Agència
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Universitaris
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Memòria justificativa de recerca de les convocatòries BCC, BE, BP, CTP-AIRE, DEBEQ, FI, INEFC, NANOS i PIV

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Nom de la convocatòria

BP

Llegenda per a les convocatòries:

BCC	Convocatòria de beques per a joves membres de comunitats catalanes a l'exterior
BE	Beques per a estades per a la recerca fora de Catalunya
BP	Convocatòria d'ajuts postdoctorals dins del programa Beatriu de Pinós
CTP-AIRE	Ajuts per accions de cooperació en el marc de la comunitat de treball dels Pirineus. Ajuts de mobilitat de personal investigador.
DEBEQ (Modalitat A3)	Beques de Cooperació Internacional i Desenvolupament
FI	Beques predoctorals per a la formació de personal investigador
INEFC	Beques predoctorals i de col·laboració, dins de l'àmbit de l'educació física i l'esport i les ciències aplicades a l'esport
NANOS	Beques de recerca per a la formació en el camp de les nanotecnologies
PIV	Beques de recerca per a professors i investigadors visitants a Catalunya

Títol del projecte: ha de sintetitzar la temàtica científica del vostre document.
Combinatorial Synthesis and Biological Evaluation of Inhibitors of D-Ala-D-Ala-Ligase

Dades de l'investigador o beneficiari

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Agència
de Gestió d'Ajuts
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Número d'expedient
2005BP-A 10117

Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
Kinase inhibitors, D-Ala-D-Ala-Ligase, cysteine alkylation, Ras, membrane subdomains

Data de presentació de la justificació
04-12-2008

Nom i cognoms i signatura
del/de la investigador/a

Vistiplau del/la responsable de la sol·licitud

Resum del projecte: cal adjuntar dos resums del document, l'un en anglès i l'altre en la llengua del document, on s'esmenti la durada de l'acció

Resum en la llengua del projecte (màxim 300 paraules)

L'estada postdoctoral s' ha centrat en dos temes. En primer lloc la identificació de nous lligands per a proteïnes gràcies a una nova aproximació que combina l'ús de mètodes computacionals amb el desenvolupament de llibreries basades en productes naturals.

Segons aquesta aproximació, la similitud structural existent entre diferents proteïnes pot ser utilitzada com a punt de partida per l'obtenció de nous lligands. Així, un lligand conegut d'una proteïna pot servir com a punt de partida per a l'obtenció de lligands per altres proteines que formin part del mateix "cluster".

Aquest projecte que requereix l'ús de mètodes multidisciplinars com la síntesi orgànica, el desenvolupament d'assaigs in vitro i l'expressió i purificació de proteïnes entre d' altres, ha permès trobar nous inhibidors per a l'enzim DD-ligasa en base a la seva similitud estructural amb diferents kinases com p56lck o cdc2.

El segon projecte dur a terme durant aquesta estada postdoctoral s´ha basat en el desenvolupament de nous mètodes de síntesi de proteines lipidades per a utilitzar-les posteriorment com a eina química en estudis bioquímics, biofísics o de l'àmbit de la biologia cel.lular.

Concretament el projecte s' ha centrat en el desenvolupament de nous mètodes de síntesi de la proteina Ras lipidada així com el posterior estudi de la distribució de Ras en els diferents microdominis de membranes que componen les cèl.lules, mitjançant espectroscopia de fluorescència o microscopi de forca atòmica, entre d'altres.

Resum en anglès (màxim 300 paraules)

The work carried out during this postdoctoral stage was focused on two different projects: Identification of D-Ala D-Ala Inhibitors and the development of new synthethic approaches to obtain lipidated peptides and proteins and the use of these lipidated proteins in biological and biophysical studies.

In the first project, new D-Ala D-Ala inhibitors were identified by using structural alignments of the ATP binding sites of the bacterial ligase DDl and protein and lipid kinases in complex with ATP analogs. We tested a series of commercially available kinase inhibitors and found LFM-A13 and Tyrphostine derivatives to inhibit DDl enzyme activity. Based on the initial screening results we synthesized a series of malononitrilamide and salicylamide derivatives and were able to confirm the validity of these scaffolds as inhibitors of DDl. From this investigation we gained a better understanding of the structural requirements and limitations necessary for the preparation of ATP competitive DDl inhibitors. The compounds in this study may serve as starting points for the development of bi-substrate inhibitors that incorporate both, an ATP competitive and a substrate competitive moiety. Bisubstrate inhibitors that block the ATP and D-Ala binding sites should exhibit enhanced selectivity and potency profiles by preferentially inhibiting DDl over kinases

Resum en anglès (màxim 300 paraules) – continuació -.
In the second project, an optimized synthesis for tha alkylation of cysteins using the thiol ene reaction was establisehd. This new protocol allowed us to obtain large amounts of hexadecylated cysteine that was required for the synthesis of differently lipidated peptides.
Afterwards the synthesis of various N-ras peptides bearing different lipid anchors was performed and the peptides were ligated to a truncated N-ras protein. The influence of this differently lipidated N-ras proteins on the partioning and association of N-Ras in model membrane subdomains was studied using Atomic Force Microscopy.

