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Original article

Tertiary amides with a five-membered heteroaromatic ring as new probes for the translocator protein

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ABSTRACT

In this study novel ligands of the translocator protein (TSPO), characterized by a five-membered aromatic heterocycle (i.e. oxazole, isoxazole, oxadiazole), a phenyl ring, and an amide side chain of carboxy or acetic type, were designed using a previously reported pharmacophore/topological model. Most of compounds showed significant TSPO binding affinity (K_i values in the nanomolar/submicromolar range), the highest being displayed by oxazolacetamides **6**. A number of compounds were tested for their ability to inhibit the proliferation/viability of human glioblastoma cell line U87MG. The dose-time dependent cell response to treatment with **6d** demonstrated the specificity of the observed effect. The ability of **6d** to induce mitochondrial membrane dissipation ($\Delta\Psi m$) substantiates the intracellular pro-apoptotic mechanism activated by ligand binding to TSPO.

1. Introduction

The 18 kDa translocator protein (TSPO) in the cell is primarily localized in the outer/inner mitochondrial membrane as a component of the megachannel responsible for mitochondrial permeability transition (MPT). As a major component of the mitochondrial membrane, TSPO mediates various mitochondrial processes including cholesterol transport and steroid hormone synthesis, mitochondrial respiration, calcium homeostasis, lipid metabolism, regulation of immune functions, apoptosis and cell proliferation [1,2]. It has been observed that cancer cells (*eg* from breast, ovary, colon, prostate and brain) express high levels of TSPO and result sensible to apoptotic cell death induced by binding of ligands to this protein, thus suggesting TSPO as a potential anti-cancer drug target. Actually, classic TSPO ligands as the isoquinolinecarboxamide derivative PK 11195 [3] and the benzodiazepine Ro5-4864 [4], as well as our previously synthesized 2-phenylindolglyoxylylamide derivative PIGA [5,6], are able to produce MPT-pore opening that lead to dissipation of the mitochondrial membrane potential ($\Delta\Psi m$), one of the early events of the apoptotic cascade activation [7,8].

In this view, the development of TSPO ligands characterized by suitable physical properties may provide both useful tools for studying MPT-pore functioning and identifying potential therapeutic agents against tumours.

Although human, bovine, and murine TSPOs have been isolated, cloned and sequenced [9], the three-dimensional structure of TSPO has not yet been determined, as its close association to the membrane makes the purification and crystallization processes very difficult to accomplish. Models of the secondary and tertiary structures of the TSPO have been proposed [10,11], which hypothesize five α-helices composed of 21 residues, the N-terminus and C-terminus being exposed to the mitochondrial and cytoplasmic spaces, respectively. Furthermore, site-directed mutagenesis studies demonstrated that the portion of the receptor which recognizes ligands such as PK 11195 is located on the first cytoplasmic loop [11].

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Analysis of the TSPO sequence clearly shows that this protein is not homologous to any other known protein, apart from a significant relationship with CrtK, a member of the protein family responsible for the carotenoid biosynthesis [12,13]. Unfortunately, the CrtK three-dimensional structure has not yet been solved and such a circumstance makes unfeasible homology building approaches to construction of TSPO three-dimensional models. This implies that the rational design of new TSPO ligands, as done in the present work, must necessarily rely on ligand-based methods.

Some years ago we proposed a pharmacophore/topological model of TSPO ligands after superimposing the structures of PK 11195 and Ro5-4864, and some pyrrolobenzothiazepine derivatives (Fig. 1) [14]. The model (see Fig. 2) is made up by a ligand heteroatom accepting an H-bond from a receptor site (H1 site), and three lipophilic pockets: the first one (L1 site) hosting an aromatic ring and the latter two (L3 and L4 sites) accommodating either aromatic or aliphatic substituents. Starting from the results obtained at the date, we designed several novel classes of TSPO ligands [5,15–21], among which the 2-phenylindolylglyoxylamides, including the dichlorosubstituded derivative PIGA, recently emerged for their high affinity and selectivity (Fig. 1).

In this paper, we report the synthesis, the characterization, and the binding data of new probes for TSPO of general formula **I** and **II** (Fig. 2), where "Het" is a five-membered aromatic heterocycle (oxazole, isoxazole, oxadiazole), and the amide side chains are carboxy and acetic type, respectively.

According to our model, the designed structures should accept an H-bond from the H1 site via the carbonyl oxygen, orient their pendant phenyl ring into the L1 pocket and fill the L3 and L4 pockets with the R_1 and R_2 substituents. The choice of such heteroaromatic rings as scaffolds was dictated by their capability of conferring to structures of type **I** and **II** a drug-like character according to the principles of Lipinski's rule [22]. In terms of physical properties, two or three heteroatoms in the aromatic scaffold contribute to increase the water solubility of these otherwise extremely hydrophobic structures. The lack of H-bond donor functions in the selected heteroaromatic rings significantly simplified the synthetic strategies whose last step is a reaction between an acyl chloride or a carboxylic acid with a secondary amine.

The type **I** series of compounds was firstly synthesized (Fig. 3). Substituents R_1 and R_2 and X were selected by taking into account the structure–affinity relationships reported in literature for various TSPO ligands. With regard to the side chain, we synthesized mostly "symmetric" ($R_1 = R_2$ = linear alkyl chains) as well as

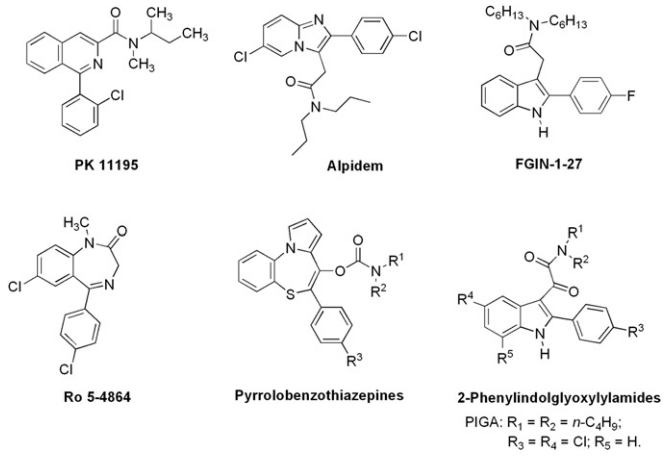


Fig. 1. High affinity ligands to TSPO.

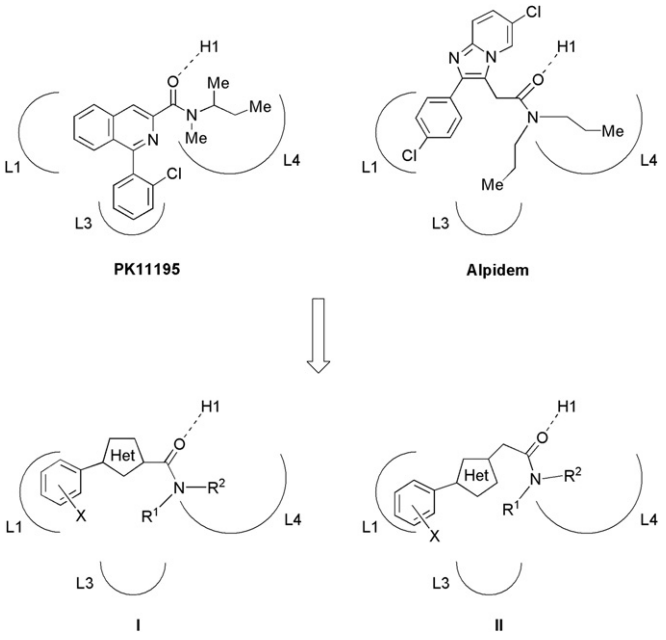


Fig. 2. Design of structures **I** and **II** guided by a pharmacophore/topological model of TSPO ligands.

a few "asymmetric" ($R_1 \neq R_2$) amides substituted by groups formerly emerged as significant in the SAR studies of 2-phenylindolglyoxylylamides [15], such as methyl/*n*-butyl and ethyl/benzyl.

Compounds type **II** (Fig. 4) feature a methylene spacer between the heterocyclic ring and the amide nitrogen in analogy to alpidem and FGIN-1-27 (Fig. 1). The set of R_1 and R_2 substituents of the new acetic derivatives **5** and **6** was limited to identical alkyl chains with a length comprised in the range C_3–C_6. Given the "explorative" character of our work, the X substituents at the *para* position were restricted to H, Cl, F, and CH_3.

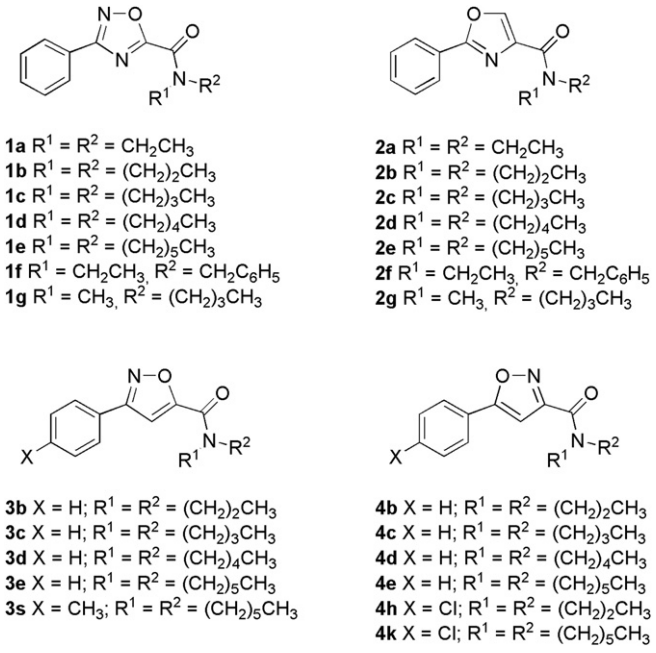


Fig. 3. Type **I** structures.

