2-oxo-1,2-dihydroquinolin-3-carboxamido)-6-chlorobenzoate (**24**).* **Yield**: 36%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.44 (bs, 1 H), 12.73 (bs, 1H), 8.21 (d, 1H, *J* = 8.6 Hz), 8.04 (d, 2H, *J* = 8.4 Hz), 7.76 (d, 2H, *J*=8.4 Hz), 7.62 (q, 1H, *J* = 8.6 Hz), 7.29 (t, 1H, *J* = 9.2 Hz), 4.27-4.19 (m, 4H), 1.82-1.71 (m, 4H), 1.39-1.34 (m, 6H).

^{13}C NMR: (100 MHz, $CDCl_3$): δ 167.2, 165.9, 163.8, 163.0, 141.9, 133.73, 130.29, 124.70, 124.70, 119.98, 115.60, 66.07, 43.73, 21.71, 20.54, 10.82, 10.09.

LC/MS: RT 5.35 min; 443 [M+H]+.

Purity: 98%.

Anal calcd for C23H23ClN2O5 C, H, N: C 62.37, H 5.23, Cl 8.00, N 6.33, O 18.06; found C 62.40, H 5.25, N 6.34.

*Propyl 4-(1-propyl-4-hydroxy-N-methyl-2-oxo-1,2-dihydroquinolin-3-carboxamido)-6-methylbenzoate (**25**).* **Yield**: 55%.

^{1}H NMR: (400 MHz, $CDCl_3$): δ 16.29 (bs, 1H), 12.86 (bs, 1H), 8.01 (d, 2H, *J* = 8.2 Hz), 7.74 (d, 2H, *J* = 8.2 Hz), (d, 1H, *J* = 7.4 Hz), 7.22 (d, 2H, *J* = 8.2 Hz), 4.26-4.17 (m, 4H), 2.38 (s, 3H), 1.80-1.72 (m, 2H), 1.25-0.98 (m, 6H).

LC/MS: RT 6.05 min; 446 [M+Na]+.

Purity: 97%.

Anal (C24H26N2O5) C, H, N: **calc.** C 68.23, H 6.20, N 6.63, O 18.94; found C 68.25, H 6.19, N

6.66.

*Propyl 3-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate (**26**).* **Yield**: 52% **^{1}H NMR** (400 MHz, $CDCl_3$): 16.21 (bs, 1H), 13.12 (bs, 1H), 8.28 (t, 1H, *J* = 6.8 Hz), 7.98 (d, 1H, *J* = 6.8 Hz), 7.82 (d, 1H, J = 8Hz), 7.69-6.57 (m, 2H), 7.42 (t, 1H, *J* = 8 Hz), 7.35-7.24 (m, 2H), 4.38 (q, 2H, *J* =8 Hz), 4.25 (q, 2H, *J* = 6.4 Hz), 1.80-1.71 (m, 2H), 1.47-1.33 (m, 6H), 1.09 (t, 3H, *J* = 6.8 Hz), 0.92 (t, 3H, *J* = 7.3 Hz).

^{13}C NMR (100 MHz, $CDCl_3$) d: 172.09, 134.01, 125.80, 122.45, 114.37, 42.55, 31.55, 27.67, 26.70, 22.62, 14.01, 10.52.

ES-MS: 450 [M+H]+, 473 [M+Na]+.

M.p.: 103-108 °C

LC/MS: RT 5.32 min; 473 [M+Na]+.

Purity: 98%.

Anal calcd for C26H30N2O5 C, H, N: C 69.31, H 6.71, N 6.22, O 17.76; found C 69.28, H 6.67, N 6.20.

*Propyl 3-methyl-5-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate (**27**).*

Yield: 48%.

^{1}H NMR (200 MHz, $CDCl_3$): d 17.01 (bs, 1H), 12.45 (bs, 1H), 8.30 (d, 1H, *J* = 4.6 Hz), 8.22

(d, 1H, *J* = 3.8 Hz), 7.89 (s, 1H), 7.67 (t, 2H, *J* = 4 Hz), 7.34-7.24 (m, 2H), 4.34 (q, 2H, J = 6.8 Hz), 4.25 (t, 2H, *J* = 6.5 Hz), 2.47 (s, 3H), 1.80-1.68 (m, 2H), 1.59-1.32 (m, 8H), 1.10-0.80 (m, 6H).

^{13}C NMR (50 MHz, $CDCl_3$): d 172.05, 169.71, 140.12, 134.02, 131.67, 128.23, 125.77, 122.39, 121.33, 114.37, 66.38, 42.30, 31.50, 27.56, 26.59, 22.60, 22.13, 18.47, 14.01, 10.53.

ES-MS: 465 [M+H]+, 487 [M+Na]+.

M.p.: 120-122 °C.

LC/MS: RT 5.98 min; 465 [M+H]+, 487 [M+Na]+.

Purity: 99%.

Anal calcd for C27H32N2O5 C, H, N: C 69.81, H 6.94, N 6.03, O 17.22; found C 69.84, H 6.95, N 6.02.

*tert-Butyl 3-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate (**28**).* Yield:

32%.

^{1}H NMR (400 MHz, $CDCl_3$): d 16.45 (bs, 1H), 12.39 (bs, 1H), 8.80 (d, 1H, *J*= 6.8 Hz), 7.91 (d, 1H, *J* = 7.6 Hz), 7.75 *(*d, 1H *J* = 7.6 Hz), 7.66 (t, 1H, *J* = 7.6 Hz), 7.39 (t, 1H, *J* = 8 Hz), 7.30 (d, 1H, *J* = 1.2 Hz), 7.26 (dd, 2H, *J1* = 8 Hz, *J2* = 7.6 Hz,), 4.24 (t, 2H, *J* = 6.4 Hz), 1.58-1.33 (m, 6H), 1.23 (s, 9H), 0.89 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): d 172.04, 169.63, 165.33, 165.33, 162.51, 138.20, 137.31, 133.98, 132.94, 128.82, 125.76, 125.67, 125.17, 122.41,122.14, 116.30, 114.35, 97.03, 81.24, 42.52, 31.54, 30.89, 29.70, 28.22, 27.66, 26.69, 22.62, 14.02.

ES-MS: 465 [M+H]+, 487 [M+Na]+.

M.p.: 120-125 °C.

LC/MS: RT 5.72 min; 465 [M+H]+, 487 [M+Na]+.

Purity: 97%.

Anal calcd for C27H32N2O5 C, H, N: C 69.81, H 6.94, N 6.03, O 17.22; found C 69.83, H 6.97, N 6.00.

*Ethyl 3-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate (**29**)* **Yield**: 58%. **^{1}H NMR** (400 MHz, $CDCl_3$): d 16.39 (bs, 1H), 12.57 (bs, 1H), 8.28 (d, 1H, *J* = 8 Hz), 7.98 (d, 1H, *J* =8 Hz), 7.82 (d, 1H, *J* = 8 Hz), 7.68 (t, 1H, *J* = 8 Hz,), 7.42 (t, 1H, *J* = 8 Hz), 7.35-7.27 (m, 2H), 4.38 (q, 2H, *J* = 8 Hz), 4.25 (t, 2H, *J* = 8 Hz,), 1.77-1.69 (m, 4H), 1.56-1.35 (m, 7H), 0.92 (t, 3H, *J* = 8 Hz,).

^{13}C NMR (100 MHz, $CDCl_3$): d 169.65, 139.22, 134.02, 131.39, 129.02, 125.80, 125.49, 122.46, 122,16, 114.38, 97.05, 61.14, 42.56, 31.56, 27.67, 26.70, 22.62, 14.38, 14.02.

ES-MS: 437 [M+H]+, 459 [M+Na]+.

M.p.: 70-73 °C.

LC/MS: RT 5.07 min; 437 [M+H]+, 459 [M+Na]+.

Purity: 97%.

Anal calcd for C25H28N2O5 C, H, N: C 68.79, H 6.47, N 6.42, O 18.33; found C 68.82, H 6.45, N 6.41.

*Propyl 2-chloro-5-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate **(30)**.*

Yield: 46%.

^{1}H NMR (400 MHz, $CDCl_3$): d 16.21 (bs, 1H), 13.12 (bs, 1H), 8.23 (d, 1H, *J* = 8 Hz), 7.85 (t,

1H, *J* = 12 Hz), 7.67 (t, 1H, *J* = 7.64 Hz), 7.58 (d, 2H, J = 8 Hz), 7.34-7.24 (m, 2H), 4.23 (q, 4H, J = 7.2 Hz), 1.82-1.69 (m, 2H), 1.58-1.30 (m, 6H), 1.03 (t, 3H, J = 6.8 Hz,), 0.89 (t, 3H, J = 7.6 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): d 172.10, 162.47, 141.10, 139.22 134.93, 134.27, 132.50, 125.83, 125.34,122.72, 118.41, 114.42, 97.00, 67.00, 42.63, 31.53, 27.63, 26.68, 22.61, 22.05, 14.02, 10.62. ES-MS:485 [M+H]+, 507 [M+Na]+.

M.p.: 110-113 °C.

LC/MS: RT 4.38 min; 485 [M+H]+, 507 [M+Na]+.

Purity: 99%.

Anal calcd for C26H29ClN2O5 C, H, N: C 64.39, H 6.03, Cl 7.31, N 5.78, O 16.50; found C 64.38, H

6.05, N 5.75.

Propyl 3-trifluoromethyl-4-(1-hexyl-4-hydroxy-2-oxo-1,2dihydroquinolin-3-carboxamido)-benzoate

***(31)*. Yield**: 56%.

^{1}H NMR ($CDCl_3$, 200 MHz): d 16.21 (bs, 1H), 12.31 (bs, 1H), 8.47 (s, Hz, 1H), 8.46 (s, 1H), 8.39 (d, 1H, *J*= 4.4 Hz), 8.33 (s, 1H), 8.20 (d, 1H, *J* = 4.4 Hz), 7.68 (t, 1H, *J* = 4.6 Hz), 7.34-724 (m, 1H), 4.39-4.25 (m, 4H), 1.82-1.87 (m, 2H), 1.46-1.18 (m, 2H), 1.05-1.0 (m, 6H), 0.90-0.87 (m, 3H), 0.86-0.83 (m, 3H).