2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

Synthesis and Biological Evaluation of Inhibitors of D-Ala-D-Ala-Ligase

DDl as potential target for new Antibiotics

D-Alanine:D-alanine ligase (DDl) (E.C. 6.3.2.4) catalyzes the ATP-dependent assembly of the dipeptide D-alanyl-D-alanine (D-Ala-D-Ala) which is an essential building block for bacterial cell wall biosynthesis. D-Ala-D-Ala is common to both Gram-negative and Gram-positive organisms and highly conserved among prokaryotes and has no eukaryotic counterparts. Therefore, DDl has emerged as an attractive target to develop novel antibiotics. D-cycloserine, a structural analog of D-Ala, is the only small molecule known to inhibit DDl activity and to result in an antibiotic effect. D-cycloserine inhibits enzyme activity as a D-Ala competitive inhibitor with a reported K_i of 27 μM and is proposed to bind to the first D-Ala binding site. However, its poor selectivity and toxicity limits its use, which stresses the importance of having access to further inhibitors.

A link between protein and lipid kinases and DDl

In order to find new inhibitor scaffolds for DDl, we drew from earlier investigations and based our search on the similarity that exists between the ATP binding domains of various kinases and the glutathione synthase fold (ATP-grasp) to which DDl belongs. Preliminary studies reported similarities between DDl and protein kinases, such as casein kinase (CK1), AMPc, cyclin dependent kinase 2 (CDK2), the Src family tyrosine kinase Hck, the insulin receptor

tyrosine kinase (IRK), phosphorylase kinase (PhK), the extra cellular signal regulated protein kinase (ERK2) and lipid kinases such as PI3K. Structural alignments of the ATP binding sites of DDl and different classes of kinases highlight a conserved topology of the co-factor binding site as well as the same relative orientation of the adenine part of ATP (Figure 1). These common features served as starting points towards our investigations of novel DDl inhibitors.

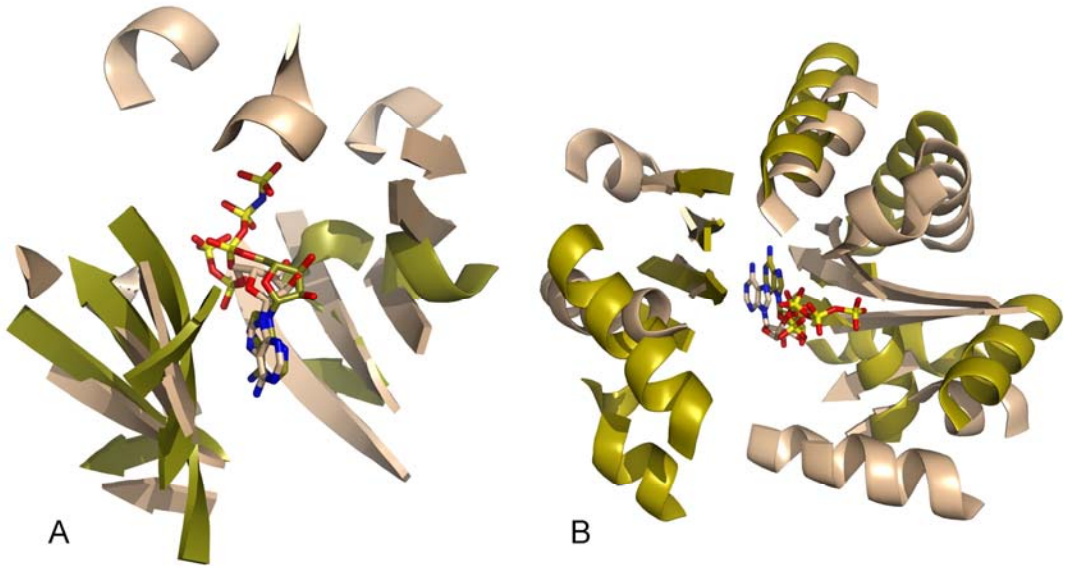


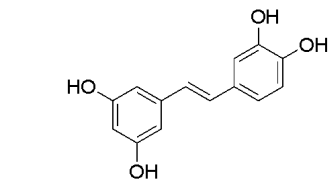
Figure 1. Structural alignments of the ATP binding sites of DDl and various kinases. (A) Structural alignment of the ATP binding sites of DDl (PDB code 2DLN) [9] in salmon and the protein kinase Hck (PDB code 1AD5) in green. (B) Structural alignment of the ATP binding sites of DDl (PDB code 2DLN) in salmon and lipid kinase PI3K-γ (PDB code 1E8X) in green. The alignments highlight similar binding modes for the ligands ADP (DDl), AMP-PNP (Hck) and ATP (PI3K-γ). Structural alignments were performed using DaliLite. Images were generated using PyMol (http://www.pymol.org).

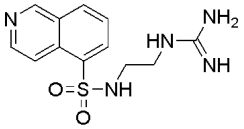
Kinase inhibitors as starting points for new inhibitors of DDl

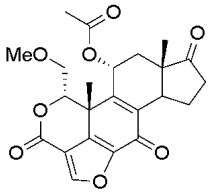
Over the last decades, kinases have evolved as a major class of target proteins in medicinal chemistry and chemical biology. In order to shut down unwanted kinase activities, a plethora of small organic molecules have since been developed to inhibit kinase function by targeting the ATP binding pocket of the kinase domain. Typically, such inhibitors bind to the ATP binding site of the kinase through the formation of 2-3 hydrogen bonds and through hydrophobic interactions in and around the region occupied by the adenine ring of ATP. Although some selective ATP competitive kinase inhibitors have been developed, the highly conserved nature of the ATP binding region often results in inhibitor scaffolds with poor kinase selectivity. This conserved nature of ATP binding sites among other protein classes and the wealth of ATP competitive kinase inhibitors could serve as starting point for the development of inhibitors that target proteins other than kinases.