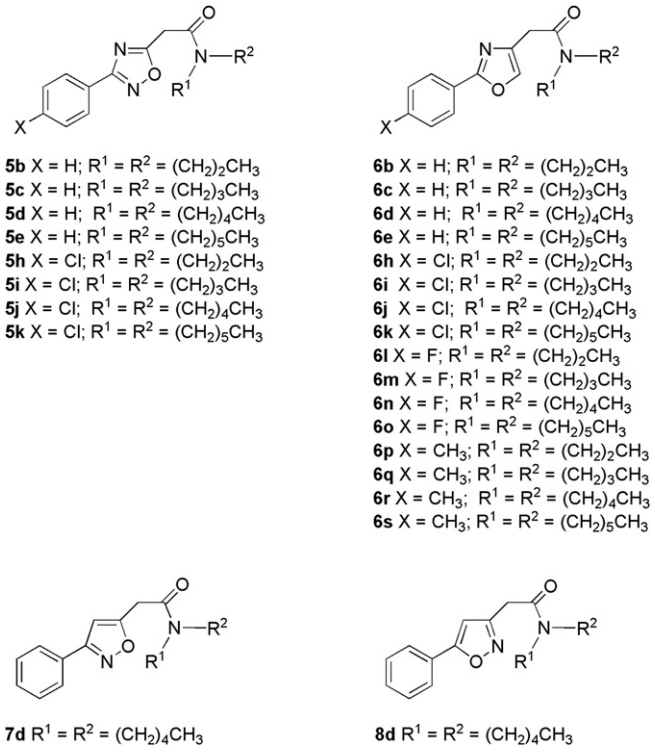
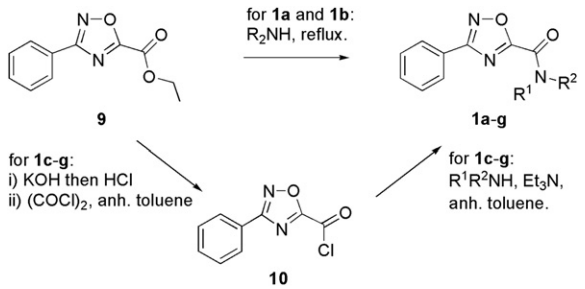


Fig. 4. Type **II** structures.

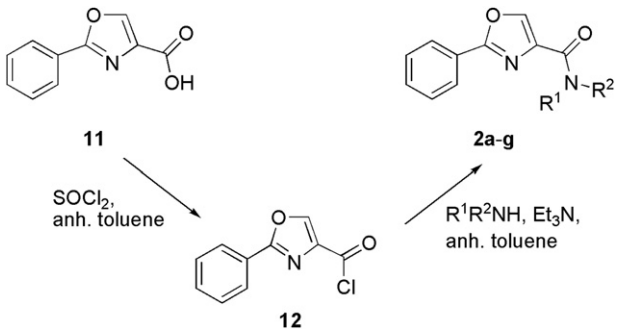
2. Chemistry

The synthetic pathway followed to obtain 1,2,4-oxadiazolecarboxamides **1a**–**g** is outlined in Scheme 1; the key intermediate was the ethyl 3-phenyl-1,2,4-oxadiazole-5-carboxylate (**9**), prepared in accordance with a reported procedure [23]. The ester **9** was directly converted to the desired final amides **1a**–**b** by heating at reflux with a large excess of diethylamine and dipropylamine, respectively, easily removed by distillation under reduced pressure during the work-up of the reaction. Derivatives **1c**–**g**, characterized by bulkier side chains, were synthesized through the oxadiazole-5-acyl chloride **10** [24], with a stoichiometric equivalent of the secondary amine, in anhydrous toluene, at room temperature, in the presence of triethylamine.

For the synthesis of oxazolecarboxamides **2a**–**g** (Scheme 2), the 2-phenyloxazole-4-carboxylic acid (**11**) was prepared following a described procedure starting from the commercially available hippuric acid [25,26]. The acid **11** was converted into the corresponding acyl chloride (**12**) and then reacted with the appropriate secondary amines in anhydrous toluene, in the presence of triethylamine, to give the oxazolecarboxamides **2a**–**g**.



Scheme 1. For R_1R_2NH see Fig. 3.



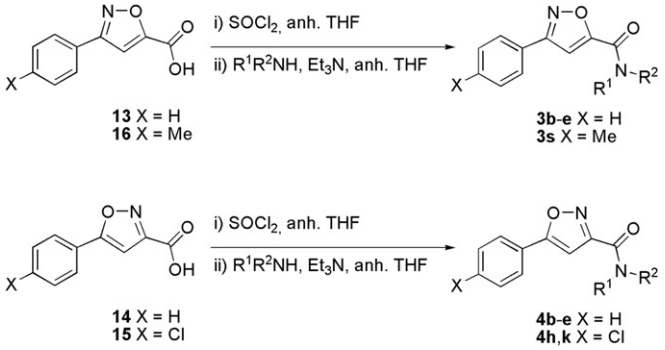
Scheme 2. For R_1R_2NH see Fig. 3.

Compounds **3b**–**e** and **4b**–**e**,**h**,**k** were prepared starting respectively from commercially available 3-phenylisoxazole-5-carboxylic acid (**13**) or 5-phenylisoxazole-3-carboxylic acid (**14**), or 5-(4-chlorophenyl)isoxazole-3-carboxylic acid (**15**) via preliminary conversion into their correspondent non-isolated acyl-chlorides and successive reaction with the appropriate amine (Scheme 3).

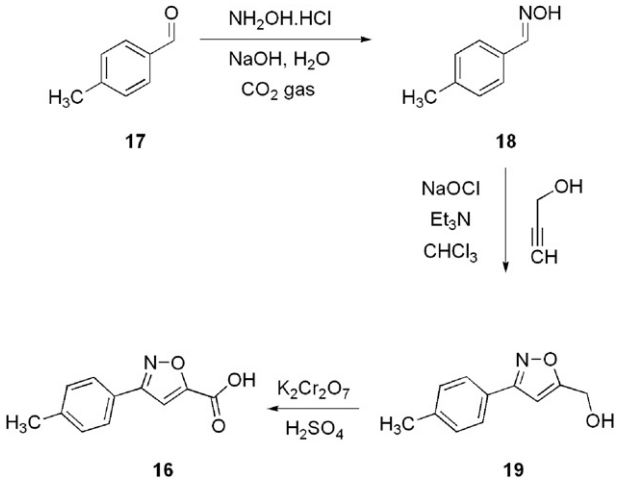
The [3 + 2] cycloaddition of alkenes/alkynes with *in situ* generated nitrile oxides is the most convenient method for the synthesis of five-membered heterocycles as isoxazoles [27–31]. So, the 3-(4-methylphenyl)isoxazole-5-carboxylic acid (**16**) was obtained as depicted in Scheme 4: the 4-methylbenzaldehyde (**17**) was converted into the corresponding oxime **18** that, by reaction with prop-2-yn-1-ol, gave the [3-(4-methylphenyl)isoxazol-5-yl] methanol (**19**); subsequent oxidation with $K_2Cr_2O_7$ gave the desired **16** (Scheme 4), which furnished, analogously, the amide **3s** (Scheme 3).

The synthetic route to obtain compounds characterized by an oxadiazoleacetamide moiety **5b**–**e**,**h**–**k** (Fig. 4) required the preparation of the corresponding unsubstituted and 4-chlorophenylsubstituted 2-aryloxadiazol-4-yl acetic acids **20** and **21**, performed adopting our previously reported procedure [32]. The acids **20** and **21** were condensed with the appropriate secondary amines in anhydrous tetrahydrofuran solution and in the presence of carbonyldiimidazole (CDI), to give the target products **5b**–**e**,**h**–**k** (Scheme 5).

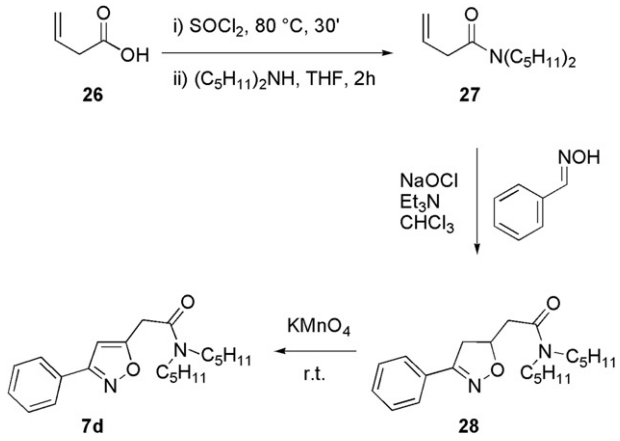
The synthesis of oxazoleacetamides **6b**–**e**,**h**–**s**, depicted in Scheme 5, consisted initially in the preparation of (2-phenyl-1,3-oxazol-4-yl)acetic acid (**22**) [32], and of 4-chloro-, 4-fluoro, 4-methyl-phenyl analogues (**23**) [33], (**24**) [32], and (**25**) [33], respectively, according to previously reported procedures. Then, the acids **22**–**25** furnished the final amides **6b**–**e**,**h**–**s** via the



Scheme 3. For R_1R_2NH see Fig. 3.



Scheme 4. Synthesis of the 5-carboxylic acid **16**.



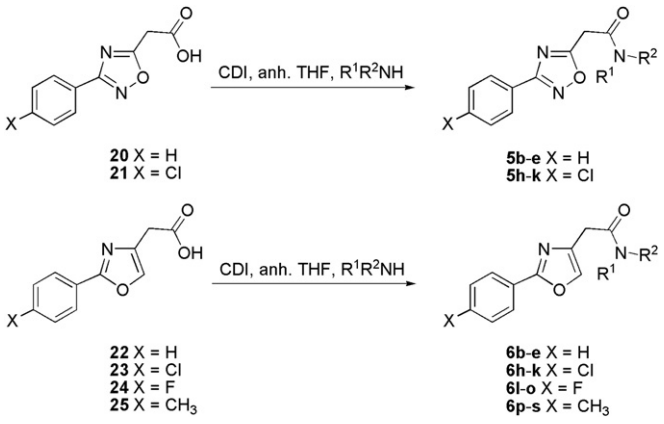
Scheme 6. Synthesis of the acetamide **7d**.

reaction with the opportune amines, under the same conditions used to synthesize oxadiazoleacetamides **5b**–**e**,**h**–**k**.

The preparation of *N*,*N*-di-*n*-pentyl-2-(3-phenylisoxazol-5-yl) acetamide (**7d**) and *N*,*N*-di-*n*-pentyl-2-(5-phenylisoxazol-3-yl) acetamide (**8d**) was not carried out starting from the corresponding isoxazolylacetic acids, due to the difficulty of their obtainment through the 1,3-cycloaddition reaction.

In brief, in our hands, the 1,3-cycloaddition reaction between the *in situ* generated nitrile oxide and the vinylacetic acid **26** did not give the desired Δ^2-isoxazoline. The procedures selected for the synthesis of **7d** and **8d** are depicted in Schemes 6 and 7. The amide **27**, obtained by the vinylacetic acid **26** via the relevant acylchloride, was the dipolarophile for the cycloaddition reaction with the benzaldoxime (Scheme 6). The Δ^2-isoxazoline **28** was oxidized to the desired isoxazole **7d** with potassium permanganate. The oxidizing agent, although did not give a satisfactory yield, was however the best one among the attempted (Scheme 6).