^{13}C NMR ($CDCl_3$, 50 MHz): d 172.09, 170.31, 165.09, 162.40, 139.46, 138.67, 134.30, 133.56, 127.86, 127.76, 126.57, 125.82, 125.28, 124.87, 122.44, 115.98, 114.45, 97.05,

83.97, 82.97, 81.95, 81.03, 67.72, 67.28, 66.95, 42.25, 31.49, 31.49, 29.68, 27.52, 26.54, 22.58, 22.07, 13.98, 10.46, 10.41.

ES-MS: 519 [M+H]+.

M.p.: 85-89 °C.

LC/MS: RT 4.23 min; 519 [M+H]+.

Purity: 98%.

Anal calcd for C27H29F3N2O5 C, H, N: C 62.54, H 5.64, F 10.99, N 5.40, O 15.43; found C

62.50, H 5.65, N 5.37.

*4-(1-Hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamido)benzoic acid.(**15**)*

To a solution of compound **14** (46 mg, 0.1 mmol) in CH_2Cl_2 dry (10 mL), trifluoroacetic acid (TFA, 114 μL, 1.5 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h. Pure **31** (37 mg, 92%) was obtained after evaporation under reduced pressure. An analytical sample was purified by flash chromatography (eluent EtOAc).

^{1}H NMR: (400 MHz, CD_3OD): δ 16.37 (bs, 1H), 15.82 (bs, 1H), 8.24 (d, 1H, *J* = 8.0 Hz), 7.87 (d, 2H, *J* = 8.4 Hz), 7.77 (t, 1H, *J* = 8.4 Hz), 7.44-7.24 (m, 2H), 6.52 (d, 2H, *J* = 8.8 Hz), 4.27 (t, 2H = 7.6 Hz), 2.85-2.80 (m, 2H), 1.49-1.23 (m, 8H), 0.89 (t, 3H, *J* = 6.8 Hz).

^{13}C NMR: (100 MHz, CD_3OD): δ 197.21, 173.05, 170.67, 169.23, 139.13, 134.58, 130.92, 125.99,

123.06, 72.53, 42.302, 30.85, 29.71, 26.11, 19.13, 13.49.

ES-MS: 407 [M-H]-.

LC/MS: RT 3.98 min; 407 [M-H]-.

Purity: 98%.

Anal calcd for (C23H24N2O5) C, 67.63; H, 5.92; N, 6.86; O, 19.58; found C 67.58, H 5.90, N 6.87.

3.2 HH ANTAGONISTS

*(E)-4-styrylbenzoic acid. **(45)*** **Yield**: 87%

To a solution of p-iodobenzoic acid **44** (1g, 4.03mmol) in DMF/Et_3N 7ml/7ml, styrene (700µL, 6.06mmol), PPh_3 (34mg, 0.13mmol) and $Pd(OAc)_2$ were added, and the mixture was refluxed and stirred under N_2 atmosphere for 12 h. The mixture was acidified (pH: 1-2) with HCl 1N and the resulting yellow precipitate was extracted with EtOAc (10ml) and washed with Brine (10ml). The organic layer was dried over Na_2SO_4, and after the evaporation of the solvent the desired compound was obtained as a yellow solid, with 87% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (dd, *J* = 18.1, 8.9 Hz, 2H), 7.55 (dd, *J* = 21.4, 7.7 Hz, 2H), 7.47 – 7.24 (m, 6H), 7.23 – 7.06 (m, 1H).

Methyl (E)-4-styrylbenzoate. **Yield**: 75%

To a solution of compound **45** (789mg, 3.52mmol) in CH_3OH (26ml), AcCl (2.1ml, 28.21mmol) was added. The mixture refluxed and stirred for 12 h, then cooled down to room temperature and diluted with CH_2Cl_2 (10ml). The solvent was evaporated under reduced pressure and the crude mixture is purified on silica gel (Büchi) with PE/EtOAc 3:7. The desired product was obtained with 75% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.4 Hz, 2H), 7.52 (dd, *J* = 12.7, 8.0 Hz, 4H), 7.35 (t, *J* = 7.5 Hz, 2H), 7.28 (d, *J* = 7.3 Hz, 1H), 7.17 (s, 1H), 7.10 (d, *J* = 16.4 Hz, 1H), 3.89 (d, *J* = 3.6 Hz, 3H).

Methyl 4-phenethylbenzoate. ***(46)*** **Yield**: >99%

To a solution of the methyl (E)-4-styrylbenzoate (230mg, 0.96mmol) in CH_3OH (20ml) under N_2 atmosphere, Pd/C 10% (51mg, 0.048mmol) was added and the mixture was stirred at room temperature under H_2 atmosphere for 12 h and monitored by TLC until the complete conversion. After the filtration on celite washing with CH_3OH, the solvent was evaporated and the crude product was purified by flash chromatography with PE/EtOAc 9:1. The desired compound was obtained in quantitative yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.2 Hz, 2H), 7.27 (t, *J* = 7.4 Hz, 2H), 7.21 (dd, *J* = 4.7, 3.4 Hz, 3H), 7.20 – 7.12 (m, 2H), 3.89 (s, 3H), 2.95 (dd, *J* = 8.7, 4.0 Hz, 4H).

*4-phenethylbenzoic acid. **(47)*** **Yield**: >99%

To a solution of the ester **46** (340mg, 1.41mmol) in CH_3OH (9ml), a solution of NaOH 5M (1.75ml) was added, and the mixture was refluxed and stirred for 12 h. After cooling down to room temperature, the solvent was evaporated and the resulting white solid is diluted with H_2O (10ml) and extracted with PE (10ml). The acqueous layer was acidified with HCl 1N (pH: 2) to give the precipitation of the product as a white solid which was filtered on Büchner washing with iced water, and dried under vacuum.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.0 Hz, 2H), 7.20 (ddd, *J* = 28.1, 16.1, 7.3 Hz, 7H), 3.13 – 2.84 (m, 4H).

4-phenethylbenzoyl chloride. ***(48)*** **Yield**: >99%

$SOCl_2$ (3.65ml, 1.46mmol) was added slowly and in portion to the acid **47** (333mg, 1.46mmol) under N_2 atmosphere, and the resulting solution was refluxed and stirred for 3 h and monitored by ES-MS of the corresponding methyl ester. $SOCl_2$ was evaporated under reduced pressure in the presence of Na_2CO_3 and the desired product was obtained as crude in quantitative yield.

ES-MS: 263 [M+Na]+.

*General procedure for the synthesis of the nitro adducts **37a-c**.*

To a solution of the 2-methyl-5-nitroaniline **35** previously crystallized, (0.4mmol) in CH_2Cl_2 dry (5ml) under N_2 atmosphere and cooled down to 0 °C, the opportune chloride **36a-c** and Py (0.8mmol) were added (0.48mmol), and the mixture was stirred at room temperature for 12 h, then diluted with CH_2Cl_2 (3ml) and washed with HCl 1N (3ml) and $NaHCO_{3ss}$ (3ml). The organic layer was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure to give the desired compounds **37a-c** as crude products with 65% to quantitative yields.

*N-(2-methyl-5-nitrophenyl)-[1,1'-biphenyl]-4-carboxamide (**37a**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 – 7.84 (m, 4H), 7.70 (d, *J* = 8.1 Hz, 2H), 7.61 (d, *J* = 7.8 Hz, 2H), 7.46 (t, *J* = 7.6 Hz, 2H), 7.38 (dd, *J* = 21.0, 7.6 Hz, 2H), 2.43 (s, 3H).

*(E)-N-(2-methyl-5-nitrophenyl)-4-styrylbenzamide (**37b**)* **Yield**: 64%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 – 7.82 (m, 4H), 7.62 – 7.53 (m, 2H), 7.51 – 7.42 (m, 3H), 7.32 (d, *J* = 8.3 Hz, 3H), 7.21 (d, *J* = 7.1 Hz, 2H), 2.39 (s, 3H).

*N-(2-methyl-5-nitrophenyl)-4-phenethylbenzamide. **(37c)*** **Yield**: >99%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (t, *J* = 8.3 Hz, 1H), 7.88 – 7.68 (m, 1H), 7.35 (d, *J* = 8.3 Hz, 1H), 7.33 – 7.26 (m, 1H), 7.27 – 7.09 (m, 2H), 2.97 (dd, *J* = 17.8, 7.4 Hz, 1H), 2.41 (s, 1H).

*General procedure for the reduction of the nitro adducts **37a-c**.*

To a solution of the nitro derivatives **36a-c** (0.25mmol) in CH_3OH (10ml) under N_2 atmosphere, Pd/C (0.012mmol) was added, and the mixture was stirred at room temperature, under H_2 atmosphere for 12 h. After filtration on celite, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (Büchi) with PE/EtOAc 1:1. The desired compounds **38a-c** were obtained with 40% to 50% yield.

*N-(5-amino-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**38a**)* **Yield**: 44%

^{1}H NMR (400 MHz, MeOD) δ 8.02 (dd, *J* = 20.4, 8.1 Hz, 1H), 7.73 (d, *J* = 8.1 Hz, 1H), 7.66 (d, *J* = 7.3 Hz, 1H), 7.44 (t, *J* = 7.3 Hz, 1H), 7.36 (t, *J* = 6.9 Hz, 1H), 6.99 (d, *J* = 8.1 Hz, 1H), 6.78 (s, 1H), 6.59 (d, *J* = 8.1 Hz, 1H), 2.15 (s, 1H).

*(E)-N-(5-amino-2-methylphenyl)-4-styrylbenzamide (**38b**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 7.8 Hz, 2H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.49 (dt, *J* = 25.3, 7.3 Hz, 4H), 7.28 – 7.20 (m, 2H), 7.15 (dd, *J* = 17.3, 7.1 Hz, 1H), 6.95 (d, *J* = 8.0 Hz, 1H), 6.47 – 6.35 (m, 1H), 6.04 (d, *J* = 12.8 Hz, 1H), 3.53 (d, *J* = 13.3 Hz, 1H), 2.18 (d, *J* = 7.6 Hz, 3H).

*General procedure for the preparation of the chloride salt of the anilines **39a-c**.*

To a solution of the anilines **38a-c** (0.11mmol) in CH_3OH under N_2 atmosphere, HCl in CH_3OH was added (0.25mmol) and the reaction mixture was stirred at room temperature for 12 h and monitored by TLC until the complete conversion. After the evaporation of the solvent under reduced pressure, the desired salts were obtained in quantitative yield as solids.