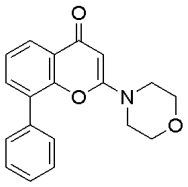
Biochemical screening

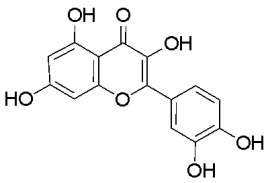
Based on the described similarities between the ATP binding sites of DDl and protein and lipid kinases we developed a new approach to find new inhibitor scaffolds binding to the ATP binding site of DDl. A collection of 27 different ATP competitive kinase inhibitors was selected and screened at mM concentrations against DDl measuring residual activity. ATP competitive kinase inhibitors identified as DDl inhibitors are given in Table 1

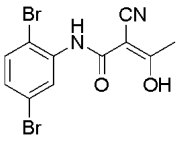

Piceatannol **(1)**

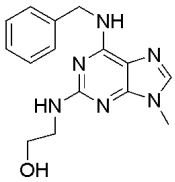

H-1004 **(2)**

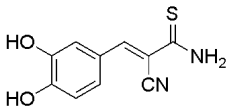

Wortmannin **(3)**

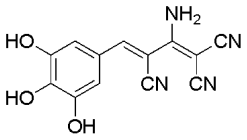

LY 294002 **(4)**


Quercetin **(5)**


LFM-A13 **(6)**


Olomoucine **(7)**


Tyrphostin 47 **(8)**


Tyrphostin 51 **(9)**

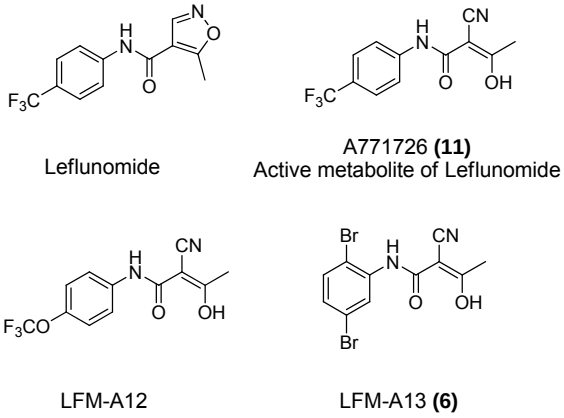
Compound	Target	IC_{50} values of Kinase Inhibition	Scaffold	DDl Residual Activity	*Ki* values for DDl Inhibition
1	$p56^{lck}$, syk		I	84.63 ± 7.5	
2	PKA, PKG		II	85.3 ± 12.86	
3	PI3K, PKC		III	81.37 ± 4.4	
4	PI3K CK2	10 µM 6.9 µM	IV	83.94 ± 3.5	1.4 mM (ATP competitive)
5	PI3K, PKC		IV	77.00 ± 14.9	
6	BTK Jak , Tec , Plk	2.5 µM	V	41.19 ± 15.5	185µM (ATP competitive)
7	CDK2/cycB,A,E ERK1/MAPK	7 µM 25 µM	VI	79.7 ± 10.3	1.8 mM (ATP competitive)
8	EGFR, PDGFR IRK	2.4-3.5 µM 640 µM	VII	51.79 ± 19.08	290 µM (ATP competitive)
9	EGFR	0.8 µM	VII	61.92 ± 2.44	Mixed inhibition

Table 1. Kinase inhibitors that showed inhibition of DDl. (Solubility of compounds could be increased by adding BSA (1mg/ml) or chaps instead of 0.01% of the detergent NP-40). *Ki* values were determined for the best inhibitors.

Under the conditions tested, DDl was better inhibited by the EGFR kinase inhibitors Tyrphostin-47 (T-47), Tyrphostin-51 (T-51) and by the Bruton's tyrosine kinase inhibitor LFM-A13. Slight inhibition could be detected for the AMPc inhibitor HA-1004, the CDK2 inhibitor

Olomoucine and the Src family tyrosine kinase inhibitor Piceatannol. Interestingly, the known PI3K inhibitors Wortmannin, and LY294002 were also found to inhibit DDI.

Detailed inhibition studies were then performed for the most potent DDI inhibitors identified in the initial screen. DDI enzyme inhibition was measured at different inhibitor and ATP concentrations in order to determine K_i values and the type of inhibition. For K_i determinations, parallel series of experiments were made under the same conditions in the presence of various inhibitor concentrations and data were analyzed by using Lineweaver-Burk plots (Table 1). Tyrphostin T-47, Olomoucine, LY29004 and LFM-A13 were found to be ATP competitive inhibitors of DDI. The malononitrilamide LFM-A13 belongs to a series of compounds that were synthesized based on the active Leflunomide metabolite A771726. (Scheme 2)

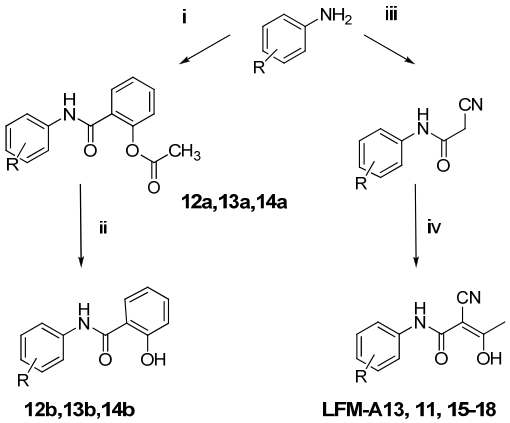


Scheme 2. Leflunomide, active metabolites of Leflunomide A771726 and analogs

Leflunomide is an antirheumatic drug (Arava®) known to inhibit dihydroorotate dehydrogenase. However, its metabolite A771726 (**11**) has been hypothesized to exert immunosuppressive activity by the inhibition of several protein kinases such as PDGFR, EGFR and to prevent phosphorylation of Jak1 and Jak3 that are necessary for Interleukin-2 receptor signalling. Several analogs of A771726 such as LFM-A12 and LFM-A13 were also reported to inhibit EGFR, Bruton´s Tyrosine kinase (BTK), Jak2, Tec and Polo-like kinases (Plk).