The 3-alkylsubstituted-5-arylisoxazoles are typically obtained by a nitroalkyl derivative and phenylacetylene by using Mukaiyama's procedure [34], but in our case this procedure or a number of its modifications were not fully satisfactory. First of all, also in this case, the carboxylic group is not compatible with the cycloaddition reaction. We performed the synthesis of amide **30** analogously to the precedent amide **27** with similar yield (Scheme 7). On the contrary, the subsequent cycloaddition reaction of **30** with the phenylacetylene to obtain **8d** showed a very low yield; the reported one is the best result of our attempts in which we changed the reaction conditions.



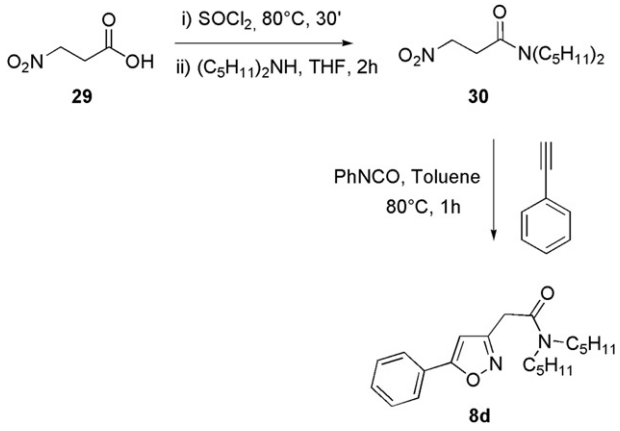
Scheme 5. For R_1R_2NH see Fig.4.

3. Biology

The binding affinity to TSPO of all the newly synthesized compounds **1**–**8** was determined by analyses of their competition on the [^{3}H]PK 11195 binding in membrane homogenates derived from kidney, a tissue rich in TSPO. The binding affinity of compounds to TSPO, expressed as K_i values, are reported in Tables 1 and 2. The subset of amides **6b**–**e** was also evaluated for the binding affinity to the central benzodiazepine receptor (CBR), using [^{3}H]Ro15-1788 as the radioligand. The compounds, tested at 10 μM concentration, showed no binding properties in this last assay, with inhibition percentages <25%, demonstrating their selectivity for TSPO (data not shown).

The compounds showing the best TSPO binding affinity, namely compounds **6d** (K_i 32 nM), **6j** (K_i 45 nM), **6r** (K_i 68 nM), **6n** (K_i 11 nM) and **8d** (K_i 51 nM), were selected to evaluate their ability to influence the proliferation/viability of the human glioblastoma cell line U87MG. As initial screening, U87MG cells were exposed for 24 h at a single concentration of each compound, corresponding to 1000 times the K_i value. On the basis of the results obtained, compound **6d** was in depth investigated to assess whether the observed effect was dose-and time-dependent, by exposing the cells to a single incubation time (24 h) and increasing **6d** concentrations (dose-dependent treatment) and to a single concentration of **6d** for different times (time-dependent treatment).

Finally, being depolarisation of mitochondrial membrane the early intracellular event of apoptosis activated by TSPO ligands, the efficacy of **6d** to induce mitochondrial membrane dissipation ($\Delta\Psi$m) of U87MG cells was evaluated.



Scheme 7. Synthesis of the acetamide **8d**.

Table 1
Receptor binding affinity of compounds **1**–**4** (structures **I**) for TSPO.

Cmpd	X	R^1	R^2	K_i (nM)[a] or % of inhibition at 10 μM.
1a	H	CH_2CH_3	CH_2CH_3	>10,000
1b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	>10,000
1c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	772 ± 54
1d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	914 ± 87
1e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	4193 ± 125
1f	H	CH_2CH_3	$CH_2C_6H_5$	4873 ± 310
1g	H	CH_3	$(CH_2)_3CH_3$	>10,000
2a	H	CH_2CH_3	CH_2CH_3	>10,000
2b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	>10,000
2c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	3462 ± 260
2d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	613 ± 45
2e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	4601 ± 370
2f	H	CH_2CH_3	$CH_2C_6H_5$	5487 ± 455
2g	H	CH_3	$(CH_2)_3CH_3$	>10,000
3b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	>10,000
3c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	651 ± 56
3d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	379 ± 25
3e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	7000 ± 568
3s	4–CH_3	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	2700 ± 158
4b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	>10,000
4c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	620 ± 47
4d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	382 ± 25
4e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	5800 ± 568
4h	4-Cl	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	>10,000
4k	4-Cl	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	>10,000
PK11195				9.3 ± 0.5
Ro 5-4864				23 ± 3.1

[a] The concentration of tested compounds that inhibited [^{3}H]PK11195 binding at rat kidney mitochondrial membranes (IC_{50}) by 50% was determined with six concentrations of the displacers, each performed in triplicate. K_i values are the means ± SEM of three determinations.

4. Discussion

Tables 1 and 2 list the binding affinities at the TSPO of the heteroarylcarboxamides **I** and heteroarylacetamides **II**, respectively, expressed as K_i values or percentages of inhibition of [^{3}H]PK 11195 binding.

Within compounds of type **I**, several amides showed low or moderate affinity to TSPO (Table 1). The affinity trend was similar for oxadiazole, oxazole and isoxazole series, with the best results (micromolar K_i values) being achieved with $R_1 = R_2 =$ *n*-butyl, *n*-pentyl; in particular, derivatives **1c, 2d**, **3d**, **4d** were the most active in their respective subsets.

Compounds with one, or both amide alkyl chains shorter than 4 carbon atoms showed to be ineffective (carboxamides **1a**, **1b**, **1g**; **2a**, **2b**, **2g**; **3b**; **4b**).

N-Ethyl-*N*-benzyl amides, namely **1f**, **2f**, did not perform better than their "symmetric" counterparts of similar molecular weight (**1e** and **2e**).

The K_i values of **1d**, **2d**, **3d** and **4d**, all bearing two *n*-pentyl groups on the amide nitrogen and differing only in the heterocyclic ring, vary from 379 nM to 914 nM. Such a narrow range of potency suggested that the four different heterocyclic rings were more or less equivalent without possessing any particular element in interacting with the TSPO binding cleft.

The introduction of a chlorine or a methyl group at the *para* position of the phenyl ring of a few derivatives did not give any appreciable improvement in affinity (compare **3s** *vs* **3e**, **4h** *vs* **4b** and **4i** *vs* **4e**). Thus, further attempts in optimizing potency in series **1**–**4** by the introduction of other substituents (X) were not made.

Although affinity was low throughout the series of the synthesized compounds, the best performing amides could be considered a starting point for the design of new TSPO ligands, thus the subsequent step performed was the modification of the amide side chain.

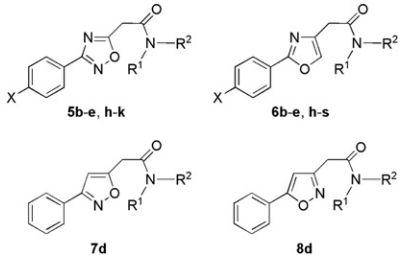
Compared with compounds of series **I**, those of type **II** feature a methylene spacer between the heterocyclic ring and the amide nitrogen. Since symmetrical di-*n*-butyl- and di-*n*-pentyl-carboxamides exhibited the best affinity within the series **1**–**4**, the synthesis of the new acetic derivatives **5** and **6** was focused on compounds bearing R_1 and R_2 with a length comprised in the range C_3–C_6.

The replacement of the carboxamide with an acetamide side chain resulted in a significant general enhancement of affinity (Table 2), both in oxadiazoles **5b**–**e** and in oxazoles **6b**–**e**, with compound **6d** ($R_1 = R_2 =$ *n*-pentyl) showing $K_i = 32$ nM.

As several of the most potent TSPO ligands (PK 11195, Ro 5-4864, alpidem, and the indolylglyoxylamide PIGA - Fig. 1) possess a chlorine on their pendant phenyl ring, both oxadiazoleacetamides and oxazoleacetamides were likewise functionalized to give derivatives **5h**–**k**, **6h**–**k** with X = Cl. Actually, such a modification increased affinity only for compounds with short *N*-alkyl chains (compare **5h** *vs*. **5b**, and **5i** *vs*. **5c**), whereas it was slightly disadvantageous for compounds with longer *N*-alkyl chains (compare **5j** *vs*. **5d**, and **5k** *vs*. **5e**).

Further attempts to improve affinity were focused on oxazoleacetamides **6** by inserting at the *para* position of the phenyl ring a small electron-withdrawing or electron-donating group (X = F,

Table 2
Receptor binding affinity of compounds **5**–**8** (structures **II**) for TSPO.



Cmpd	X	R^1	R^2	K_i (nM)[a]
5b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	4340 ± 370
5c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	489 ± 36
5d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	133 ± 10
5e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	1100 ± 100
5h	4-Cl	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	3400 ± 250
5i	4-Cl	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	267 ± 18
5j	4-Cl	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	152 ± 11
5k	4-Cl	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	3200 ± 235
6b	H	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	1698 ± 150
6c	H	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	346 ± 25
6d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	32 ± 4
6e	H	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	151 ± 12
6h	4-Cl	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	942 ± 85
6i	4-Cl	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	177 ± 15
6j	4-Cl	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	45 ± 3
6k	4-Cl	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	704 ± 56
6l	4-F	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	1630 ± 190
6m	4-F	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	88 ± 3
6n	4-F	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	11 ± 1
6o	4-F	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	157 ± 14
6p	4–CH_3	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	2200 ± 188
6q	4–CH_3	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	487 ± 41
6r	4–CH_3	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	68 ± 7
6s	4–CH_3	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	877 ± 65
7d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	257 ± 20
8d	H	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	51 ± 5
PK11195				9.3 ± 0.5
Ro5-4864				23 ± 3.1

[a] The concentration of tested compounds that inhibited [^{3}H]PK11195 binding at rat kidney mitochondrial membranes (IC_{50}) by 50% was determined with six concentrations of the displacers, each performed in triplicate. K_i values are the means ± SEM of three determinations.

CH_3). Affinity was increased by the presence of a *p*-fluorine (**6n**, $K_i = 11$ nM), whereas it was slightly diminished by a *p*-methyl.

Taken together, the above results suggest that: i) the pendant phenyl ring of our ligands might be involved in a π-stacking interaction with an electron-rich aromatic moiety of the TSPO binding cavity; ii) the X substituent face the steric boundaries of the L1 pocket. Both hypotheses are consistent with the electron-withdrawing and steric properties of the X substituent beneficial and, respectively, unfavourable to potency in series **6**. Thus, the *p*-fluorine, electron-withdrawing and small, is the best one; the methyl electron-donating and relatively bulky is the worst one; the hydrogen and the *p*-chlorine exert weaker effects on affinity according to their intermediate electronic properties and dimensions.