*N-(5-amino-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide hydrochloride (**39a**)* **Yield:** 98%

^{1}H NMR (400 MHz, MeOD) δ 8.03 (d, *J* = 8.0 Hz, 2H), 7.76 (d, *J* = 8.1 Hz, 2H), 7.70 – 7.64 (m, 2H), 7.50 – 7.40 (m, 4H), 7.40 – 7.30 (m, 1H), 7.19 (d, *J* = 8.9 Hz, 1H), 2.35 (s, 3H).

*(E)-N-(5-amino-2-methylphenyl)-4-styrylbenzamide hydrochloride (**39b**)* **Yield**: >99%

^{1}H NMR (400 MHz, MeOD) δ 7.94 (d, *J* = 7.5 Hz, 2H), 7.84 (d, *J* = 7.9 Hz, 1H), 7.55 (d, *J* = 5.9 Hz, 2H), 7.49 (t, *J* = 7.4 Hz, 2H), 7.44 – 7.33 (m, 2H), 7.27 (d, *J* = 7.9 Hz, 1H), 7.19 (dd, *J* = 11.3, 6.9 Hz, 2H), 7.11 (d, *J* = 7.5 Hz, 1H), 2.93 (dd, *J* = 16.3, 6.6 Hz, 1H), 2.31 (d, *J* = 4.4 Hz, 3H).

*N-(5-amino-2-methylphenyl)-4-phenethylbenzamide hydrochloride (**39c**)* FP20

ES-MS: 331 [M+H]+

*Synthesis of the acids **51a-c** for the preparation of the EPMF derivatives.*

General procedure for the di-benzyl protection.

To a suspension of the opportune benzoic acid **49a-c** (1.01mmol) in Acetone dry, K_2CO_3 (10.1mmol) was added under N_2 atmosphere and the mixture was stirred at room temperature for 20 min. after the addition of BnBr (4.03mmol) the resulting mixture was stirred for 7 h, then filtered to remove K_2CO_3 washing with acetone. The solvent was evaporated and the crude product was purified on silica gel (Büchi) with PE/EtOAc 4:1 to give the desired compounds with 90% yield.

*Benzyl 4-(benzyloxy)-3-methoxybenzoate (**50a**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.58 (d, *J* = 1.8 Hz, 1H), 7.43 – 7.39 (m, 4H), 7.34 (ddd, *J* = 16.7, 8.5, 4.1 Hz, 6H), 6.87 (d, *J* = 8.4 Hz, 1H), 5.32 (s, 2H), 5.19 (s, 2H), 3.91 (s, 3H).

*Benzyl 4-(benzyloxy)-3,5-dimethoxybenzoate (**50b**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.41 (m, 3H), 7.40 – 7.32 (m, 3H), 7.30 – 7.21 (m, 5H), 5.34 (s, 2H), 5.07 (s, 2H), 3.84 (s, 6H).

*Benzyl 3-(benzyloxy)-4,5-dimethoxybenzoate (**50c**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 – 7.21 (m, 12H), 5.29 (s, 2H), 5.09 (s, 2H), 3.87 (d, *J* = 0.5 Hz, 3H), 3.83 (s, 3H).

General procedure for the deprotection.

Compounds **50a-c** (0.91mmol) were solubilized in CH_3OH (5ml) and a solution of NaOH 5N (1.13ml) was added. The mixture was refluxed for 5 h, then cooled to room temperature and after evaporation of the solvent, the resulting oil is diluted with H_2O and extracted with PE (2x10ml). The aqueous layer was acidified (pH: 2) with HCl 1N to give the desired acids **51a-c** as white solids, filtered on büchner and washed with H_2O (65% of yield).

*4-(benzyloxy)-3-methoxybenzoic acid (**51a**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.59 (d, *J* = 1.7 Hz, 1H), 7.34 (ddd, *J* = 48.1, 25.4, 15.4 Hz, 4H), 6.90 (d, *J* = 8.5 Hz, 1H), 5.20 (s, 2H), 3.92 (s, 3H).

*4-(benzyloxy)-3,5-dimethoxybenzoic acid (**51b**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 6.9 Hz, 2H), 7.38 – 7.18 (m, 5H), 5.09 (s, 2H), 3.86 (s, 6H).

*3-(benzyloxy)-4,5-dimethoxybenzoic acid (**51c**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (dd, *J* = 9.2, 4.4 Hz, 3H), 7.36 – 7.29 (m, 3H), 7.29 – 7.24 (m, 1H), 5.11 (s, 2H), 3.88 (s, 3H), 3.86 (s, 3H).

*Synthesis of the cyanamide **34** and **53a-c** for the preparation of the MRT and the EPMF derivatives.*

Chloride derivatives:

The 3,4,5-trimethoxy benzoic acid or the opportune acid **51a-c** (4.35mmol) was mixed with $SOCl_2$ under N_2 atmosphere and the resulting solution was stirred at room temperature for 12 h, and monitored by ES-MS. $SOCl_2$ was removed under reduced pressure in the presence of NaOH or Na_2CO_3 and the desired product was obtained as a white solid in quantitative yield.

*3,4,5-trimethoxybenzoyl chloride (**33**)* **Yield**: >99%

ES-MS: (corresponding methyl ester) 245 [M+H]+, 267 [M+Na]+.

*3-(benzyloxy)-4,5-dimethoxybenzoyl chloride (**52c**)* **Yield**: >99%
ES-MS: (corresponding methyl ester) 303 [M+H]+, 325 [M+Na]+.

Cyanamide synthesis.
To a suspension of the chlorides **33** and **52a-c** (4.35mmol) in Et_2O (10ml), a solution of NH_2CN (4.35mmol) in NaOH 10% was added, and the mixture is stirred at room temperature for 12 h. Then, the mixture was acidified (pH: 2) with HCl 1N to give the desired product as a white precipitate, which was finally filtered on Büchner and washed with water (63% yield).

*N-cyano-3,4,5-trimethoxybenzamide (**34**)*
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (s, 2H), 3.85 (s, 9H).

*4-(benzyloxy)-N-cyano-3-methoxybenzamide (**53a**)*
^{1}H NMR (400 MHz, MeOD) δ 7.50 – 7.38 (m, 3H), 7.37 – 7.23 (m, 3H), 7.06 (d, *J* = 8.2 Hz, 1H), 5.15 (s, 2H), 3.86 (s, 3H).

*4-(benzyloxy)-N-cyano-3,5-dimethoxybenzamide (**53b**)*
^{1}H NMR (400 MHz, MeOD) δ 7.39 (d, *J* = 7.3 Hz, 2H), 7.32 – 7.22 (m, 3H), 7.17 (d, *J* = 5.0 Hz, 2H), 5.01 (d, *J* = 4.7 Hz, 2H), 3.83 (d, *J* = 5.1 Hz, 6H).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J* = 6.5 Hz, 2H), 7.36 – 7.16 (m, 4H), 7.04 (s, 1H), 5.07 (s, 2H), 3.84 (s, 6H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 153.60, 141.66, 136.83, 128.47, 128.30, 128.27, 124.61, 105.65, 76.71, 75.22, 56.37.

*3-(benzyloxy)-N-cyano-4,5-dimethoxybenzamide (**53c**)*
^{1}H NMR (400 MHz, MeOD) δ 7.38 (d, *J* = 7.4 Hz, 2H), 7.33 (s, 1H), 7.26 (dt, *J* = 15.7, 7.3 Hz, 4H), 5.03 (s, 2H), 3.78 (s, 3H), 3.74 (s, 3H).

General procedure for the synthesis of the acylguanidine core of the MRT and the EPMF compounds.

A stirred suspension of the cyanamides **34** and **53a-c** (1eq) and the anilines **39a-c** (1.1eq) in toluene (2-3ml) was refluxed and toluene was added until the complete solubilisation of the two starting materials. The resulting solution was stirred for 3 h to 24 or 48 h, depending on the substrates, and motitored by TLC and ES-MS. Once cooled to room temperature, the solvent was evaporated and the crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 9:1 to give the desired products with 25-30% yield.

*N-(5-(3-(4-(benzyloxy)-3-methoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**54**)*

^{1}H NMR (400 MHz, MeOD) δ 8.05 (t, *J* = 7.8 Hz, 2H), 7.77 (d, *J* = 8.3 Hz, 2H), 7.66 (t, *J* = 8.6 Hz, 4H), 7.55 (s, 1H), 7.49 (d, *J* = 11.6 Hz, 2H), 7.43 (d, *J* = 7.3 Hz, 2H), 7.36 (dd, *J* = 16.4, 7.9 Hz, 2H), 7.28 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 8.2 Hz, 1H), 5.20 (s, 2H), 3.92 (s, 3H), 2.37 (s, 3H).

*N-(5-(3-(4-(benzyloxy)-3,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**55**)*

^{1}H NMR (400 MHz, MeOD) δ 8.06 (d, *J* = 8.1 Hz, 2H), 7.78 (d, *J* = 8.1 Hz, 2H), 7.68 (d, *J* = 7.9 Hz, 3H), 7.45 (ddd, *J* = 29.9, 20.6, 12.9 Hz, 9H), 7.29 (d, *J* = 7.6 Hz, 3H), 5.07 (s, 2H), 3.89 (s, 6H), 2.38 (s, 3H).

*N-(5-(3-(3-(benzyloxy)-4,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**56**)*

^{1}H NMR (400 MHz, MeOD) δ 8.05 (d, *J* = 7.9 Hz, 2H), 7.77 (d, *J* = 7.9 Hz, 2H), 7.67 (d, *J* = 7.0 Hz, 3H), 7.47 (t, *J* = 14.1 Hz, 8H), 7.41 – 7.24 (m, 5H), 5.18 (s, 2H), 3.91 (s, 3H), 3.84 (s, 3H), 2.36 (s, 3H).

*General procedure for the debenzylation of the acylguanidines **54-56**.*

To a suspension of the opportune acylguanidine (0.27mmol) in CH_3OH under N_2 atmosphere, Pd/C was added (0.05eq to 0.6eq depending on the substrate) and the resulting mixture was stirred at room temperature under H_2 atmosphere for 12 h. After filtration on celite washing with CH_3OH, the crude product was purified by flash chromatography (CH_2Cl_2/CH_3OH 9.9:0.1) and the desired products were obtained with 52% to 87% yield.