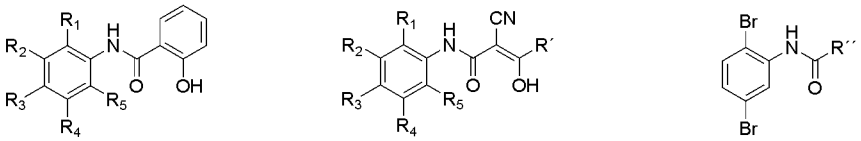
Synthesis

Based on the findings that binding of ATP to DDI can be inhibited by ATP competitive kinase inhibitors of the malononitrilamide class in the micromolar range, we developed a focused library based on LFM-A13 to establish further structure-activity relationships. To explore substitution patterns at the aniline moiety, compounds **11** and **15-18** were synthesized according to published procedures.



Scheme 3. Synthesis of Leflunomide analogs

The prepared derivatives were evaluated for their ability to inhibit recombinant DDl. Compounds were screened in a DDl assay that was carried out in the presence of 250 μM ATP and 500 μM D-Ala. Orthophosphate was detected with Malachite Green. Structure–activities are provided in Table 2.



A
Salicylic acid

B
Leflunomide metabolite
(malononitrilamides)

C

Molecule No	Class	Substitution							DDl Residual activity [%]	K_i
		R_1	**R_2**	**R_3**	**R_4**	**R_5**	**R'**	**R''**		
11	B			CF_3			CH_3		114.52 ±10.7	
12	A	Br			Br				65.43±10.7	
13	A	CH_3				CH_3			Not soluble	
14	A	CH_3		CH_3		CH_3			Not soluble	
LFM-A13	B	Br			Br		CH_3		41.19 ± 15.5	185 μM
15	B	Cl				CH_3	CH_3		46.15±3.14	215 μM
16	B	CH_3				CH_3	CH_3		95 ±13	
17	B	CH_3		CH_3		CH_3	CH_3		116.37 ±7.25	
18	B	CF_3			CF_3		CH_3		58.61± 0.3	60 μM
19	B	Br			Br		CH_2COOH		88.10± 12.45	
20	C							$COCH_3$	112.02±8.24	

Table 2. Activity of Leflunomide analogs on DDl.

The inhibitory activity of LFMA13 could be retained by the salicylic derivative **12** and slightly increased by **15, 18**. Compound **18** with CF_3-substituents in the 2- and 5-position showed

higher activity compared to LFM-A13 (bromo-substitution in 2,5-position). Similar results were obtained for the 2-chloro-6-methyl derivative **15**. However, neither A771726 (CF_3 in the 4-position) nor the dimethyl- (**16)** or trimethyl-analog **17** showed any DDl inhibiting activity. Changes in the aliphatic region of the molecules were not tolerated and the extended analogs **19** and the carbamat **20** were not active.

Conclusions

Using structural alignments of the ATP binding sites of the bacterial ligase DDl and protein and lipid kinases in complex with ATP analogs, we rationalized that inhibition of DDl activity may be achieved by ATP competitive kinase inhibitors. We tested a series of commercially available kinase inhibitors and found LFM-A13 and Tyrphostine derivatives to inhibit DDl enzyme activity. Based on the initial screening results we synthesized a series of malononitrilamide and salicylamide derivatives and were able to confirm the validity of these scaffolds as inhibitors of DDl. From this investigation we gained a better understanding of the structural requirements and limitations necessary for the preparation of ATP competitive DDl inhibitors. The compounds in this study may serve as starting points for the development of bi-substrate inhibitors that incorporate both, an ATP competitive and a substrate competitive moiety. Bisubstrate inhibitors that block the ATP and D-Ala binding sites should exhibit enhanced selectivity and potency profiles by preferentially inhibiting DDl over kinases.

Synthesis and biophysical evaluation of lipidated peptides and proteins

Introduction

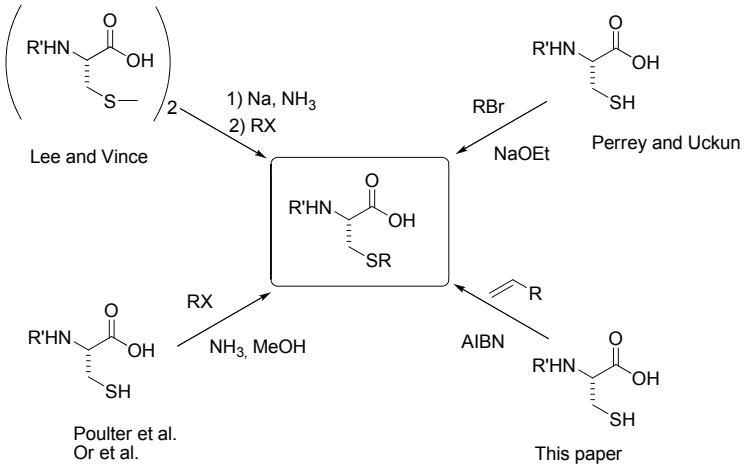
Lipid-modified proteins play important roles in numerous biological processes like signal transduction and vesicular trafficking. In order to study these roles in precise molecular detail they develop methods for the synthesis of differently lipidated peptides and proteins which may also carry further reporter groups and labels.