Inside every subset of four compounds with amide side chains ranging from C_3 to C_6 (**5b**–**e**, **5h**–**k**, **6h**–**k**, **6l**–**o**, **6p**–**s**), affinity followed the same order, that is di-*n*-pentyl > di-*n*-butyl > di-*n*-hexyl > di-*n*-propyl, with the only exception for oxazoleacetamides unsubstituted on the pendant phenyl **6b**–**e**, where the di-*n*-hexyl derivative is about two-fold more potent than the di-*n*-butyl one.

Concerning the isoxazole series, we prepared and evaluated only the acetamides **7d** and **8d** owing to a more difficult synthetic route (see Chemistry). The *n*-pentyl groups attached to the amide nitrogen of these two compounds were chosen as those best performing in the series **5** and **6**. Indeed, **8d** displayed a potency ($K_i = 51$ nM) comparable with that of **6d** ($K_i = 32$ nM).

The observation that the most active compounds were always the di-*n*-pentyl ones, both in the carboxamide and in the acetamide derivatives, suggests that these alkyl chains possess the best steric complementarity with the lipophilic pockets L3 and L4, once the basic scaffold of the molecule has been anchored to the receptor. Moreover, the heterocyclic core engages better interactions within the receptor binding cleft when the link of the side amide function is longer and more flexible (acetic type).

Differently from the results obtained with the above discussed carboxamides (Table 1), in which the nature of the heterocyclic core seemed not to be crucial in the receptor site interaction, a comparison of the K_i values of the four acetamides differing in the heterocycle ring and bearing two *n*-pentyl on the amide nitrogen (**5d**, **6d**, **7d** and **8d**) reveals that **6d** and **8d** (K_i values of 32 nM and 51 nM, respectively) are significantly more potent than **5d** and **7d** (K_i values of 133 nM and 257 nM, respectively). These findings might be attributed to the different H-bonding geometric properties of nitrogen and oxygen as acceptors in aromatic heterocycles. Nobeli and co-workers investigated the directionality and relative strengths of H-bonds between C–OH and aromatic rings containing nitrogen and/or oxygen heteroatoms, using crystal structure data and theoretical calculations [35]. These authors showed that the H-bonds in the C–OH···O(furan) system are more widely scattered around the aromatic ring plane compared with the C–OH···N(pyridine) system. More specifically, the interaction energy is destabilized up to 5.0 kJ/mol upon deviations of the C–OH hydrogen from the pyridine plane by 25–35° or from the furan plane by 50°. In the quoted article, Nobeli's group concluded that "substituting oxygen for nitrogen heterocycles will generally have a marked effect on the binding of a protein ligand" [35].

In light of the above-mentioned studies, given that the TSPO binding site might contain an H-bond donor group placed out of the plane of the ligand heterocycle, only the endocyclic oxygen of **6d** and **8d**, but not the corresponding nitrogen of **5d** and **7d**, would form a significantly attractive H-bond with the receptor protein.

Compounds **6d, 6j, 6r, 6n** and **8d** showing the best values of binding affinity to TSPO (nanomolar K_i values), were assayed *in vitro* to determine their ability to induce death in cancer cells. The human U87MG cell line of glioblastoma multiforme (GBM, Grade IV astrocytoma), characterized by high TSPO density and high degree of resistance to current conventional DNA alkylating chemotherapy, was selected as experimental model. In the initial screening, the compounds were examined for their ability to inhibit proliferation/viability of U87MG cells at the concentration of 1000 times their K_i value after 24 h incubation time (see Fig. 5). After the cell exposure with each compound the percentage of proliferating/viable cells were calculated with respect to control cells (100%) and resulted: 9.3 ± 2.1% for **6d**, 72.6 ± 2.1% for **6j**, 5.3 ± 0.9% for **6r**, 98 ± 1.2% for **6n** and 24.8 ± 1.6% for **8d** (Fig. 5). The Fig. 5 shows also the percentage of proliferating/viable cells exposed to the TSPO ligand PIGA (76.0 ± 1.7%), used as reference compound. Among the tested compounds, **6d** showed the best balance in binding affinity value and efficacy in inhibiting proliferation/viability of U87MG cells, thus it was further investigated to exclude aspecific cytotoxic effects. In particular, experiments were performed to assess whether the observed effect was dose- and time-dependent.

Concerning the dose-dependent treatment, cells were exposed to a single incubation time (24 h) and increasing **6d** concentrations, corresponding to 15, 30 and 150 times K_i value. Cell treatments with **6d** concentrations of 30 and 150 times K_i value revealed a significant reduction of living cells with respect to control ($p < 0.01$) (Fig. 6A). Concerning the time-dependent cell treatment, U87MG cells were exposed to a single concentration of **6d** (30 times of K_i value) for 24, 48 and 72 h. As shown in Fig. 6B, cell treatment with **6d** significantly inhibits the exponential growth of cells after 48 and 72 h incubation time ($p < 0.05$). In summary, **6d** was able to reduce U87MG cell proliferation/viability in a dose-time dependent manner, supporting the specificity of the effect (see Fig. 6A, B).

Finally, the ability of **6d** to cause the dissipation of mitochondrial membrane potential was studied to substantiate the intracellular pro-apoptotic mechanism activated following ligand-TSPO binding. This effect was investigated by flow cytometric analysis, with the use of the mitochondrial potentiometric probe JC-1 and the uncoupling agent carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) as a positive control. Representative examples of the cytometric analysis are given in Fig. 7A. The majority of untreated control cells (99%) showed high fluorescence emission in both channels and were found in the upper right quadrant of the plot. The remaining (1%) of the untreated control cells showed low emission of fluorescence in FL2, therefore plotting in the lower right quadrant. An increase in the percentage of the cells plotting in the lower right quadrant, consistent with the $\Delta\Psi m$ dissipation, was seen in cells exposed to **6d** (30 times of K_i value concentration). In

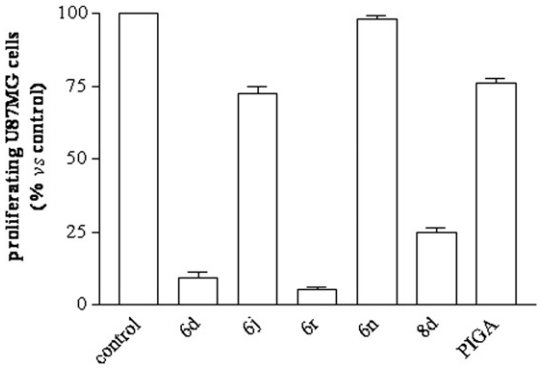


Fig. 5. Effect of the TSPO ligands on U87MG cell proliferation/viability. U87MG cells were exposed for 24 h at a single concentration of each compound (**6d**, **6j**, **6n**, **6r**, **8d**) corresponding to 1000 times the K_i value and used in the MTS assay. The percentage of proliferating cells exposed to each compound was calculated with respect to untreated control cells (100%). The figure shows data (means ± SEM) obtained from 3 experiments performed in triplicate.

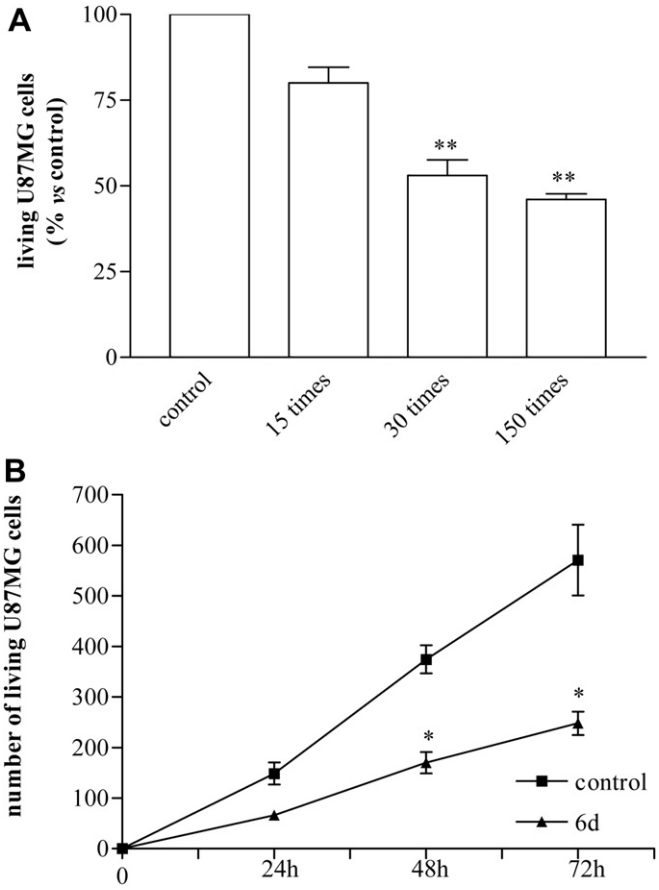


Fig. 6. Dose and time-response curve of U87MG cell proliferation/viability following treatment with compound **6d**. The dose-and time-response effect exerted by **6d** on U87MG cell proliferation/viability was estimated using trypan blue exclusion dye assay. The percentage of living cells after exposure to each treatment was calculated with respect to untreated control cells, which was assigned a value of 100%. (6A) shows the dose–response curve following cell exposure with increasing concentrations of **6d** (15, 30 and 150 times the K_i value) for 24 h. (6B) shows the time-response curve following cell exposure to a single concentration of **6d** (30 times the K_i value) for 24, 48 and 72h. The figures show data (means ± SEM) obtained from 3 experiments performed in triplicate.

particular, significant changes in $\Delta\Psi m$ were observed after treatment for 48 h (24%; $p < 0.05$), as shown in Fig. 7B.

5. Conclusion

In conclusion, a number of novel TSPO ligands **1**–**8**, featuring a five-membered heteroaromatic ring as scaffold, were synthesized and tested. The structures were designed on the basis of a pharmacophore/topological model summarizing the key pharmacophoric interactions of well-known TSPO ligands. The highest affinities were displayed by oxazolacetamides **6**, with **6n** turning out the most potent compound ($K_i = 11$ nM). The structure–affinity relationships derived from these novel compounds allowed us to probe the TSPO binding cleft in the perspective of further molecular design studies.

A subset of selected compounds (**6d**, **6j**, **6r**, **6n** and **8d**), showing the best values of TSPO binding affinity, were assayed *in vitro* to determine their ability to induce death in glioblastoma multiforme U87MG cell line. In the initial screening, **6d**, **6r** and **8d**, after 24 h incubation time, resulted to inhibit proliferation/viability of U87MG cells at the concentration of 1000 times their K_i value. Ligand **6d** was selected and further investigated to assess the specificity of the observed effect. Actually, **6d** showed to be able to reduce U87MG cell proliferation/viability in a dose-time dependent manner, and to cause the dissipation of mitochondrial membrane potential. These data substantiate the specificity of the inhibition of U87MG cell growth and the intracellular pro-apoptotic mechanism activated by ligand binding to TSPO protein.