*N-(5-(3-(4-hydroxy-3-methoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**57**)* **Yield**: 52%

^{1}H NMR (400 MHz, MeOD) δ 7.99 (d, *J* = 8.1 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.66 – 7.54 (m, 1H), 7.47 (s, 1H), 7.39 (t, *J* = 7.4 Hz, 1H), 7.30 (dd, *J* = 18.4, 7.6 Hz, 1H), 7.17 (d, *J* = 8.0 Hz, 1H), 6.74 (d, *J* = 8.2 Hz, 1H), 3.79 (s, 1H), 2.25 (s, 1H).

*N-(5-(3-(4-hydroxy-3,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**58**)* **Yield**: 52%

^{1}H NMR (400 MHz, MeOD) δ 7.97 (d, *J* = 8.2 Hz, 2H), 7.68 (d, *J* = 8.2 Hz, 2H), 7.60 (d, *J* = 7.6 Hz, 2H), 7.53 – 7.36 (m, 5H), 7.31 (t, *J* = 7.2 Hz, 1H), 7.21 (dd, *J* = 21.6, 7.9 Hz, 2H), 3.76 (s, 6H), 2.24 (s, 3H).

*N-(5-(3-(3-hydroxy-4,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide (**59**)* **Yield**: 87%

^{1}H NMR (400 MHz, MeOD) δ 8.03 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 8.1 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.51 (s, 1H), 7.43 (t, *J* = 7.5 Hz, 2H), 7.38 – 7.26 (m, 3H), 7.26 – 7.16 (m, 2H), 3.78 (d, *J* = 6.5 Hz, 6H), 3.27 (s, 3H), 2.28 (s, 3H).

General procedure for the hydrochloride salts formation.

To a solution of the proper acylguanidine (60mg, 0.11mmol) in CH_3OH under N_2 atmosphere, HCl in CH_3OH (181µL, 0.25mmol) was added, and the mixture was stirred at room temperature for 12 h. After the evaporation of the solvent, the desired salts were obtained as crude products in almost quantitative yields (90-99%).

N-(5-(3-(4-hydroxy-3-methoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide hydrochloride **EPMF08 Yield**: 90%

^{1}H NMR (400 MHz, MeOD) δ 8.86 (d, *J* = 5.6 Hz, 3H), 8.66 (t, *J* = 7.8 Hz, 2H), 8.16 – 8.03 (m, 5H), 7.77 (d, *J* = 8.2 Hz, 2H), 7.65 (dd, *J* = 16.6, 8.1 Hz, 4H), 7.46 (dd, *J* = 18.8, 8.2 Hz, 4H), 7.40 – 7.34 (m, 1H), 7.28 (d, *J* = 8.2 Hz, 1H), 6.92 (d, *J* = 8.2 Hz, 1H), 3.93 (s, 3H), 2.37 (s, 3H).

N-(5-(3-(4-hydroxy-3,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide hydrochloride **EPMF09 Yield**: >90%

^{1}H NMR (400 MHz, MeOD) δ 8.06 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H), 7.54 – 7.26 (m, 7H), 7.28 (d, *J* = 8.1 Hz, 2H), 3.92 (s, 6H), 2.37 (s, 3H).

^{13}C NMR (400 MHz, MeOD) δ 147.89, 144.89, 139.66, 137.23, 132.30, 128.69, 128.07, 126.81, 126.77, 106.11, 55.87, 16.55.

N-(5-(3-(3-hydroxy-4,5-dimethoxybenzoyl)guanidino)-2-methylphenyl)-[1,1'-biphenyl]-4-carboxamide hydrochloride **EPMF10 Yield:** 99%

^{1}H NMR (400 MHz, MeOD) δ 8.07 (d, *J* = 8.0 Hz, 2H), 7.78 (d, *J* = 8.0 Hz, 2H), 7.68 (d, *J* = 7.6 Hz, 2H), 7.47 (dd, *J* = 16.6, 8.5 Hz, 5H), 7.41 – 7.32 (m, 1H), 7.33 – 7.16 (m, 3H), 3.93 (s, 3H), 3.86 (s, 3H), 2.38 (s, 3H).

3.3 MRN INHIBITORS

*3-isobutyl-2-thioxothiazolidin-4-one (**69**)*

To a stirred suspension of bis-carboxymethyl trithiocarbonate **68** (2g, 8.84mmol) in DME (12ml), TEA (883µL, 8.84mmol) and isobutylamine (1.84ml, 13.2mmol) were added, and the mixture was heated under MW irradiation at 90 °C for 30 min in a capped tube. The solvent was evaporated and the crude product was purified on silica gel (Büchi) PE/EtOAc 1:1 to give the desired compound with 79% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (s, 2H), 3.59 (d, *J* = 7.5 Hz, 2H), 2.03 (dt, *J* = 13.7, 6.9 Hz, 1H), 0.70 (d, *J* = 6.8 Hz, 6H).

*3-(sec-butyl)-2-thioxothiazolidin-4-one (**70**)*

To a stirred suspension of *sec*-butylamine (145µL, 1.7mmol) in H_2O (2ml) NaOH (149mg, 3.74mmol) and CS_2 (112µL, 1.87mmol) were added, and the mixture was heated under MW irradiation at 100 °C for 5 min. After cooling down to 40 °C, chloroacetic acid (177mg, 1.87mmol) was added and a second cycle under microwaves is completed. Again, the mixture was cooled down to 40 °C, HCl (3ml for 10mmol) is added, and the resulting mixture was irradiated under microwaves at 120 °C for 30 min. The solution was extracted with EtOAc and the organic layer was dried over Na_2SO_4, filtered and after the solvent evaporation, the crude product was purified on silica gel (Büchi) PE/EtOAc 2:1 and the desired compound was obtained in 48% of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.91 (dd, *J* = 13.8, 6.8 Hz, 1H), 3.76 (s, 2H), 2.08 – 1.93 (m, 1H), 1.80 – 1.66 (m, 1H), 1.34 (d, *J* = 7.0 Hz, 3H), 0.77 (t, *J* = 7.5 Hz, 3H).

Tert-butyl (4-(hydroxymethyl)phenyl)carbamate. *(**65**)*

The *p*-aminobenzyl alcohol (PABA, 500mg, 3.64mmol) was solubilized in THF dry under N_2 atmosphere, DIPEA (633µL, 3.64mmol) and boc-anhydride (795mg, 3.64mmol) were added and the resulting mixture was refluxed and stirred for 12 h. After cooling down, the solvent was evaporated and the residue was diluted with EtOAc and washed with HCl 0.1 N (2x10ml). The organic layers were collected, dried on Na_2SO_4, filtered and evaporated under

reduced pressure. The crude product was purified on silica gel (Büchi) PE/EtOAc 1:1 and the desired compound was obtained in 63% of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, *J* = 6.9 Hz, 2H), 7.25 – 7.18 (m, 2H), 6.45 (s, 1H), 4.55 (d, *J* = 2.1 Hz, 2H), 1.45 (s, 9H).

*Tert-butyl (4-formylphenyl)carbamate (**66**)*

To a solution of compound **65** in CH_2Cl_2 dry under N_2 atmosphere, MnO_2 was added and the mixture was stirred at room temperature for 12 h, then filtered on celite washing with CH_2Cl_2 and the solvent was evaporated. The desired product was obtained pure as crude with 43% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (s, 1H), 7.79 – 7.68 (m, 2H), 7.47 (d, *J* = 7.8 Hz, 2H), 6.84 (s, 1H), 1.45 (d, *J* = 1.1 Hz, 9H).

General procedure for the Knoevenagel reaction for the rodanine derivatives (PFM01 and PFM03)

To a solution of the opportune rhodanine (**69**-**70**) (0.53mmol) in DME (3ml) and TEA (0.53mmol) is added, followed by the *p*-OH-benzaldehyde (0.53mmol). The mixture was heated at 110 °C for 40min, under microwaves irradiation in open vessel. The solvent was evaporated and the crude product was purified by flash chromatography eluting with PE/EtOAc 7:3 or PE/Toluene/EtOAc 1:1:1 depending on the substrate, and furnished the desired compounds PFM01 and PFM03 with good yields.

*(Z)-5-(4-hydroxybenzylidene)-3-isobutyl-2-thioxothiazolidin-4-one **PFM01*** **Yield:** 90%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (s, 1H), 7.34 (d, *J* = 8.5 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 5.98 (s, 1H), 3.90 (d, *J* = 7.5 Hz, 2H), 2.27 (dt, *J* = 13.7, 6.8 Hz, 1H), 0.88 (d, *J* = 6.7 Hz, 8H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.00, 116.55, 26.83, 25.09, 20.07.

*(Z)-3-(sec-butyl)-5-(4-hydroxybenzylidene)-2-thioxothiazolidin-4-one **PFM03*** **Yield**:73%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 8.3 Hz, 2H), 6.92 (d, *J* = 8.4 Hz, 2H), 5.15 (s, 1H), 2.17 (s, 3H), 1.84 (td, *J* = 14.2, 7.1 Hz, 1H), 1.49 (d, *J* = 6.8 Hz, 2H), 0.86 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (50 MHz, CDCl3, 323 K, δ ppm, J Hz) 168.6; 158.1; 133.1; 126.2; 119.9; 116.5; 114.7; 52.3; 26.8; 20.0; 15.9. ES/MS: m/z 292 [M-1]- (SI) *Mol Cell*. **2014**; *53*(1): 7–18.

General procedure for the Knoevenagel reaction for the pseudothiohydantoin derivatives (Mirin and PFM39)

To a stirred suspension of the opportune aldehyde (12.93mmol) and the 2-iminothiazolidin-4-one **60** (8.82mmol) in glacial AcOH (10ml), AcONa (34.5mmol) was added and the mixture was refluxed for 4 h or 12 h depending on the substrates. The reaction was monitored by TLC and ES-MS. The product was obtained as a yellow-orange precipitate which was filtered on Büchner washing with PE, H_2O and PE again to give the pure final compound in discrete yields.

*(Z)-5-(4-hydroxybenzylidene)-2-iminothiazolidin-4-one (**Mirin**)* **Yield**:68%

^{1}H NMR (400 MHz, DMSO) δ 7.42 (s, 1H), 7.33 (d, *J* = 8.4 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H).