In order to study the biochemical properties and the biological functions of the Ras/Rab proteins they have developed methods that provide amounts of prenylated proteins with new functionalities such as fluorescence, photoreactivity, spin-labeled groups, non hydrolizable palmioyl analogs, or isoprenoid groups at non-native positions. By combining molecular biology techniques with organic synthetic methods, biologically active Ras and Rab proteins were built up and the influence of lipidation on their biological activity was studied in more detail.

The small GTPases H-, K-, and N-Ras control, for example, cell proliferation and differentiation. Mutations in this class of proteins lead to uncontrolled cell growth and cancer.[4] The Ras proteins are posttranslationally modified *via* lipidation of their C-termini. This creates a hydrophobic peptide stretch which confers membrane affinity to the proteins and is essential for their localization and functioning. N-ras is posttranslationally modified by farnesylation and palmitoylation.

Farnesylation is a stable, non-reversible, modification, but palmitoylation is a dynamic process due to the lability of the thioester moiety and corresponding palmitoylating and depalmitoylating enzymes. The palmitoylation process, its regulation and its influence on protein localization and functioning still poses open questions. In order to investigate these phenomena in detail we have previously prepared Ras proteins, both native and modified, *via* a combination of peptide synthesis, molecular biology and protein ligations. This has given important insights in the above listed processes. These studies have involved the synthesis of lipidated peptides featuring several differently lipidated cysteine building blocks. These included cysteines featuring the natural farnesyl and palmitoyl modifications, but also cysteines with non-natural modifications such as the hexadecylated cysteine **4**, which bears a hydrolysis-resistant hexadecyl thioether. This building block has been used for the synthesis of lipidated Ras-proteins whose palmitoyl functionality can not be hydrolysed anymore.

The typical methods used up to now by us and others for the preparation of these alkylated cysteine building blocks are based on the selective alkylation of the cysteine thiol functionality under basic conditions (Scheme 4).



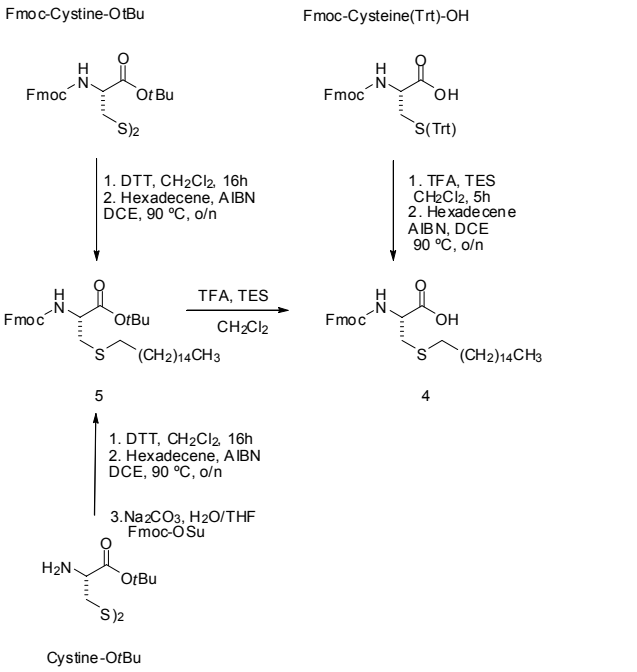
Scheme 4. Different strategies for the synthesis of alkylated cysteines.

The preparation of alkylated cysteines and the hexadecylated cysteine **4** specifically has, however, typically been characterized by low conversion under these nucleophilic conditions and difficult purification procedures. The addition of a thiyl radical to a multiple bond, also known as thiol-ene reaction, allows the formation of thioethers through radical-induced addition of thiols to alkenes. This reaction follows a mechanism in which a thiyl radical formed by a radical source adds to an alkene double bond, producing a carbon radical, which in turn can abstract a new hydrogen from another thiol and thus propagating the cycle. The results of our studies as well as the applicability of this reaction for the synthesis of differently substituted cysteines are discussed below.

Thiol functionalities adjacent to an ester group have been described to be more reactive in the thiol-ene reaction than those with a free carboxylic acid. Therefore, we used the conveniently protected cysteine derivative *N*-Fmoc, *O*-*tert*-butyl protected cystine (Fmoc-cystine-O*t*Bu). After reduction of the disulfide bond with dithiothreitol (DTT), the reaction of the resulting free thiol with hexadecene using AIBN as the radical initiator followed by removal of the *tert*-butyl group under acidic conditions afforded the hexadecylated cysteine **4** in an overall yield of 42% after 3 steps (Scheme 5 and Table 3, entry 1). The yield of the reaction sequence increased to 57% (in 4 steps) when first the radical alkylation was performed on reduced cystine-O*t*Bu, followed by Fmoc protection of the amino group (Table 3, entry 2). The analysis of these hexadecylated cysteines after the *tert*-butyl deprotection by chiral HPLC showed >99% enantiomeric excess, hereby proving the suitability of this method with respect to racemization free synthesis (Table 3, entry 5).