6. Experimental section

6.1. Chemistry

Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Routine nuclear magnetic resonance spectra were recorded on a Varian $Mercury_{plus}$ 400 spectrometer operating at 400 MHz or on a Varian Gemini 200 spectrometer operating at 200 MHz. Mass spectra were obtained on a ThermoQuest Finnigam GCQplus spectrometer using a direct injection probe and an electron beam energy of 70 eV. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Silica gel 60 (230–400 mesh) was used for column flash-chromatography. Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%. The acid **13** is commercially available from Bionet Research, Cornwell. The acids **14** and **15** are from Sigma–Aldrich. The commercially available products were used as received without additional purification. Anhydrous solvents (Toluene, THF) were freshly distilled over sodium.

6.2. Synthesis

6.2.1. General procedure for the synthesis of N,N-dialkyl-3-phenyl-1,2,4-oxadiazole-5-carboxamides *1a–b*

3-Phenyl-1,2,4-oxadiazole-5-carboxylate **9** [23] (0.44 g, 2.0 mmol) was refluxed in a large excess of diethylamine or di-*n*-propylamine (40.0 mmol) for 2 h (TLC analysis); the resulting solution was evaporated to dryness to yield crude **1a**–**b**. Product **1a** was a white solid purified by crystallization; compound **1b** was a colourless oil, purified by column chromatography.

*6.2.1.1. N,N-Diethyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1a**).* Yield: 20%, mp: 45.0–46.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.08–8.03 (m, 2H, H-Ar); 7.65–7.55 (m, 3H, H-Ar); 3.57–3.45 (m, 4H, 2×CH_2); 1.30–1.14 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1; 167.2; 154.7; 131.9; 129.3; 127.1; 125.3; 43.0 (39.9); 14.1 (12.3). IR cm^{-1}: 1682; 1651; 1566; 1354; 1293; 1135; 1118; 862; 715; 691; 613. EI-MS *m*/*z* (%): 131 (100); 246 (M^++1). Anal.: calcd for $C_{13}H_{15}N_3O_2$: 63.66%; 6.16%, 17.13%, found: C 64.02%; 6.31%, 17.50%.

*6.2.1.2. 3-Phenyl-N,N-di-n-propyl-1,2,4-oxadiazole-5-carboxamide (**1b**).* Yield: 36%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.08–8.03 (m, 2H, H-Ar); 7.65–7.56 (m, 3H, H-Ar); 3.48–3.40 (m, 4H, 2×CH_2); 1.71–1.58 (m, 4H, 2×CH_2); 0.95–0.79 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.3; 167.4; 155.3; 132.0; 129.4; 127.2; 125.4; 49.7 (47.2); 21.6 (20.0); 11.1 (10.7). IR cm^{-1}: 1662; 1443; 1300; 1214; 1139; 1100; 715; 692. EI-MS *m*/*z* (%): 131 (100); 274 (M^++1). Anal.: calcd for $C_{15}H_{19}N_3O_2$: 65.91%; 7.01%, 15.37%, found: C 66.30%; 7.31%, 15.22%.

6.2.2. General procedure for the synthesis of N,N-dialkyl-3-phenyl-1,2,4-oxadiazole-5-carboxamides *1c–g* and N,N-dialkyl-2-phenyl-1,3-oxazole-4-carboxamides *2a–g*

A solution of the appropriate secondary amine (1.0 mmol) in 2 ml of anhydrous toluene was added dropwise to a stirred

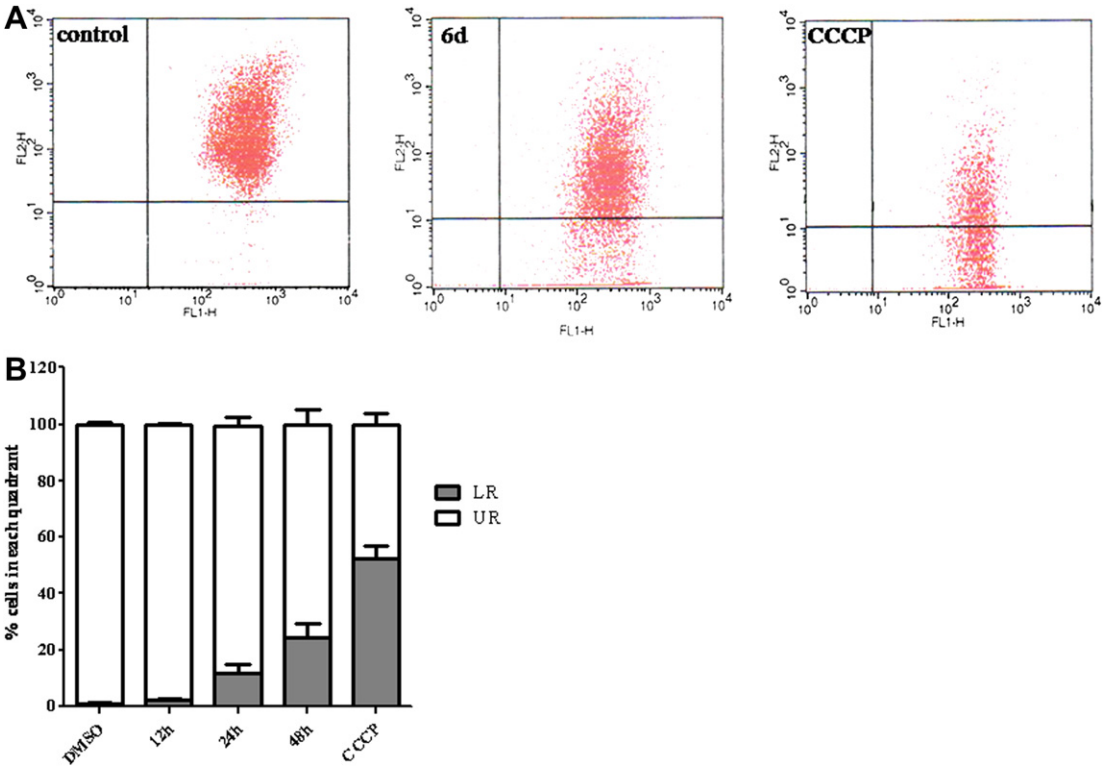


Fig. 7. Flow cytometry analyses of mitochondrial membrane potential dissipation by **6d** in U87MG cells. Following U87MG cell treatment with a single concentration of **6d** (30 times the K_i value) for various incubation times (12, 24 and 48 h), the cells were stained with JC-1 and analysed by FACS. 7**A**: Representative examples of dot-blots of the fluorescence pattern of DMSO-treated (control) or **6d**-, and CCCP-treated cells (positive control) stained with JC-1. Cells with polarized mitochondria are found in the upper right quadrant of plots, corresponding in high emission in both FL 1 (*x*-axis) and FL 2 (*y*-axis) channels. After treatment, mitochondrial depolarization is evident as a decrease in the fluorescence emission in the FL 2 and an increase in FL 1 channels, lower right (LR) quadrant. 7B: Histograms show mean values of cell percentages either in the upper right (polarized mitochondria) and lower right quadrant of $\Delta\Psi$m analysis plots derived from three independent experiments.

solution, cooled at 0 °C, of 3-phenyl-1,2,4-oxadiazol-5-carbonylchloride **10** [24] or 2-phenyloxazolcarbonylchloride **11** [25,26] (1.0 mmol) in 10 ml of the same solvent, followed by the dropwise addition of a solution of triethylamine (0.14 ml, 1.0 mmol) in 2 ml of anhydrous toluene. The reaction mixture was stirred at room temperature for 3–20 h (1,2,4-oxadiazolcarboxamides **1c**–**g**) or 1–2 h (oxazolecarboxamides **2a**–**g**) (TLC analysis). Triethylamine hydrochloride was removed by filtration and the toluene solution was washed with water, dried on $MgSO_4$ and evaporated to dryness to yield crude **1c**–**g**, **2a**–**g**, successively purified by flash-chromatography or crystallization.

*6.2.2.1. N,N-Di-n-butyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1c**).* Yield: 34%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.07–8.02 (m, 2H, H-Ar); 7.64–7.60 (m, 3H, H-Ar); 3.51–3.43 (m, 4H, 2×CH_2); 1.70–1.56 (m, 4H, 2×CH_2); 1.42–1.15 (m, 4H, 2×CH_2); 0.96–0.79 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1; 167.2; 154.7; 131.9; 129.2; 127.0; 125.3; 47.9 (45.3); 30.4 (28.7); 19.5 (19.1); 13.6 (13.4). IR cm^{-1}: 1659; 1455; 1300; 1204; 1137; 953; 722; 692. EI-MS *m*/*z* (%): 131 (100); 302 (M^++1). Anal.: calcd for $C_{17}H_{23}N_3O_2$: 67.75%; 7.69%, 13.94%, found: C 68.11%; 7.93%, 13.58%.

*6.2.2.2. N,N-Di-n-pentyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1d**).* Yield: 46%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.07–8.02 (m, 2H, H-Ar); 7.66–7.57 (m, 3H, H-Ar); 3.50–3.41 (m, 4H, 2×CH_2); 1.72–1.54 (m, 4H, 2×CH_2); 1.32–1.15 (m, 8H, 4×CH_2); 0.92–0.78 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1; 167.2; 155.0; 131.9; 129.2; 127.0; 125.3; 48.1 (45.5); 28.3 (28.0); 27.9 (26.3); 21.8 (21.6); 13.8 (13.7). IR cm^{-1}: 1661; 1446; 1279; 1187; 1135; 774; 719; 692. EI-MS *m*/*z* (%): 132 (100); 330 (M^++1). Anal.: calcd for $C_{19}H_{27}N_3O_2$: 69.27%; 8.26%, 12.76%, found: C 68.89%; 8.04%, 12.38%.