ES-MS: 219 [M-H]-

Tert-butyl (Z)-(4-((2-imino-4-oxothiazolidin-5-ylidene)methyl)phenyl)carbamate. ***(67)*** **Yield:** 82%

^{1}H NMR (400 MHz, DMSO) δ 7.51 (d, *J* = 8.6 Hz, 2H), 7.47 – 7.35 (m, 3H), 3.22 (s, 1H), 1.40 (s, 9H).

ES-MS: 320 [M+H]+

*(Z)-5-(4-aminobenzylidene)-2-iminothiazolidin-4-one 2,2,2-trifluoroacetate (**PFM39**)* **Yield**: >99%

^{1}H NMR (400 MHz, DMSO) δ 7.41 (s, 1H), 7.25 (d, *J* = 8.0 Hz, 2H), 6.71 (d, *J* = 7.9 Hz, 2H).

ES-MS: 220 [M+H]+

3.4 LINKERS FOR BIOCONJUGATION

Kad-type1

*(1r,4r)-4-(((E)-3-carboxyacrylamido)methyl)cyclohexane-1-carboxylic acid (**75**)*

To a suspension of the trans-4-(aminomethyl)cyclohexane carboxylic acid (1g, 6.37mmol) in glacial AcOH under N_2 atmosphere, a solution of maleic anhydride (624mg, 6.37mmol) in AcOH was added. The mixture was stirred at room temperature for 4 h, with the formation of a white precipitate, which was filtered on büchner to give the desired product with 89% yield. *Synthetic Communications*, **2008**, *38*: 303–308.

^{1}H NMR (400 MHz, MeOD) δ 6.19 – 5.95 (m, 2H), 2.98 (t, *J* = 9.9 Hz, 2H), 2.07 (ddd, *J* = 12.2, 7.9, 3.5 Hz, 1H), 1.85 (d, *J* = 13.4 Hz, 2H), 1.71 (d, *J* = 13.2 Hz, 2H), 1.47 – 1.34 (m, 1H), 1.26 (ddd, *J* = 25.5, 13.0, 3.2 Hz, 2H), 0.99 – 0.78 (m, 2H).

*4-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)cyclohexane-1-carboxylic acid (**72**)*

To a solution of **75** (300mg, 1.17mmol) in DMA (10ml), acetic anhydride (Ac_2O) (223µL, 2.35mmol) and AcONa (24mg, 0.29mmol) were added, and the mixture was heated to 100 °C, stirred for 2 h and monitored by TLC until the complete conversion. Iced water (50ml) was added to promote the precipitation of the product and system put in an ice-bath for 4-5 h. The resulting precipitate was filtered on büchner and the acqueous layer was extracted with CH_2Cl_2 to give the desired product with 88% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (s, 1H), 3.34 (d, *J* = 6.9 Hz, 2H), 2.22 (ddd, *J* = 12.2, 7.8, 3.4 Hz, 1H), 1.99 (d, *J* = 13.4 Hz, 2H), 1.79 – 1.59 (m, 3H), 1.35 (qd, *J* = 13.1, 3.0 Hz, 2H), 1.00 (dd, *J* = 17.5, 7.8 Hz, 2H).
^{13}C NMR (400 MHz, $CDCl_3$) δ 180.87, 170.60, 133.55, 76.90, 76.58, 76.26, 43.13, 42.28, 35.85, 29.14, 27.56.

*4-((3-((2-((tert-butoxycarbonyl)amino)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylic acid. (**77**)*

A solution of compound **72** (1.23g, 5.2mmol) and the 2-(bocamino) ethanethiol **73** (1.75ml, 10.4mmol) in CH_3CN (9ml) under N_2 atmosphere was stirred at room temperature. After 12

h, the solvent was evaporated and the crude product was purified on silica gel (Büchi) with PE/EtOAc 1:1 to give the desired product with 63% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.27 (s, 1H), 3.77 (s, 1H), 3.35 (d, *J* = 6.5 Hz, 4H), 3.12 (dd, *J* = 15.9, 7.5 Hz, 2H), 2.79 (dd, *J* = 13.4, 6.6 Hz, 1H), 2.48 (d, *J* = 18.7 Hz, 1H), 2.32 – 2.11 (m, 2H), 2.01 (d, *J* = 11.6 Hz, 3H), 1.71 (d, *J* = 10.8 Hz, 4H), 1.42 (s, 9H), 1.00 (d, *J* = 12.4 Hz, 2H).

*4-((3-((2-aminoethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylic acid (triflate) (**71**)*

To a solution of compound **77** (50mg, 0.12mmol) in CH_2Cl_2 (3ml), TFA was added (96.2µL, 1.2mmol) and the solution was stirred at room temperature for 12 h. Then the solvent was evaporated under reduced pressure in the presence of NaOH to remove the TFA. The desired product is obtained in quantitative yield (>99%).

^{1}H NMR (400 MHz, MeOD) δ 3.98 (dd, *J* = 9.1, 4.0 Hz, 1H), 3.40 – 3.10 (m, 7H), 3.08 – 2.85 (m, 1H), 2.51 (dd, *J* = 18.6, 4.0 Hz, 1H), 2.29 – 2.05 (m, 1H), 1.97 (d, *J* = 12.5 Hz, 2H), 1.79 – 1.49 (m, 3H), 1.43 – 1.14 (m, 2H), 1.02 (dd, *J* = 23.4, 11.0 Hz, 2H).

<u>Kad-type2</u>

*Benzyl 4-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)cyclohexane-1-carboxylate. (**103**)*

Compound **72** (5g, 21.1mmol) is solubilized in DMF dry (15ml) under N_2 atmosphere. BnBr (3ml, 25.31mmol) and DIPEA (4.43ml, 25.31mmol) are added and the mixture is stirred at 25 °C for 12 h, then diluted with Et_2O (100ml), washed with HCl 1N (50ml) and the organic layer is dried on Na_2SO_4. The solvent is evaporated and the crude product is purified on silica gel (Büchi) with PE/EtOAc 1:1 to give the pure compound as a white solid in 50% of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.25 (m, 5H), 6.66 (s, 2H), 5.07 (s, 2H), 3.34 (d, *J* = 6.9 Hz, 2H), 2.25 (tt, *J* = 12.2, 3.6 Hz, 1H), 2.05 – 1.95 (m, 2H), 1.67 (ddd, *J* = 11.5, 9.9, 4.5 Hz, 3H), 1.45 – 1.32 (m, 2H), 1.05 – 0.90 (m, 2H).

*Benzyl 4-((3-((2-hydroxyethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylate (**104**)*

A solution of compound **103** (1.91g, 5.84mmol) and the 2-mercaptoethan-1-ol (820µL, 11.7mmol) in CH_3CN (25ml) under N_2 atmosphere was stirred at room temperature. After 12 h the solvent was evaporated and the crude product was purified on silica gel (Büchi) with PE/EtOAc 3:7 to give the desired product with 55% of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.22 (m, 5H), 5.04 (s, 2H), 3.82 (ddd, *J* = 7.6, 3.9, 1.9 Hz, 4H), 3.31 (d, *J* = 6.7 Hz, 2H), 3.09 (ddd, *J* = 20.3, 13.0, 7.1 Hz, 3H), 2.89 – 2.78 (m, 2H), 2.49 (dd, *J* = 18.7, 3.8 Hz, 1H), 2.29 – 2.19 (m, 1H), 1.97 (d, *J* = 14.5 Hz, 3H), 1.67 (d, *J* = 10.9 Hz, 4H), 1.36 (q, *J* = 12.4 Hz, 2H), 0.96 (q, *J* = 12.6 Hz, 2H).

Kad-type3

*(E)-6-(3-carboxyacrylamido)hexanoic acid. (**81**)*

To a stirred solution of caproic acid **80** (2.6g, 19.9mmol) in glacial acetic acid (15ml) under N_2 atmosphere, a solution of maleic anhydride in acetic acid was added in portions. The resulting mixture was stirred at room temperature for 3 h, then diluted with Et_2O and the precipitate was filtered on Büchner to give the desired product in 95% of yield.

*6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid. (**82**)*

To a solution of compound **81** (4.35g, 20.4mmol) in DMA (20ml), Ac_2O (3.85ml, 40.75mmol) and AcONa (418mg, 5.09mmol) were added, and the mixture is heated at 100 °C and stirred for 2 h. After cooling down to room temperature, the resulting mixture was washed with HCl 0.5N (3x10ml) and the organic layers were collected, dried over Na_2SO_4, filtered and the solvent was evaporated to give the desired product in 57% of yield.

^{1}H NMR (400 MHz, MeOD) δ 6.78 (s, 2H), 3.45 (t, *J* = 7.1 Hz, 3H), 2.25 (t, *J* = 7.4 Hz, 3H), 1.67 – 1.48 (m, 6H), 1.38 – 1.21 (m, 3H).

*2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate. (**83**)*

Compound **82** (2.5g, 11.84mmol) is solubilized in CH_2Cl_2 dry (20ml) under N_2 atmosphere. The solution is cooled down to -15 °C (with an iced and salted bath), NHS (1.36g, 11.84mmol) and DCC (2.68g, 13.02mmol) are added and the resulting mixture is stirred at -15 °C for 12 h. After filtration to eliminate DCU, the solvent is evaporated and the crude

product is purified on silica gel (Büchi) with PE/EtOAc 1:4 to give the desired compound in 70 % of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.59 (s, 2H), 4.00 (q, *J* = 7.1 Hz, 1H), 3.41 (t, *J* = 7.1 Hz, 2H), 2.72 (s, 3H), 2.49 (t, *J* = 7.4 Hz, 2H), 1.73 – 1.61 (m, 2H), 1.52 (dt, *J* = 14.8, 7.2 Hz, 3H), 1.30 (dt, *J* = 18.8, 8.0 Hz, 3H), 1.14 (t, *J* = 7.1 Hz, 2H).