The thiol-ene reaction was also studied using Fmoc-cysteine(Trt)-OH, to determine the effects of the unprotected acid functionality on the reaction. Although in this case the yield obtained was lower than in the case of the cysteine methyl ester, the hexadecylated cysteine **4** could be isolated after two reaction steps in a 55% yield from Fmoc-cysteine(Trt)-OH (Table 3, entry 4). Lower yields but in a similar range were obtained when dichloroethane (DCE) was replaced by hexane/isopropanol as a solvent system (Table 3, entry 3). The yield decreased

to 43% when instead of three equivalents only one equivalent of the hexadecene was used, showing that an excess of one of the two reagents is beneficial.



DTT: dithiothreitol; AIBN: azobisisobutyronitrile; TES: triethylsilane; DCE: dichloroethane

Scheme 5. Different synthesis approaches to alkylated cysteine 4, based on the thiol-ene reaction.

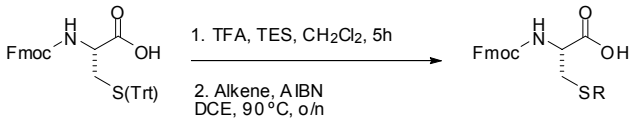
Table 3. Cysteine reagents and conditions for the synthesis of hexadecylated cysteine 4.

Entry	Thiol	Solvent	Overall yield of **4** (%)
1	Fmoc-Cystine-O*t*Bu	DCE	42
2	Cystine-O*t*Bu	DCE	57
3	Fmoc-Cysteine(Trt)-OH	Hexane/*i*-PrOH	47
4	Fmoc-Cysteine(Trt)-OH	DCE	55
5	Fmoc-Cysteine(Trt)-OH	DCE (1 eq. of alkene)	43

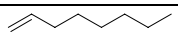
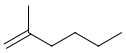
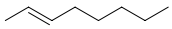
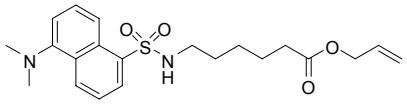
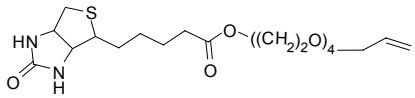
In order to demonstrate the general applicability of the method, a selected number of other alkenes were also reacted with the mercapto-group of the cysteine under the established conditions (Table 4). The thiol-ene reaction with Fmoc-cysteine(Trt)-OH proceeded in high yields both with the shorter 1-octene (Table 4, entry 1) and the branched 2-methyl-1-hexene (Table 4, entry 2). The internal alkene *trans*-2-octene also reacted under the same conditions although in lower yields (Table 4, entry 3). This reaction was not regioselective and yielded the two regioisomers **8a** and **8b**. The reaction is tolerant to a range of biologically relevant functional elements. It could be successfully applied for the attachment of the fluorescent dansyl derivative (**10)** (Table 4, entry 4) and a biotin marker (**12)** (Table 4, entry 5). These

reactions proceeded in satisfying yields taking into account that these were performed with equimolar amounts of the cysteine and the allyl functionalized dansyl or biotin derivative.

Table 4. Alkylation of Fmoc-cysteine(Trt)-OH with a variety of alkenes, using the alkene AIBN reaction.



TES:triethylsilane; AIBN: Azobisisobutyronitrile; DCE: dichloroethane

Entry	Alkene	Cp	Yield
1		**6**	57
2		**7**	91
3		**8a, 8b**	28
4	 **9**	**10**	35
5	 **11**	**12**	43

Concluding, herein we described a convenient and practical methodology for the synthesis of alkylated cysteines under non-racemizing conditions. The approach is widely applicable, to various alkyl groups and additional functional substituents. The use of the thiol-ene reaction has allowed the generation of several alkylated cysteines in satisfying yields. Complex substituents can be introduced onto the sulphur such as for compounds **10** and **12**, which would be difficult to perform using classic methodologies. Moreover, the experimental procedure is simple and easy to perform, uses inexpensive starting materials and avoids toxic reagents.

Influence of the Lipidation Motif on the Partitioning and Association of N-Ras in Model Membrane Subdomains

Introduction

The lipidation is believed to play an important role in Ras regulatory functions, e.g. by mediating protein protein and protein-lipid interactions. The association with different membrane microenvironments, including ordered raft domains, has been proposed to further regulate Ras signalling. The lateral organization and intermolecular interactions of lipids and proteins in biological membranes are issues under heavy scrutiny in the fields of membrane biochemistry, biophysics and cell biology. The existence of membrane subdomains with different lipid composition and the relationship between lipid-domain formation and the conformation and functional properties of membrane associated proteins is one of the central questions in this field. The Ras proteins are lipidated membrane bound GTPases which in their active GTP-bound form mediate numerous growth factor related signaling events. Misregulated Ras signaling is involved in the establishment of ca 30% of human cancers. It has been suggested that raft domains play a role in a wide range of important biological processes, including signal transduction pathways. Such raft domains could also act as "signaling platforms" that couple events on the outside of the cell with signaling pathways inside the cell. In addition, recent theoretical and experimental work suggests that the interfacial line tension between domains may play a major role affecting membrane organization, but there has been little evidence how this could affect lipoprotein partitioning and nanoclustering in membranes. So far, essentially imaging approaches on intact plasma membranes have been used, and the structural properties of the lipid anchor systems have been proposed to control the partitioning properties in membrane sub-compartments. In an effort towards a molecular level understanding of the interaction of the Ras proteins with lipid domains, our focus has been to carry out biophysical studies aiming at analyzing the distribution of Ras lipoproteins with modified anchor systems in multiphasic artificial membrane structures. By using semisynthetic fully functional lipidated proteins, e.g. a fluorescence labeled N-Ras bearing a farnesyl group and a hexadecyl moiety as a non-hydrolyzable mimic of the palmitoyl anchor (N-Ras HD/Far), the distribution of Ras between liquid-disordered and liquid-ordered (i.e., raft-like) subdomains and the orientation at the lipid interface was studied. This led us to the formulation of a rule for preferred insertion of N-Ras HD/Far into membrane subdomains. Recent NMR-spectroscopic determination of the structure of the Ras protein bound to a one-component phospholipid bilayer revealed a detailed picture describing how the entire protein is embedded into a lipid bilayer.