*6.2.2.3. N,N-Di-n-hexyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1e**).* Yield: 39%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 98:2 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.07–8.02 (m, 2H, H-Ar); 7.65–7.57 (m, 3H, H-Ar); 3.50–3.42 (m, 4H, 2×CH_2); 1.73–1.52 (m, 4H, 2×CH_2); 1.38–1.11 (m, 12H, 6×CH_2); 0.91–0.75 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1; 167.2; 155.0; 131.9; 129.2; 127.0; 125.3; 48.1 (45.6); 30.8 (30.7); 28.2 (26.6); 25.9 (25.4); 22.0 (21.9); 13.8 (13.7). IR cm^{-1}: 1661; 1525; 1351; 1296; 1228; 1180; 1139; 1105; 1030; 712; 688. EI-MS *m*/*z* (%): 131 (100); 358 (M^++1). Anal.: calcd for $C_{21}H_{31}N_3O_2$: 70.55%; 8.74%, 11.75%, found: C 70.59%; 8.70%, 11.76%

*6.2.2.4. N-Benzyl-N-ethyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1f**).* Yield: 58%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 98:2 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.09–7.97 (m, 2H, H-Ar); 7.63–7.57 (m, 3H, H-Ar); 7.40–7.28 (m, 5H, H-Ar); 4.83 and 4.75 (2s, $CH_2C_6H_5$); 3.57–3.43 (m, 2H, CH_2); 1.26–1.07 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1 (168.1); 167.5 (167.4); 155.5 (155.3); 136.4 (136.3); 132.1 (132.0); 129.4 (129.3); 128.6; 127.8 (127.7); 127.6 (127.5); 127.3 (127.2); 125.4; 51.2 (47.9); 43.2 (40.8); 13.90 (11.8). IR cm^{-1}: 3066; 3037; 1663; 1600; 1567; 1354; 1281; 1180; 1126; 962; 720. EI-MS *m*/*z* (%): 132 (100); 308 (M^++1). Anal.: calcd for $C_{18}H_{17}N_3O_2$: 70.34%; 5.58%, 13.67%, found: C 70.73%; 5.15%, 13.81%.

*6.2.2.5. N-n-Butyl-N-methyl-3-phenyl-1,2,4-oxadiazole-5-carboxamide (**1g**).* Yield: 63%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.08–8.02 (m, 2H, H-Ar); 7.65–7.55 (m, 3H, H-Ar); 3.55–3.46 (m, 2H, CH_2); 3.19 and 3.07 (2s, 3H, CH_3); 1.71–1.52 (m, 2H, CH_2); 1.43–1.14 (m, 2H, CH_2); 0.97–0.81 (m, 3H, CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 169.1 (169.0); 167.2; 155.0 (154.8); 131.9 (131.8); 129.3 (129.2); 127.1 (127.0); 125.3 (125.2); 49.8 (47.1); 35.9 (33.1); 29.7 (28.1); 19.4 (19.0); 13.6 (13.4). IR cm^{-1}: 3018; 1665; 1597; 1559; 1538; 1412; 1347; 1303; 1197; 1139; 767; 719; 702. EI-MS *m*/*z* (%): 131 (100); 260 (M^++1). Anal.: calcd for $C_{14}H_{17}N_3O_2$: 64.85%; 6.61%, 16.20%, found: C 65.47%; 7.11%, 15.84%.

*6.2.2.6. N,N-Diethyl-2-phenyl-1,3-oxazole-4-carboxamide (**2a**).* Yield: 99%. Colourless oil by column chromatography (petroleum ether:-ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.61 (s, 1H, H-5); 8.02–7.97 (m, 2H, H-Ar); 7.60–7.53 (m, 3H, H-Ar); 3.70–3.40 (m, 4H, 2×CH_2); 1.22–1.04 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.5; 159.7; 142.6; 137.5; 130.9; 129.1; 126.2; 126.0; 42.2; (40.1); 14.6 (12.7). IR cm^{-1}: 3153, 3106, 3065, 1621, 1556, 1334, 1109, 856, 712, 692. EI-MS *m*/*z* (%): 72 (100); 244 (M^+). Anal.: calcd for $C_{14}H_{16}N_2O_2$: 70.34%; 5.58%, 13.67%, found: C 70.73%; 5.15%, 13.81%.

*6.2.2.7. 2-Phenyl-N,N-dipropyl-1,3-oxazole-4-carboxamide (**2b**).* Yield: 68%. Colourless oil by column chromatography (petroleum ether:-ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.61 (s, 1H, H-5); 8.01–7.97 (m, 2H, H-Ar); 7.59–7.55 (m, 3H, H-Ar); 3.64 (t, 2H, CH_2); 3.34 (t, 2H, CH_2); 1.67–1.53 (m, 4H, 2×CH_2); 0.89–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.7; 159.7; 143.0; 137.7; 130.9; 129.1; 126.3; 125.9; 49.3 (47.5); 22 (20.4); 11.2 (10.8). IR cm^{-1}: 3167; 1624; 1556; 1330; 1105; 712; 692. EI-MS *m*/*z* (%): 190 (100); 272 (M^+). Anal.: calcd for $C_{16}H_{20}N_2O_2$: 70.56%; 7.40%, 10.29%, found: C 70.21%; 7.49%, 10.31%.

*6.2.2.8. N,N-Dibutyl-2-phenyl-1,3-oxazole-4-carboxamide (**2c**).* Yield: 65%. Colourless oil by column chromatography (petroleum ether:-ethyl acetate = 99:1 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.60 (s, 1H, H-5); 8.00–7.92 (m, 2H, H-Ar); 7.59–7.54 (m, 3H, H-Ar); 3.66 (t, 2H, CH_2); 3.36 (t, 2H, CH_2); 1.65–1.51 (m, 4H, 2×CH_2); 1.32–1.21 (m, 4H, 2×CH_2); 0.94–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.6; 159.6; 143.0; 137.7; 130.9; 129.1; 126.3; 125.9; 47.4 (45.5); 31.0 (29.3); 19.7 (19.4); 13.7 (13.6). IR cm^{-1}: 3160; 3065; 1624; 1559; 1334; 1108; 712; 689. EI-MS *m*/*z* (%): 190 (100); 300 (M^+). Anal.: calcd for $C_{18}H_{24}N_2O_2$: 71.97%; 8.05%, 9.33%, found: C 72.09%; 8.19%, 9.03%.

*6.2.2.9. N,N-Dipentyl-2-phenyl-1,3-oxazole-4-carboxamide (**2d**).* Yield: 89%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 98:2 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.61 (s, 1H, H-5); 8.02–7.96 (m, 2H, H-Ar); 7.59–7.55 (m, 3H, H-Ar); 3.67 (t, 2H, CH_2); 3.37 (t, 2H, CH_2); 1.64–1.56 (m, 4H, 2×CH_2); 1.40–1.18 (m, 8H, 4×CH_2); 0.91–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.6; 159.6; 143.0; 137.7; 130.9; 129.1; 126.3; 125.9; 47.7 (45.7); 28.6 (28.5); 28.3 (26.8); 21.8 (21.7); 13.8 (13.7). IR cm^{-1}: 3106; 1638; 1559; 1331; 1105; 774; 712; 689. EI-MS *m*/*z* (%): 190 (100); 328 (M^+). Anal.: calcd for $C_{20}H_{28}N_2O_2$: 73.14%; 8.59%, 8.53%, found: C 73.13%; 8.99%, 8.20%.

*6.2.2.10. N,N-Dihexyl-2-phenyl-1,3-oxazole-4-carboxamide (**2e**).* Yield: 55%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 98:2 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.60 (s, 1H, H-5); 8.00–7.94 (m, 2H, H-Ar); 7.58–7.53 (m, 3H, H-Ar); 3.67–3.36 (m, 4H, 2×CH_2); 1.70–1.50 (m, 4H, 2×CH_2); 1.38–1.15 (m, 12H, 6×CH_2); 0.86–0.79 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.6; 159.6; 143.0; 137.8; 130.9; 129.0; 126.3; 125.9; 47.7 (45.8); 31.0 (30.9); 28.9 (27.1); 26.1 (25.8); 22.0 (21.9); 13.8 (13.7). IR cm^{-1}: 3099; 1628; 1563; 1331; 1105; 712; 689. EI-MS *m*/*z* (%):356 (100, M^+). Anal.: calcd for $C_{22}H_{32}N_2O_2$: 74.12%; 9.05%, 7.86%, found: C 73.87%; 9.12%, 8.23%.

*6.2.2.11. N-Benzyl-N-ethyl-2-phenyl-1,3-oxazole-4-carboxamide (**2f**).* Yield: 27%. mp: 110.0–111.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.71 (s, 1H, H-5); 8.00–7.86 (m, 2H, H-Ar); 7.67–7.21 (m, 8H, H-Ar); 5.07 and 4.67 (2s, $CH_2C_6H_5$); 3.66–3.25 (m, 2H, CH_2); 1.22–1.07 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 161.4 (160.9); 160.0 (159.9); 143.5 (143.3); 138.0 (137.8); 137.5 (137.3); 131.1; 129.2; 129.1; 128.4; 127.7; 127.4 (127.1); 126.1; 50.4 (47.8); 42.5 (40.4); 14.2 (12.2). IR cm^{-1}: 3126; 3092; 1611; 1559; 1098; 951; 740; 709. EI-MS *m*/*z* (%): 201 (100); 306 (M^+). Anal.: calcd for $C_{19}H_{18}N_2O_2$: 74.49%; 5.92%, 9.14%, found: C 74.36%; 6.03%, 9.19%.

*6.2.2.12. N-Butyl-N-methyl-2-phenyl-1,3-oxazole-4-carboxamide (**2g**).* Yield: 53%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.62 (s, 1H, H-5); 8.00–7.98 (m, 2H, H-Ar); 7.88–7.55 (m, 3H, H-Ar); 3.77–3.47 (m, 2H, CH_2); 3.27 and 2.97 (2s, 3H, CH_3); 1.67–1.51 (m, 2H, CH_2); 1.32–1.22 (m, 2H, CH_2); 0.95–0.83 (m, 3H, CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 160.9; 159.7; 142.9 (142.6); 137.4; 130.9; 129.1; 126.2; 125.9; 49.3 (47.0); 35.9 (33.5); 30.3 (28.5); 19.5 (19.2); 13.6 (13.4). IR cm^{-1}: 3099; 1631; 1556; 1334: 1109; 931; 781; 709; 692. EI-MS m/z (%): 190 (100); 258 (M^+). Anal.: calcd for $C_{15}H_{18}N_2O_2$: 69.74%; 7.02%, 10.84%, found: C 69.54%; 6.99%, 10.46%

*6.2.3. General procedure for the synthesis of N,N-dialkyl-3-arylisoxazole-5-carboxamides **3b–e,s** and N,N-dialkyl-5-arylisoxazole-3-carboxamides **4b–e,h,k***

Thionyl chloride (1.2 mL) was added at 0 °C to the 3-phenylisoxazole-5-carboxylic acid **13** or the 5-phenylisoxazole-3-carboxylic acid **14** or the 5-(4-chlorophenyl)isoxazole-3-carboxylic acid **15** or the 3-(4-methylphenyl)isoxazole-5-carboxylic acid **16** (0.3 g, 1.58 mmol). The obtained suspension was stirred and heated at reflux for 16 h and then cooled at 0 °C. At this temperature, a new addition of thionyl chloride (1.2 mL) was followed by another heating at reflux for 2 h. The reaction mixture was cooled at room temperature and the thionyl chloride excess was evaporated to dryness in vacuo. Anhydrous THF (2 mL) was added to the crude product. To the resulting solution, cooled at −5 °C, was added dropwise a solution of appropriate amine (3.16 mmol) in dry THF (2 mL). The reaction mixture was stirred at room temperature for ca. 90 min (TLC, petroleum ether/ethyl acetate) and then the solid mass was filtered off and washed with THF. The filtrate was evaporated to dryness; the residue was treated with saturated sodium bicarbonate solution (20 mL) and extracted with dichloromethane (3×15 mL). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness to give the crude product, purified by flash-chromatography to give the desired amide.