*4-((6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)methyl)cyclohexane-1-carboxylic acid. (**84**)*

To a solution of compound **83** (2.46g, 7.98mmol) in glacial acetic acid (25ml) under N_2 atmosphere, trans-4-(aminomethyl)cyclohexane carboxylic acid **74** (2.38g, 15.17mmol) was added. The mixture was stirred at room temperature for 12 h, and monitored by TLC and ES-MS. The solvent was evaporated in the presence of hexane to create an azeotrope and promote the evaporation (this operation is repeated 3 times). CH_2Cl_2 was added to give a white precipitate, which was filtered on Büchner. The resulting solution was evaporated, diluted with CH_2Cl_2 (10ml) and washed with H_2O (3ml). The organic layer was dried over Na_2SO_4 and the crude product was purified on silica gel (Büchi) PE/EtOAc 1:9 to give the desired compound with 21% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.57 (s, 2H), 3.45 – 3.32 (m, 3H), 2.70 (s, 3H), 2.47 (t, *J* = 7.4 Hz, 2H), 2.19 (dd, *J* = 10.1, 4.7 Hz, 1H), 1.62 (dd, *J* = 15.2, 7.5 Hz, 2H), 1.49 (dt, *J* = 15.1, 7.6 Hz, 4H), 1.34 – 1.07 (m, 3H).
ES-MS: 349 [M+H]+

<u>Ad-type</u>

*Ethyl 7-((1-((1-(4-(hydroxymethyl)phenoxy)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoate (**87**)* **Yield:** 67%

To a solution of compound **86** (200mg, 0.45mmol) in THF dry (30ml) under N_2 atmosphere, PABA (83mg, 0.67mmol), HOBt (121mg, 0.9mmol) and HBTU (341mg, 0.9mmol) were added. After cooling down to 0 °C DIPEA (312µL, 1.8mmol) was added, and the resulting solution was stirred at room temperature for 12 h. The mixture was diluted with $NaHCO_{3ss}$ (5ml) and extracted with THF/EtOAc 10ml/5ml, then with EtOAc and finally with $CHCl_3/CH_3OH$ 13ml/2ml. The organic layers were collected and dried over Na_2SO_4, the solvent was evaporated under reduced pressure and the crude product was purified by flash

chromatography with CH_2Cl_2/CH_3OH 8:2. The desired product was obtained as a white-yellow solid in almost good yield (67%).

*Ethyl 7-((1-((1-(4-(bromomethyl)phenoxy)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoate. (**92**)*

A solution of compound **87** (50mg, 0.09mmol) in THF dry (3ml) under N_2 atmosphere was cooled down to 0 °C and PBr_3 (12.5µL, 0.13mmol) was added. The resulting solution was stirred for 2 h, the solvent was evaporated and the crude mixture was purified by flash chromatography with CH_2Cl_2/CH_3OH 9:1 to give the desired product in quantitative yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.0 Hz, 2H), 7.32 (d, *J* = 8.1 Hz, 2H), 4.44 (s, 2H), 4.09 (d, *J* = 6.7 Hz, 3H), 3.84 (dd, *J* = 28.8, 10.5 Hz, 2H), 3.61 (s, 3H), 3.41 (d, *J* = 5.9 Hz, 3H), 3.33 (d, *J* = 6.6 Hz, 3H), 3.12 (dd, *J* = 18.8, 9.0 Hz, 1H), 2.60 – 2.47 (m, 1H), 2.16 (t, *J* = 12.1 Hz, 1H), 1.93 (d, *J* = 6.6 Hz, 5H), 1.82 (s, 3H), 1.66 (d, *J* = 12.2 Hz, 3H), 1.42 – 1.18 (m, 2H), 0.95 (d, *J* = 12.5 Hz, 2H).

ES-MS: 612 [M+H]+, 636 [M+Na]+

*Ethyl 7-((1-((1-(4-(((2-((tert-butoxycarbonyl)amino)ethyl)thio)methyl)phenoxy)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoate. (**95**)*

To a solution of the bromide **92** (62mg, 0.1mmol) in THF dry (1ml) under N_2 atmosphere the 2-(bocamino)-ethanethiol **73** (17µL, 0.1mmol) and CH_3ONa (6mg, 0.11mmol) were added, and the mixture was stirred at room temperature for 12 h. The solvent was evaporated and the crude product was purified by flash chromatography with CH_2Cl_2/CH_3OH 9:1 to give the desired product with 40% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, *J* = 8.3 Hz, 2H), 7.20 (d, *J* = 8.2 Hz, 2H), 4.01 – 3.90 (m, 3H), 3.67 (s, 4H), 3.50 – 3.36 (m, 3H), 2.58 (dd, *J* = 18.8, 3.5 Hz, 1H), 2.22 (dd, *J* = 16.4, 7.9 Hz, 2H), 1.97 (s, 4H), 1.67 (dd, *J* = 38.1, 9.3 Hz, 4H), 1.49 – 1.19 (m, 6H), 1.01 (q, *J* = 39.2 Hz, 3H).

7-((1-((1-(4-(((2-((tert-butoxycarbonyl)amino)ethyl)thio)methyl)phenoxy)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoic acid. ***(96)***
Yield: >99%

Compound **95** (27mg, 0.04mmol) is solubilized in a solvent mixture of THF/H_2O/EtOH in 1:1:1 ratio (2ml/2ml/2ml) and LiOH.H_2O (4.54mg, 0.11mmol) is added. The resulting solution is stirred at 25 °C for 12 h monitoring by TLC and ES-MS, then diluted with H_2O, acidified (pH: 1-3) with HCl 1N, and extracted with EtOAc (2x10ml). The collected organic layers are dried over Na_2SO_4, filtered and the desired product is obtained as crude in quantitative yield.

ES-MS: 728 $[M+Li]^+$, 744 $[M+Na]^+$, 720 [M-H]-

*7-((1-((1-(4-(((2-aminoethyl)thio)methyl)phenoxy)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoic acid (triflate)(**97**)*

To a solution of compound **96** (150mg, 0.22mmol) in CH_2Cl_2 dry (7ml), Et3SiH (147µL, 0.92mmol) and TFA (92µL, 12mmol) are added and the mixture is stirred at room temperature for 12 h. The solvent is evaporated and the crude mixture is purified by flash chromatography with CH_2Cl_2/CH_3OH 9:1. The desired compound is obtained in very good yield (94%).

ES-MS: 603 [M+Na]+

3.5 BIOCONJUGATES

General procedure for the drug and the linker's activation.

A solution of the opportune cytotoxic compound or linker **104** (0.14mmol) and PNP-chloroformate (0.50mmol) in CH_2Cl_2 dry (7ml) under N_2 atmosphere is cooled down at 0 °C. DMAP (0.77mmol) was added, and the mixture was stirred at room temperature for 1 h and then diluted with CH_2Cl_2 (15ml) and washed with HCl 0.1 N (5ml). The organic layer was dried on Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude mixture was purified by flash chromatography with CH_2Cl_2/CH_3OH or PE/EtOAc to give the desired product in almost good yields.

(S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl (4-nitrophenyl) carbonate. ***(98)*** **Yield:** 86%

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (s, 1H), 8.24 (d, *J* = 8.6 Hz, 1H), 8.21 – 8.16 (m, 2H), 7.94 (d, *J* = 8.2 Hz, 1H), 7.84 (dd, *J* = 8.4, 7.0 Hz, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.49 – 7.35 (m, 3H), 5.68 (d, *J* = 17.3 Hz, 1H), 5.39 (d, *J* = 17.3 Hz, 1H), 5.36 – 5.21 (m, 2H), 2.34 (dd, *J* = 14.1, 7.4 Hz, 1H), 2.21 (dd, *J* = 14.1, 7.5 Hz, 1H), 1.64 (s, 2H), 1.04 (t, *J* = 7.5 Hz, 3H).

(Z)-2-methoxy-5-(3,4,5-trimethoxystyryl)phenyl (4-nitrophenyl) carbonate. ***(101)*** **Yield:** 70%

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 – 8.31 (m, 2H), 8.22 – 8.15 (m, 1H), 7.50 (d, *J* = 9.2 Hz, 2H), 7.26 – 7.20 (m, 2H), 6.95 (dd, *J* = 16.7, 8.7 Hz, 3H), 6.61 – 6.51 (m, 5H), 3.94 (s, 3H), 3.90 (s, 3H), 3.75 (s, 6H).

Benzyl 4-((3-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylate. ***(105)*** **Yield:** 40%

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 – 8.21 (m, 2H), 7.41 – 7.23 (m, 7H), 5.06 (s, 2H), 4.66 – 4.55 (m, 1H), 4.51 – 4.41 (m, 1H), 3.84 (dd, *J* = 9.0, 3.5 Hz, 1H), 3.48 – 3.30 (m, 4H), 3.12 (dd, *J* = 18.7, 9.0 Hz, 1H), 3.02 (dt, *J* = 14.4, 6.3 Hz, 1H), 2.46 (dd, *J* = 18.7, 3.5 Hz, 1H), 2.24 (ddd, *J* = 12.2, 7.9, 3.5 Hz, 1H), 1.99 (d, *J* = 11.3 Hz, 3H), 1.77 – 1.63 (m, 4H), 1.38 (td, *J* = 12.8, 2.5 Hz, 3H), 1.08 – 0.92 (m, 2H).