In a combined chemical biological and biophysical approach using time-lapse tapping-mode atomic force microscopy, we studied the partitioning of differently lipidated N-Ras proteins with various membrane-localization motifs into lipid domains of canonical model raft mixtures. The results provide direct evidence that partitioning of N-Ras occurs preferentially into liquid-disordered lipid domains, independent of the lipid anchor system. N-Ras proteins bearing at

least one farnesyl group have a comparable membrane partitioning behavior and show diffusion of the protein into the liquid disordered/ liquid-ordered phase boundary region, thus leading to a decrease of the unfavorable line tension between domains. In addition, except for the monofarnesylated N-Ras, strong intermolecular interactions foster self-association and formation of nanoclusters at the domain boundaries and may serve as an important vehicle for association processes and nanoclustering, which has also been observed in *in vivo* studies. No significant changes of the localization between GDP- and GTP-loaded N-Ras could be detected. Conversely, the non-biological dual-hexadecylated N-Ras exhibits a timeindependent incorporation into the bulk liquid-disordered phase to maintain high conformational entropy of its lipid chains.

By using time-lapse tapping-mode AFM, we were able to detect the partitioning of N-Ras lipoproteins with various membrane-localization motifs into lipid domains of canonical model raft mixtures. The results provide direct evidence that partitioning of GDP-loaded N-Ras occurs preferentially into liquid disordered lipid domains, independent of the lipid anchor system (Far/Far, HD/Far, HD/HD, Far). Upon incorporation of the single- and double-farnesylated N-Ras proteins, the thickness of the fluid bilayer decreases due to membrane disordering. Furthermore, we were able to demonstrate that GDP-loaded N-Ras proteins bearing at least one farnesyl anchor (N-Ras Far/Far, N-Ras HD/Far, N-Ras Far) have a comparable membrane partitioning behavior and show diffusion of the protein into the lo/ld phase boundary region with time. We may propose that the farnesyl anchor is largely responsible for the clustering of N-Ras proteins in the interfacial regions of membrane domains (Figure 1), thus leading to a decrease of the line energy (tension) between domains. Upon insertion at the interfacial domain boundaries, the line tension between domains decreases, which in fact depends quadratically on the phase height mismatch.

Notably, no pronounced clustering is observed for the monofarnesylated N-Ras Far, which is less stably inserted into the lipid membrane. Hence, line tension is likely to be one of the key parameters controlling not only the size and dynamic properties of rafts, but also of signaling platforms. Owing to the prevalence of interfaces in natural membranes, this general physico-chemical line tension effect that we have observed is likely to be extendable to the plasma membrane situation as well. Such an interfacial adsorption effect can generally be expected in many-phase lipid systems for inserting proteins that have no particular preference for any particular phase − for example due to hydrophobic mismatch and/or due to entropic reasons − so that the proteins are expelled to the boundary. It is clear that the localization and accumulation of proteins in the domain interfaces of a lipid bilayer increases the effective concentration and may provide particularly strong and direct protein protein interactions and hence may serve as an important vehicle for association processes of signalling proteins in membranes.

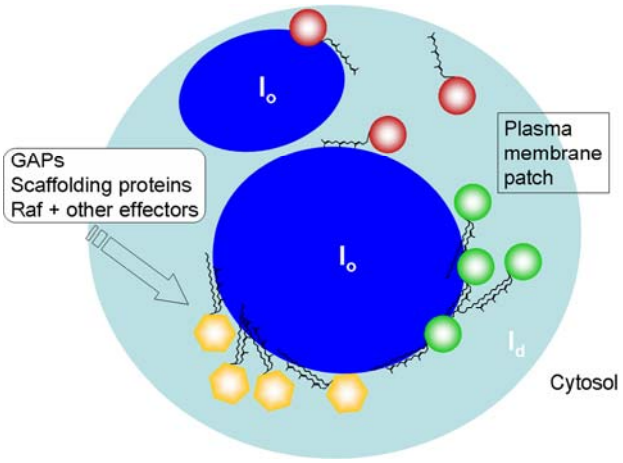


Figure 1. Model for N-Ras localization in heterogeneous membranes with liquid-disordered (ld, light blue) and liquid-ordered (lo, blue) domains. GDP-loaded farnesylated Ras (red) partitions into the fluidlike phase of the membrane and subsequently diffuses to the ld/lo phase boundaries of the subcompartments. When Ras is farnesylated and palmitoylated (green), strong intermolecular interactions foster self-association and formation of nanoclusters at the domain boundaries, which might bserve as reaction platform for GTP activation (yellow) and for recruitment of further membranous and cytosolic regulators as well as downstream effectors of the Ras signaling pathway, such as Gap, and Raf.29,46,47 In addition, scaffolding proteins might be involved in this process. Conversely, the dual-hexadecylated N-Ras exhibits a time-independent incorporation into the bulk liquid-disordered phase.