*6.2.3.1. 3-Phenyl-N,N-dipropylisoxazole-5-carboxamide (**3b**).* Yield: 60%. Purified by column chromatography (petroleum ether:ethyl acetate = 4:1 v/v as eluant). mp: 46.5–47.0 °C (from petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.84–7.81 (m, 2H, H-Ar); 7.49–7.46 (m, 3H, H-Ar); 7.08 (s, 1H, H-4); 3.51–3.44 (m, 4H, 2×CH_2); 1.75–1.67 (m, 4H, 2×CH_2); 0.97 (t, 3H, CH_3); 0.93 (t, 3H, CH_3).^{13}C NMR (100 MHz, $CDCl_3$) δ: 165.7; 162.3; 158.0; 130.4; 129.0; 128.2; 126.85; 106.2; 50.3; 48.5; 22.6; 20.6; 11.4; 11.0. Anal.: calcd

for $C_{16}H_{20}N_2O_2$: 70.56%; 7.40%, 10.29%, found: C 70.23%; 7.48%, 10.32%.

*6.2.3.2. N,N-Dibutyl-3-phenylisoxazole-5-carboxamide (**3c**).* Yield: 21%. Purified by column chromatography (petroleum ether:ethyl acetate = 5:1 v/v as eluant). mp: 56.9–57.4 °C (from petroleum ether). ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.84–7.80 (m, 2H, H-Ar); 7.48–7.44 (m, 3H, H-Ar); 7.07 (s, 1H, H-4); 3.54–3.46 (m, 4H, 2×NCH_2); 1.68–1.61 (m, 4H, 2×CH_2); 1.40–1.32 (m, 4H, 2×CH_2); 0.97 (t, 3H, J = 7.4, CH_3); 0.93 (t, 3H, J = 7.5, CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 165.7; 162.3; 157.9; 130.4; 129.0; 128.2; 126.9; 106.2; 48.5; 46.7; 31.5; 29.4; 20.2; 19.9; 13.9; 13.7. ESI-MS: 323.2 (M + Na^+). Anal.: calcd for $C_{18}H_{24}N_2O_2$: 71.97%; 8.05%, 9.33%, found: C 72.07%; 8.17%, 9.05%.

*6.2.3.3. N,N-Dipentyl-3-phenylisoxazole-5-carboxamide (**3d**).* Yield: 35%. Purified by column chromatography (petroleum ether:ethyl acetate = 6:1 v/v as eluant). White wax (from petroleum ether). ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.84–7.80 (m, 2H, H-Ar); 7.48–7.46 (m, 3H, H-Ar); 7.07 (s, 1H, H-4); 3.53–3.45 (m, 4H, 2×NCH_2); 1.70–1.63 (m, 4H, 2×CH_2); 1.40–1.26 (m, 8H, 4×CH_2); 0.92 (t, 3H, J = 6.7, CH_3); 0.89 (t, 3H, J = 7.2, CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 165.7; 162.3; 157.8; 130.4; 129.0; 128.2; 126.8; 106.2; 48.7; 46.9; 29.1; 29.0; 28.8; 27.0; 22.4; 22.3; 14.0; 13.9. Mass (ESI): 351.2 (M + Na^+). Anal.: calcd for $C_{20}H_{28}N_2O_2$: 73.14%; 8.59%, 8.53%, found: C 73.15%; 8.97%, 8.25%.

*6.2.3.4. N,N-Dihexyl-3-phenylisoxazole-5-carboxamide (**3e**).* Yield: 33%. Colourless oil by column chromatography (petroleum ether:-ethyl acetate = 9:1 v/v as eluant). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.84–7.82 (m, 2H, H-Ar); 7.49–7.46 (m, 3H, H-Ar); 7.08 (s, 1H, H-4); 3.53–3.46 (m, 4H, 2×CH_2N); 1.67–1.64 (m, 4H, 2×CH_2); 1.38–1.28 (m, 12H, 6×CH_2); 0.92–0.86 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 165.7; 162.3; 157.8; 130.4; 129.0; 128.2; 126.85; 106.2; 48.7; 46.9; 31.6; 31.4; 29.4; 27.3; 26.7; 26.4; 22. 6; 22.5; 14.0; 13.9. Anal.: calcd for $C_{22}H_{32}N_2O_2$: 74.12%; 9.05%, 7.86%, found: C 73.91%; 9.10%, 8.20%.

*6.2.3.5. N,N-Dihexyl-3-(4-methylphenyl)isoxazole-5-carboxamide (**3s**).* Yield: 40%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 4:1 v/v as eluant). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.72 (AA' part of the system AA'XX', 2H, H-Ar); 7.28 (XX' part of the system AA'XX', 2H, H-Ar); 7.05 (s, 1H, H-4); 3.52–3.45 (m, 4H, 2×CH_2N); 2.41 (s, 3H, CH_3-Ar); 1.67–1.65 (m, 4H, 2×CH_2); 1.39–1.22 (m, 12H, 6×CH_2); 0.927–0.86 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 165.5; 162.2; 157.9; 140.6; 129.7; 126.7; 125.3; 106.1; 48.7; 46.9; 31.6; 31.4; 29.4; 27.3; 26. 7; 26.3; 22. 6; 22.5; 21.4; 14.0; 13.9. Anal.: calcd for $C_{23}H_{34}N_2O_2$: 74.55%; 9.25%, 7.56%, found: C 74.59%; 9.18%, 7.45%.

*6.2.3.6. 5-Phenyl-N,N-dipropylisoxazole-3-carboxamide (**4b**).* Yield: 25%. Purified by column chromatography (petroleum ether:ethyl acetate = 4:1 v/v as eluant). mp: 55.3–55.8 °C (from petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.82–7.79 (m, 2H, H-Ar); 7.49–7.47 (m, 3H, H-Ar); 6.81 (s, 1H, H-4); 3.59–3.56 (m, 2H, CH_2); 3.49–3.46 (m, 2H, CH_2); 1.72–1.67 (m, 4H, 2×CH_2); 0.98 (t, 3H, CH_3); 0.88 (t, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.0; 160.8; 159.8; 130.5; 129.1; 126.9; 125.9; 100.8; 50.6; 48.1; 22.4; 20.7; 11.4; 11.0. Anal.: calcd for $C_{16}H_{20}N_2O_2$: 70.56%; 7.40%, 10.29%, found: C 70.62%; 7.48%, 10.14%.

*6.2.3.7. N,N-Dibutyl-5-phenylisoxazole-3-carboxamide (**4c**).* Yield: 30%. Purified by column chromatography (petroleum ether:ethyl acetate = 5:1 v/v as eluant). mp: 65.9–66.7 °C (from petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.82–7.75 (m, 2H, H-Ar); 7.49–7.42 (m, 3H, H-Ar); 6.81 (s, 1H, H-4); 3.63–3.58 (m, 2H, NCH_2); 3.53–3.48 (m, 2H, NCH_2); 1.69–1.60 (m, 4H, 2×CH_2); 1.40 (sext, 2H, J = 7.4, CH_2); 1.29 (sext, 2H, J = 7.4, CH_2); 0.98 (t, 3H, J = 7.4, CH_3); 0.90 (t, 3H, J = 7.4, CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 169.9; 160.7; 159.7; 130.5; 129.1; 126.9; 125.9; 100.7; 48.7; 46.2; 31.3; 29.5; 20.3; 19.8; 13.9; 13.7. ESI-MS: 323.1 (M + Na^+). Anal.: calcd for $C_{18}H_{24}N_2O_2$: 71.97%; 8.05%, 9.33%, found: C 72.13%; 8.16%, 9.22%.

*6.2.3.8. N,N-Dipentyl-5-phenylisoxazole-3-carboxamide (**4d**).* Yield: 79%. Purified by column chromatography (petroleum ether:ethyl acetate = 6:1 v/v as eluant). White wax (from petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.81–7.77 (m, 2H, H-Ar); 7.48–7.45 (m, 3H, H-Ar); 6.80 (s, 1H, H-4); 3.61–3.56 (m, 2H, NCH_2); 3.51–3.46 (m, 2H, NCH_2); 1.69–1.59 (m, 4H, 2×CH_2); 1.42–1.19 (m, 8H, 4×CH_2); 0.92 (t, 3H, J = 6.8, CH_3); 0.87 (t, 3H, J = 7.0, CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 169.9; 160.6; 159.7; 130.4; 129.0; 126.9; 125.9; 100.7; 48.9; 46.4; 29.2; 28.8; 28.7; 27.1; 22.4; 22.2; 14.0; 13.9. ESI-MS: 350.9 (M + Na^+). Anal.: calcd for $C_{20}H_{28}N_2O_2$: 73.14%; 8.59%, 8.53%, found: C 73.20%; 8.76%, 8.41%.

*6.2.3.9. N,N-Dihexyl-5-phenylisoxazole-3-carboxamide (**4e**).* Yield: 50%. Colourless oil by column chromatography (petroleum ether:-ethyl acetate = 4:1 v/v as eluant). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.81–7.79 (m, 2H, H-Ar); 7.49–7.47 (m, 3H, H-Ar); 6.80 (s, 1H, H-4); 3.61–3.57 (m, 2H, CH_2N); 3.51–3.47 (m, 2H, CH_2N); 1.67–1.63 (m, 4H, 2×CH_2); 1.35–1.24 (m, 12H, 6×CH_2); 0.92–0.84 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 169.9; 160.7; 159.8; 130.5; 129.1; 127.0; 125.9; 100.7; 49.0; 46.5; 31.6; 31.3; 29.2; 27.4; 26.7; 26.3; 22.6; 22.5; 14.0; 13.9. Anal.: calcd for $C_{22}H_{32}N_2O_2$: 74.12%; 9.05%, 7.86%, found: C 73.93%; 9.12%, 8.10%.

*6.2.3.10. 5-(4-Chlorophenyl)-N,N-dipropylisoxazole-3-carboxamide (**4h**).* Yield: 51%. Purified by column chromatography (petroleum ether:ethyl acetate = 4:1 v/v as eluant). mp: 96.6–97.1 °C (from petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, 2H, H-Ar); 7.46 (d, 2H, H-Ar); 6.80 (s, 1H, H-4); 3.57 (dd, 2H, CH_2); 3.47 (dd, 2H, CH_2); 1.69 (m, 4H, 2×CH_2); 0.97 (t, 3H, CH_3); 0.88 (t, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 168.8; 160.5; 159.9; 136.6; 129.4; 127.1; 125.4; 101.1; 50.5; 48.1; 22.4; 20.7; 11.4; 11.0. Anal.: calcd for $C_{16}H_{19}ClN_2O_2$: 62.64%; 6.24%, 9.13%, found: C 62.55%; 6.01%, 9.20%.