3.5.1 CAMPTOTHECIN Payloads

Kad-Camptothecin

*4-((3-((2-(((((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)carbonyl)amino)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylic acid. (**99**)*

Compound **71** (26mg, 0.06mmol) is solubilized in CH_2Cl_2 dry (4ml) under N_2 atmosphere. A solution of **98** (30mg, 0.06mmol) in CH_2Cl_2 dry is added followed by TEA (17µL, 0.12mmol) and the solution is stirred at room temperature for 12 h. The solvent is evaporated and the crude mixture is purified by flash chromatography eluting with CH_2Cl_2/MeOH 9:1 to give the desired product with 90% of yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.05 (d, *J* = 4.3 Hz, 1H), 8.42 – 8.19 (m, 2H), 7.96 – 7.82 (m, 1H), 7.81 – 7.65 (m, 2H), 7.31 (s, 1H), 5.48 (d, *J* = 3.5 Hz, 2H), 5.34 (s, 1H), 5.11 (dd, *J* = 22.0, 14.2 Hz, 2H), 4.11 (d, *J* = 9.2 Hz, 1H), 4.03 – 3.85 (m, 1H), 3.53 (s, 1H), 3.40 (d, *J* = 6.5 Hz, 3H), 2.63 – 2.40 (m, 3H), 2.26 (dd, *J* = 25.4, 13.3 Hz, 1H), 2.05 (d, *J* = 12.4 Hz, 2H), 1.76 (s, 3H), 1.41 (dd, *J* = 15.2, 8.8 Hz, 3H), 1.21 – 0.87 (m, 5H).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (t, *J* = 8.2 Hz, 1H), 8.33 – 8.10 (m, 2H), 7.80 (dd, *J* = 15.9, 8.4 Hz, 1H), 7.66 (dd, *J* = 16.3, 7.2 Hz, 2H), 5.36 (d, *J* = 6.2 Hz, 3H), 5.11 – 4.95 (m, 2H), 4.05 (dd, *J* = 8.1, 4.8 Hz, 1H), 3.91 (ddd, *J* = 16.9, 9.0, 4.1 Hz, 2H), 3.34 (dd, *J* = 19.1, 5.8 Hz, 4H), 3.11 (dt, *J* = 23.7, 9.2 Hz, 2H), 3.03 – 2.96 (m, 1H), 2.88 (dd, *J* = 13.3, 4.6 Hz, 1H), 2.81 – 2.61 (m, 1H), 2.55 – 2.35 (m, 3H), 2.17 (dd, *J* = 25.8, 13.6 Hz, 2H), 1.98 (d, *J* = 11.4 Hz, 3H), 1.68 (s, 4H), 1.41 – 1.30 (m, 3H), 1.21 (s, 1H), 1.10 – 0.86 (m, 7H).

Ad-Camptothecin

*7-((1-((1-((4-(((2-(((((R)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H Pyrano [3',4':6,7] indolizino [1,2-b]quinolin-4-yl)oxy)carbonyl)amino)ethyl)thio)methyl)phenyl)amino)-1-oxo-4-ureidobutan-2 yl) amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoic acid. (**100**)*

To a solution of the linker **97** (40mg, 0.07mmol) in DMF dry (2ml) under N_2 atmosphere, TEA (19.2µL, 0.14mmol) and **98** (43mg, 0.07mmol) were added and the mixture was stirred at room temperature for 2 h and monitored by TLC and ES-MS. The solution was diluted with CH_2Cl_2 (30ml) and washed with HCl 1N (2x10ml). The organic layer was dried over

Na_2SO_4 and after the evaporation of the solvent, the crude product was purified by flash chromatography with CH_2Cl_2/MeOH 9:1 to give the desired product with 45% yield.

ES-MS: 1076 [M+Na]+
R_f: 0.24 (CH_2Cl_2/MeOH 9:1)

3.5.2 Combretastatin Payload

Kad-Combretastatin

*(Z)-4-((3-((2-(((2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)carbonyl)amino)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylic acid. **(102)*** **Yield:**75%
To a solution of compound **71** (32mg, 0.07mmol) in CH_2Cl_2 dry/DMF dry (4ml/1ml), compound **101** (37mg, 0.07mmol) and TEA (19µl, 0.14mmol) were added, and the resulting mixture was stirred at room temperature for 12 h. The mixture was diluted with CH_2Cl_2 (7ml) and extracted with HCl 1N (2ml). The organic layer was dried over Na2SO4 and filtered. The crude was purified by flash chromatography with CH_2Cl_2/CH_3OH 9:1 to give the desired product with 75% yield.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 – 7.03 (m, 2H), 6.85 (d, *J* = 8.2 Hz, 1H), 6.53 (s, 2H), 6.46 (s, 2H), 3.90 – 3.77 (m, 6H), 3.72 (s, 5H), 3.65 – 3.47 (m, 2H), 3.38 (d, *J* = 6.2 Hz, 2H), 3.17 (dd, *J* = 19.0, 8.9 Hz, 2H), 2.97 (s, 1H), 2.90 (s, 2H), 2.52 (d, *J* = 18.5 Hz, 1H), 2.24 (t, *J* = 11.7 Hz, 2H), 2.03 (d, *J* = 12.4 Hz, 3H), 1.75 (d, *J* = 11.6 Hz, 2H), 1.40 (dd, *J* = 24.6, 12.2 Hz, 2H).

3.5.3 HSP90 Payload

Kad-HSP90

*Benzyl 4-((3-((2-(((4-(1-(2,4-bis(benzyloxy)-5-isopropylphenyl)-4-(ethylcarbamoyl)-1H-1,2,3-triazol-5-yl)benzyl)(cyclohexylmethyl)carbamoyl)oxy)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl) cyclohexane-1-carboxylate. (**106**)*

A solution of compound **105** (425mg, 0.74mmol) and HSP90 (prodrug) (500mg, 0.74mmol) in CH_2Cl_2 dry (10ml) was stirred at room temperature for 48 h. After the evaporation of the solvent, the crude mixture was purified on silica gel (Büchi) with PE/EtOAc 1:1 to give the desired product with 45% yield.

^{1}H NMR (400 MHz, MeOD) δ 7.51 (d, *J* = 7.8 Hz, 2H), 7.39 (d, *J* = 7.7 Hz, 2H), 7.06 (s, 1H), 6.53 (s, 1H), 4.67 (d, *J* = 3.8 Hz, 2H), 4.50 (d, *J* = 42.7 Hz, 2H), 4.10 (d, *J* = 30.1 Hz, 1H), 3.91 (s, 1H), 3.64 – 3.47 (m, 6H), 3.45 – 3.14 (m, 6H), 2.62 (t, *J* = 20.7 Hz, 1H), 2.38 (t, *J* = 11.9 Hz, 1H), 2.24 – 2.11 (m, 3H), 2.05 (s, 1H), 2.00 – 1.74 (m, 10H), 1.63 – 1.45 (m, 4H), 1.45 – 1.32 (m, 6H), 1.24 (dd, *J* = 25.6, 9.5 Hz, 8H), 1.09 (d, *J* = 8.3 Hz, 3H).

*4-((3-((2-(((cyclohexylmethyl)(4-(1-(2,4-dihydroxy-5-isopropylphenyl)-4-(ethylcarbamoyl)-1H-1,2,3-triazol-5-yl)benzyl)carbamoyl)oxy)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylic acid. (**107**)*

To a solution of compound **106** (100mg, 0.09mmol) in EtOAc/CH_2Cl_2/CH_3OH 10:1:1 under N_2 atmosphere, $Pd(OH)_2/C$ (50% H_2O) (0.1eq) was added and the mixture was stirred at room temperature under H_2 atmosphere for 16 h, then filtered to remove the residual $Pd(OH)_2/C$ and the solvent evaporated. The desired product was obtained with 48% yield as crude.

^{1}H NMR (400 MHz, MeOD) δ 8.06 (s, 1H), 7.52 (d, *J* = 8.0 Hz, 3H), 7.39 (d, *J* = 7.9 Hz, 3H), 7.06 (s, 1H), 6.53 (s, 1H), 5.00 (s, 7H), 4.67 (d, *J* = 4.1 Hz, 3H), 4.59 – 4.38 (m, 3H), 4.18 – 4.02 (m, 1H), 3.65 – 3.48 (m, 6H), 3.44 – 3.15 (m, 8H), 2.64 (d, *J* = 20.2 Hz, 1H), 2.39 (dd, *J* = 16.3, 7.8 Hz, 1H), 2.16 (d, *J* = 12.2 Hz, 3H), 1.99 – 1.74 (m, 4H), 1.61 – 1.47 (m, 4H), 1.39 (dd, *J* = 13.5, 6.3 Hz, 2H), 1.23 (dd, *J* = 25.6, 9.6 Hz, 9H), 1.09 (d, *J* = 9.8 Hz, 4H).

3.5.4 Payload's Activation

General activation procedure for lysine's conjugation

A solution of the opportune conjugate (0.14mmol) in CH_2Cl_2 dry (5ml) under N_2 atmosphere was cooled down to -15 °C (with ice and NaCl), NHS (0.14mmol) and DCC (0.15mmol) were added and the resulting mixture was stirred at -15 °C for 12 h. The solvent was evaporated and the crude product was purified by flash chromatography with PE/EtOAc or CH_2Cl_2/MeOH to give the desired products in good yields.

2,5-dioxopyrrolidin-1-yl 4-((3-((2-(((((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H pyrano [3',4':6,7] indolizino [1,2-b]quinolin-4-yl)oxy)carbonyl)amino)ethyl)thio)-2,5 dioxopyrrolidin-1 yl)methyl)cyclohexane-1-carboxylate. ***KAD-Camptothecin-NHS*** **Yield**: 90%

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 – 9.01 (m, 1H), 8.38 – 8.19 (m, 2H), 7.89 (dd, *J* = 16.0, 8.5 Hz, 1H), 7.73 (t, *J* = 6.9 Hz, 2H), 5.44 (d, *J* = 6.1 Hz, 2H), 5.10 (t, *J* = 11.5 Hz, 2H), 4.13 (dd, *J* = 9.5, 3.8 Hz, 2H), 3.99 (ddd, *J* = 14.2, 8.9, 3.8 Hz, 2H), 3.56 – 3.34 (m, 5H), 3.18 (dd, *J* = 18.8, 9.0 Hz, 1H), 2.87 (d, *J* = 17.4 Hz, 8H), 2.66 – 2.42 (m, 4H), 2.18 (d, *J* = 16.5 Hz, 3H), 2.02 – 1.92 (m, 2H), 1.92 – 1.68 (m, 6H), 1.45 – 1.26 (m, 4H), 1.13 (dd, *J* = 13.1, 6.7 Hz, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.32, 170.09, 168.69, 153.24, 151.69, 149.19, 143.85, 141.79, 131.75, 130.28, 129.90, 127.82, 124.95, 122.37, 97.22, 89.10, 81.03, 58.68, 52.47, 48.72, 43.97, 39.90, 38.03, 37.25, 36.47, 35.79, 34.88, 34.66, 33.46, 31.52, 28.86, 27.52, 27.21, 25.15, 24.47, 7.48.