It might have been envisaged that the N-Ras HD/HD protein with its long saturated and unbranched lipid chains is prone to partition into ordered raft-like domains. This is clearly not the case. The most likely explanation is that by remaining in the fluid-like phase and being integrated in the collective flexibility and dynamics of the lipid bilayer, the high conformational entropyof the two lipid chains separated by a dynamic peptide linker is retained. As revealed by recent NMR experiments, such an effect is probably fostered by the high-amplitude dynamics of the C-terminal peptide linker region. Thus, only the more rigid, but bulkier farnesyl moiety of the N-Ras protein seems to be essential for interfacial self-association and formation of nanoclusters. Such an effective concentration of signaling proteins into discrete nanodomains in the membrane may in fact be expected to increase the efficiency and specificity of signaling events. Interestingly, besides a high population of monomers, nanoclusters of similar sizes (radius of □6−12 nm) have been found in the plasma membrane as well. It is assumed that N-Ras Far and N-Ras HD/Far resemble the depalmitoylated and palmitoylated forms of the natural N-Ras, respectively, in terms of the chemical nature of the lipid anchors. Palmitoyl and hexadecyl differ only in the thioester and thioether bond connecting the fatty acid chain with the Ras peptide; the saturation, conformation and length of the fatty acid chains are identical. Thus, similar interactions of these membrane anchors with the canonical raft membranes can be envisaged. Concerning our finding that GDP-

loaded N-Ras HD/Far and N-Ras Far display a similar membrane partitioning behavior, we propose that the main function of the natural palmitoyl anchor of N-Ras resides predominantly in the residence time in a particular cellular membrane compartment in the course of the acylation/deacylation cycle. This acylation/deacylation cycle was discovered recently and revealed that the dynamic property of the palmitate moiety, i.e. the reversibility of palmitoylation, is critical for the correct localization of N-Ras in a particular cellular membrane, such as the Golgi or plasma membrane. Farnesylation alone is not sufficient to stably anchor Ras to the plasma membrane, so loss of palmitate will lead to dissociation of Ras from the plasma membrane. The stability of palmitate attachment thereby dictates the steady-state distribution and the speed of exchange of the plasma membrane and Golgi pools. The cycle has been proposed to operate independently of the activation state of Ras. In fact, our data point to such behavior also in the heterogeneous model membrane system studied, as no significant changes of the localization between GDP- and GTP-loaded N-Ras HD/Far could be detected. GTP-bound N-Ras HD/Far differs from GDP-bound N-Ras HD/Far in showing different clustering propensities, only. These results are in contrast to previous experiments using a monopalmitoylated H-Ras as a N-Ras mimetic, which suggested that GTP/GDP-loading might influence the plasma membrane microdomain localization of N-Ras analogous to H-Ras. However, recently it was demonstrated that H-Ras contains in addition to the lipid anchor two further domains that regulate the membrane association of H-Ras: the hypervariable linker domain and the N-terminal catalytic domain, with the latter being modulated by conformational changes caused by GTP-loading. Whereas the N-terminal G-domain of H-Ras has been shown to influence the localization of H-Ras within the plane of the plasma membrane, there is evidence that the N-Ras G-domain regulates gross compartmentalization (subcellular localization). Therefore, the N-terminal domain does not seem to influence the plasma membrane microdomain localization of N-Ras, which is also supported by our recent data and previous experiments studying the membrane partitioning behavior of lipidated N-Ras peptides corresponding to the C-terminal segment of N-Ras.

To conclude, the aim of this study was to understand the mechanism of interaction of different lipidated proteins, i.e. the membrane-localization motif itself, with lipid domains of canonical model raft mixtures. This issue has been a matter of severe debate in the last years, in particular in cell biological studies, where, owing to the complexity of the system, such interaction mechanisms are difficult to reveal. Certainly, the observed interaction processes occur on a faster time scale *in vivo* due to a different dynamics in the complex biological cell compared to an AFM fluid cell. However, the underlying biophysical mechanism for the partitioning process will not be influenced by the different dynamics. Moreover, previous studies have shown that the spontaneous intermembrane transfer of dually lipid anchored Ras peptides may also occur on the order of hours. Even if the *in vivo* acylation/deacylation cycle is not present in this model system, a general understanding of the interaction mechanism of membrane anchored proteins with heterogeneous raft membranes is gained

from our study. These data also provide the basis for further studies involving different types of cellular interaction partners with Ras, like Raf and other effectors, which will aid in interpreting cell biological work on Ras signaling.

El treball dut a terme durant l´estada postdoctoral ha donat lloc fins al moment a les següents publicacions:

- G.Triola, S. Wetzel, B. Ellinger, M.A. Koch, K. Hübel, D. Rauh, H. Waldmann, ATP competitive inhibitors of d-alanine-d-alanine ligase based on protein kinase inhibitor scaffolds. *Bioorg. Med. Chem,* **2008** Feb 16 [Epub ahead of print]

- G.Triola, L. Brunsveld, H. Waldmann, Racemization-free synthesis of S-alkylated cysteines via thiol-ene reaction. *J Org Chem*. **2008**; 73(9):3646-9.

- K. Weise , G. Triola , L. Brunsveld , H. Waldmann , R. Winter; Influence of the Lipidation Motif on the Partitioning and Association of N-Ras in Model Membrane Subdomains, *J. Am. Chem. Soc*, accepted for publication (ja-2008-08691r)

I ha estat presentat en les següents conferències

- Comunicació Oral en el XI Encuentro Peptídico Ibérico, Santiago de Compostela, Març 2008.
- Poster a la EMBL Conference on Chemical Biology, Octubre 2008

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