7-((1-((1-((4-(((2-(((((R)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1pyrano [3',4':6,7] indolizino[1,2-b] quinolin-4-yl)oxy)carbonyl)amino)ethyl)thio)methyl)phenyl)amino)-1-oxo-4-ureidobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-7-oxoheptanoic acid. ***Ad-Camptothecin-NHS*** **Yield:** 90%

ES-MS: 1174 $[M+Na]^+$

R_f: 0.33 (CH_2Cl_2/MeOH 4:1)

2,5-dioxopyrrolidin-1-yl(Z)-4-((3-((2-(((2-methoxy-5-(3,4,5-trimethoxystyryl) phenoxy) carbonyl)amino)ethyl)thio) -2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylate. ***Kad-Combretastatin-NHS*** **Yield**: 82%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (s, 1H), 7.14 (dd, *J* = 13.8, 5.4 Hz, 2H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.56 (s, 2H), 6.49 (s, 2H), 5.65 (s, 1H), 3.87 (d, *J* = 4.7 Hz, 6H), 3.75 (s, 6H), 3.68 – 3.54 (m, 2H), 3.43 (d, *J* = 6.8 Hz, 2H), 3.31 – 3.16 (m, 2H), 2.88 (d, *J* = 17.9 Hz, 6H), 2.59 (ddd, *J* = 22.4, 15.5, 6.1 Hz, 2H), 2.20 (d, *J* = 11.6 Hz, 1H), 2.08 (s, 1H), 1.97 (d, *J* = 9.1 Hz, 1H), 1.92 – 1.67 (m, 6H), 1.64 – 1.51 (m, 2H), 1.30 (t, *J* = 7.1 Hz, 2H), 1.12 (d, *J* = 13.0 Hz, 3H).

2,5-dioxopyrrolidin-1-yl 4-((3-((2-(((cyclohexylmethyl)(4-(1-(2,4-dihydroxy-5-isopropylphenyl)-4-(ethylcarbamoyl)-1H-1,2,3-triazol-5-yl)benzyl)carbamoyl)oxy)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)cyclohexane-1-carboxylate. ***Kad-HSP90-NHS*** **Yield**: 69%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J* = 5.5 Hz, 2H), 7.26 (dd, *J* = 13.5, 8.2 Hz, 3H), 7.06 (d, *J* = 7.1 Hz, 3H), 6.74 (s, 1H), 6.64 (s, 1H), 6.34 (s, 1H), 6.26 (s, 1H), 4.32 (ddd, *J* = 37.4, 25.8, 12.3 Hz, 5H), 3.78 (d, *J* = 6.0 Hz, 1H), 3.66 (d, *J* = 6.2 Hz, 1H), 3.43 – 3.36 (m, 3H), 3.36 – 3.21 (m, 3H), 3.00 (ddd, *J* = 28.6, 17.1, 10.2 Hz, 6H), 2.77 (s, 6H), 2.53 (dd, *J* = 24.5, 14.4 Hz, 1H), 2.34 (t, *J* = 17.0 Hz, 1H), 2.07 (s, 3H), 1.79 – 1.54 (m, 12H), 1.47 (d, *J* = 11.4 Hz, 2H), 1.17 (dd, *J* = 20.1, 12.8 Hz, 8H), 1.09 – 0.90 (m, 11H), 0.84 (d, *J* = 10.0 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 180.91, 178.87, 174.20, 172.96, 164.27, 162.65, 160.55, 159.42, 153.37, 142.32, 141.63, 134.01, 131.10, 130.36, 129.98, 128.58, 118.95, 67.28, 66.66, 56.58, 54.43, 47.98, 43.88, 40.34, 39.93, 39.11, 38.89, 37.70, 34.34, 32.80, 31.50, 29.93, 29.69, 29.37, 29.16, 26.02, 18.35.

General activation procedure for cysteine's conjugation

To a solution of N-(2-aminoethyl)maleimido trifluoroacetic salt **110** (0.11mmol) in DMF dry (3ml) at 0 °C, the opportune conjugate (0.11mmol), HOBt (0.16mmol) HBTU (0.16mmol) and DIPEA (0.27mmol) were added. The mixture was stirred at 0 °C for 30 min, then at room temperature for 12 h. Iced H_2O was added (10ml) and the mixture was extracted with CH_2Cl_2 (20ml). The organic layer was dried over Na_2SO_4, the solvent evaporated and the crude product was purified by flash chromatography with CH_2Cl_2/CH_3OH 9:1 to give the desired products in good yields.

(S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl (2-((1-((4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)cyclohexyl)methyl)-2,5-dioxopyrrolidin-3-yl)thio)ethyl)carbamate. ***Kad-Camptothecin-Maleimide*** **Yield**: 53%

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 – 8.25 (m, 1H), 7.94 – 7.88 (m, 1H), 7.76 (d, *J* = 9.5 Hz, 1H), 7.31 (s, 1H), 6.75 (d, *J* = 2.5 Hz, 1H), 5.89 (s, 1H), 5.49 (d, *J* = 3.3 Hz, 1H), 5.11 (dd, *J* = 20.8, 9.7 Hz, 1H), 4.20 – 4.06 (m, 1H), 4.05 – 3.91 (m, 1H), 3.71 (dd, *J* = 6.5, 3.6 Hz, 1H), 3.54 – 3.45 (m, 2H), 3.39 (d, *J* = 6.7 Hz, 2H), 3.17 (dd, *J* = 18.8, 9.0 Hz, 1H), 2.85 (s, 2H), 2.64 – 2.41 (m, 2H), 2.00 (t, *J* = 12.2 Hz, 1H), 1.90 (d, *J* = 12.7 Hz, 2H), 1.77 (d, *J* = 10.1 Hz, 3H), 1.40 (d, *J* = 9.9 Hz, 2H), 1.14 (dd, *J* = 13.1, 7.1 Hz, 2H), 1.03 (d, *J* = 13.1 Hz, 2H).
ES-MS: 823 [M+Na]+

(Z)-2-methoxy-5-(3,4,5-trimethoxystyryl)phenyl (2-((1-((4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)cyclohexyl)methyl)-2,5-dioxopyrrolidin-3-yl)thio)ethyl)carbamate **Yield**: 53%

^{1}H NMR (400 MHz, Acetone) δ 7.20 – 7.03 (m, 3H), 6.96 (d, *J* = 4.8 Hz, 1H), 6.85 (d, *J* = 7.3 Hz, 1H), 6.62 (d, *J* = 7.0 Hz, 1H), 6.52 (t, *J* = 5.3 Hz, 1H), 4.10 – 3.96 (m, 1H), 3.84 (d, *J* = 7.0 Hz, 2H), 3.72 (dd, *J* = 13.9, 7.3 Hz, 6H), 3.64 – 3.55 (m, 2H), 3.57 – 3.45 (m, 1H), 3.45 – 3.37 (m, 1H), 3.33 (d, *J* = 6.6 Hz, 2H), 3.15 (dd, *J* = 13.2, 6.5 Hz, 1H), 2.92 (d, *J* = 9.3 Hz, 2H), 2.59 – 2.48 (m, 1H), 2.07 (ddd, *J* = 19.0, 10.6, 2.7 Hz, 1H), 1.78 (dd, *J* = 28.3, 11.9 Hz, 4H), 1.52 (dd, *J* = 8.9, 4.9 Hz, 2H), 1.44 – 1.29 (m, 2H), 0.99 (d, *J* = 12.1 Hz, 2H).
^{13}C NMR (101 MHz, Acetone) δ 205.42, 175.62, 174.80, 170.87, 153.23, 134.25, 132.40, 129.86, 129.36, 128.43, 127.00, 123.37, 112.43, 106.27, 78.31, 55.39, 44.63, 44.26, 37.38, 35.85.

2-((1-((4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)cyclohexyl)methyl)-2,5-dioxopyrrolidin-3-yl)thio)ethyl (cyclohexylmethyl)(4-(1-(2,4-dihydroxy-5-isopropylphenyl)-4-(ethylcarbamoyl)-1H-1,2,3-triazol-5-yl)benzyl)carbamate. ***Kad-HSP90-Maleimide*** **Yield**: 30%

^{1}H NMR (400 MHz, MeOD) δ 8.05 (dd, *J* = 12.2, 3.5 Hz, 2H), 7.92 (d, *J* = 8.2 Hz, 1H), 7.69 (dt, *J* = 15.3, 7.1 Hz, 1H), 7.51 (d, *J* = 7.6 Hz, 2H), 7.39 (d, *J* = 7.8 Hz, 2H), 7.06 (s, 1H), 6.95 (s, 2H), 6.53 (s, 1H), 5.02 (s, 6H), 4.66 (d, *J* = 2.9 Hz, 2H), 4.53 (dd, *J* = 46.7, 23.2 Hz, 2H), 3.85 – 3.68 (m, 2H), 3.53 (ddd, *J* = 11.0, 9.0, 4.3 Hz, 8H), 3.44 – 3.15 (m, 6H),

2.61 (t, *J* = 20.7 Hz, 1H), 2.19 (t, *J* = 11.8 Hz, 1H), 1.99 – 1.71 (m, 12H), 1.52 (s, 3H), 1.39 (dd, *J* = 14.5, 7.3 Hz, 6H), 1.26 (d, *J* = 6.8 Hz, 6H), 1.12 (dd, *J* = 23.1, 11.0 Hz, 5H).

^{13}C NMR (101 MHz, MeOD) δ 177.21, 177.07, 175.28, 170.71, 160.98, 156.57, 150.72, 140.01, 138.79, 137.49, 133.57, 129.67, 126.39, 126.14, 125.93, 125.35, 125.06, 116.82, 114.39, 109.62, 101.97, 77.63, 63.10, 52.70, 52.00, 49.83, 47.83, 47.61, 47.40, 47.19, 46.98, 46.76, 46.55, 44.26, 43.75, 38.26, 36.86, 36.61, 35.78, 35.30, 34.93, 33.26, 30.06, 29.05, 27.97, 25.72, 25.55, 25.10, 21.23, 13.25.

ABBREVIATIONS USED

Hh: Hedgehog

Ptch: Patched

Disp: Dispatched

Smo: Smoothened

AcTU: acylthiourea

AcU: acylurea

AcG: acylguanigine

DME: dimethoxyethane

DMF: dimethylformammide

TEA: triethylamine

r.t.: room temperature

MW: microwaves

PE: petroleum ether

EtOAc: ethyl acetate

AcOH: acetic acid

MeONa: sodium methoxylate

h: hours

mAb: monoclonal antibodies

ADC: antibody-drug conjugates

Kad: kadcyla

Ad: adcetris

EtOH: ethanol

MeOH: methanol

DCM: dichloromethane

DMA: dimethylacetamide

Ac_2O: acetic anhydride

AcONa: sodium acetate

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