*6.2.3.11. 5-(4-Chlorophenyl)-N,N-dihexylisoxazole-3-carboxamide (**4k**).* Yield: 20%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 9:1 v/v as eluant). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.74–7.72 (AA' part of the system AA'XX', 2H, H-Ar); 7.47–7.45 (XX' part of the system AA'XX', 2H, H-Ar); 6.79 (s, 1H, H-4); 3.61–3.57 (m, 2H, CH_2N); 3.50–3.46 (m, 2H, CH_2N); 1.68–1.62 (m, 4H, 2×CH_2); 1.32–1.24 (m, 12H, 6×CH_2); 0.92–0.84 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 168.8; 160.4; 159.9; 136.6; 129.4; 127.2; 125.4; 101.1; 49.0; 46.6; 31.6; 31.4; 29.2; 27.4; 26.7; 26.3; 22.6; 22.5; 14.0; 13.9.Anal.: calcd for $C_{22}H_{31}ClN_2O_2$: 67.59%; 7.99%, 7.17%, found: C 67.44%; 7.84%, 7.03%.

*6.2.4. General procedure for the synthesis of N,N-dialkyl-2-(3-aryl-1,2,4-oxadiazol-5-yl)acetamides **5b**–**e**,**h**–**k** and N,N-dialkyl-2-(2-aryl-1,3-oxazol-4-yl)acetamides **6b**–**e**,**h**–**s***

To a stirred solution of 1,2,4-oxadiazoleacetic acid **20**, **21** [32] or oxazoleacetic acid **22**–**25** [32,33] (1.33 mmol) in 20 ml of freshly distilled anhydrous THF, cooled at 0 °C and maintained in a nitrogen atmosphere, 1,1′-carbonyldiimidazole (216 mg, 1.33 mmol) was added. After 10 min a solution of an equimolar amount of the appropriate amine in anhydrous THF was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred, under nitrogen atmosphere, for 72 h on the whole. Based on TLC analysis, an excess of reagents (20–40%)

was eventually added at the reaction time of 24 and/or 48 h. The final solution was evaporated to dryness and the residue was dissolved in CH_2Cl_2. The dichloromethane solution was washed with 1N HCl, 10% $NaHCO_3$ and water, dried on $MgSO_4$ and evaporated to dryness, obtaining crude **5b-e,h-k**, **6b-e,h-s**, purified by recrystallization or flash-chromatography.

*6.2.4.1. 2-(3-Phenyl-1,2,4-oxadiazol-5-yl)-N,N-dipropylacetamide (**5b**).* Yield: 40%, mp: 89.0–90.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.03–7.98 (m, 2H, H-Ar); 7.60–7.54 (m, 3H, H-Ar); 4.34 (s, 2H, CH_2); 3.35–3.19 (m, 4H, 2×CH_2); 1.69–1.40 (m, 4H, 2×CH_2); 0.93–0.79 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.5; 167.6; 165.0; 131.5; 129.3; 126.9; 126.2; 49.1 (46.9); 32.2; 25.6 (20.4); 11.1 (10.9). IR cm^{-1}: 1637; 1597; 1327; 1231; 1088; 1023; 801; 719. Anal.: calcd for $C_{16}H_{21}N_3O_2$: 66.88%; 7.34%, 14.62%, found: C 66.70%; 7.73%, 14.25%.

*6.2.4.2. N,N-Dibutyl-2-(3-phenyl-1,2,4-oxadiazol-5-yl)acetamide (**5c**).* Yield: 38%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz; DMSO-d_6) δ: 8.04–7.99 (m, 2H, H-Ar); 7.61–7.57 (m, 3H, H-Ar); 4.33 (s, 2H, CH_2); 3.36–3.22 (m, 4H, 2×CH_2); 1.60–1.19 (m, 8H, 4×CH_2); 0.95–0.84 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.6; 167.6; 164.8; 131.5; 129.3; 126.9; 126.2; 47.3 (45.0); 32.2; 30.5 (29.3); 19.6 (19.5); 13.7 (13.6). IR cm^{-1}: 1658; 1593; 1569; 1112; 900; 726. Anal.: calcd for $C_{18}H_{25}N_3O_2$: 68.54%; 7.99%, 13.32%, found: C 68.63%; 7.82%, 12.93%.

*6.2.4.3. N,N-Dipentyl-2-(3-phenyl-1,2,4-oxadiazol-5-yl)acetamide (**5d**).* Yield: 28%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.03–7.98 (m, 2H, H-Ar); 7.60–7.54 (m, 3H, H-Ar); 4.32 (s, 2H, CH_2); 3.32–3.21 (m, 4H, 2×CH_2); 1.60–1.18 (m, 12H, 6×CH_2); 0.91–0.82 (m, 6H, 2×CH_3) ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.5; 167.5; 164.6; 131.4; 129.1; 126.8; 126.1; 47.5 (45.2); 32.2; 28.5 (28.3); 28.0 (26.7); 21.8; 13.8. IR cm^{-1}: 1651; 1597; 1135; 1115; 907; 787; 715. Anal.: calcd for $C_{20}H_{29}N_3O_2$: 69.94%; 8.51%, 12.23%, found: C 69.57%; 8.82%, 11.85%.

*6.2.4.4. N,N-Dihexyl-2-(3-phenyl-1,2,4-oxadiazol-5-yl)acetamide (**5e**).* Yield: 35%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 96:4 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.02–7.98 (m, 2H, H-Ar); 7.60–7.57 (m, 3H, H-Ar); 4.32 (s, 2H, CH_2); 3.32–3.21 (m, 4H, 2×CH_2); 1.54–1.18 (m, 16H, 8×CH_2); 0.88–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.5; 167.5; 164.6; 131.4; 129.1; 126.8; 126.1; 47.6 (45.2); 40.1; 32.2 (31.0); 28.3 (27.0); 25.9 (25.8); 22.0 (21.9); 13.8. IR cm^{-1}: 1651; 1597; 1107; 1057; 1016; 797; 716; 690. Anal.: calcd for $C_{22}H_{33}N_3O_2$: 71.12%; 8.95%, 11.31%, found: C 71.40%; 8.28%, 11.00%.

*6.2.4.5. 2-[3-(4-Chlorophenyl)-1,2,4-oxadiazol-5-yl]-N,N-dipropylacetamide (**5h**).* Yield: 37%. mp: 190.0–195.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.03–7.99 (m, 2H, H-Ar); 7.60–7.54 (m, 3H, H-Ar); 4.34 (s, 2H, CH_2); 3.35–3.19 (m, 4H, 2×CH_2); 1.69–1.40 (m, 4H, 2×CH_2); 0.93–0.79 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.8; 166.7; 164.7; 136.1; 129.3; 128.6; 124.9; 49.1 (46.8); 32.2; 21.5 (20.3); 11.1 (10.9). IR cm^{-1}: 1637; 1597; 1327; 1231; 1088; 1023; 801; 719. Anal.: calcd for $C_{16}H_{20}ClN_3O_2$: 59.72%; 6.26%, 13.06%, found: C 59.42%; 6.53%, 12.90%.

*6.2.4.6. N,N-Dibutyl-2-[3-(4-chlorophenyl)-1,2,4-oxadiazol-5-yl] acetamide (**5i**).* Yield: 17%. mp: 76.0–82.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.04–7.99 (m, 2H, H-Ar); 7.67–7.63 (m, 3H, H-Ar); 4.34 (s, 2H, CH_2); 3.32–3.18 (m, 4H, 2×CH_2); 1.60–1.19 (m, 8H, 4×CH_2); 0.95–0.84 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.9; 166.8; 164.7; 136.2; 129.4; 128.7; 125.0; 47.3 (45.0); 32.2; 30.5 (29.3); 19.5 (19.4); 13.7 (13.6). IR cm^{-1}: 1658; 1593; 1569; 1112; 900; 726. Anal.: calcd for $C_{18}H_{24}ClN_3O_2$: 61.79%; 6.91%, 12.01%, found: C 61.49%; 6.45%, 11.70%.

*6.2.4.7. 2-[3-(4-Chlorophenyl)-1,2,4-oxadiazol-5-yl]-N,N-dipentylacetamide (**5j**).* Yield: 47%. mp: 50.0–55.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.03–7.98 (m, 2H, H-Ar); 7.60–7.54 (m, 3H, H-Ar); 4.32 (s, 2H, CH_2); 3.32–3.21 (m, 4H, 2×CH_2); 1.60–1.18 (m, 12H, 6×CH_2); 0.91–0.82 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.9; 166.8; 164.7; 136.3; 129.5; 128.7; 125.0; 47.6 (45.2); 32.2; 28.5 (28.3); 28.0 (26.7); 21.9 (21.8); 13.9 (13.8). IR cm^{-1}: 1651; 1597; 1135; 1115; 907; 787; 715. Anal.: calcd for $C_{20}H_{28}ClN_3O_2$: 63.56%; 7.47%, 11.12%, found: C 63.88%; 7.90%, 11.16%.

*6.2.4.8. 2-[3-(4-Chlorophenyl)-1,2,4-oxadiazol-5-yl]-N,N-dihexylacetamide (**5k**).* Yield: 44%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 95:5 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.02–7.98 (m, 2H, H-Ar); 7.60–7.57 (m, 3H, H-Ar); 4.32 (s, 2H, CH_2); 3.32–3.21 (m, 4H, 2×CH_2); 1.54–1.18 (m, 16H, 8×CH_2); 0.88–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 175.9; 166.8; 164.7; 136.3; 129.4; 128.7; 125.0; 47.6 (45.3); 32.2; 31.0 (30.9); 28.3 (27.0); 25.9 (25.8); 22.0 (21.9); 13.8 (13.7). IR cm^{-1}**:** 1651; 1597; 1107; 1057; 1016; 797; 716; 690. Anal.: calcd for $C_{22}H_{32}ClN_3O_2$: 65.09%; 7.95%, 10.35%, found: C 65.30%; 8.27%, 9.97%.

*6.2.4.9. 2-(2-Phenyl-1,3-oxazol-4-yl)-N,N-dipropylacetamide (**6b**).* Yield: 13%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 96:4 v/v as eluant). ^{1}HNMR (200 MHz, DMSO-d_6) δ: 8.01 (s, 1H, H-5); 7.97–7.92 (m, 2H, H-Ar); 7.56–7.51 (m, 3H, H-Ar); 3.67 (s, 2H, CH_2); 3.25–3.17 (m, 4H, 2×CH_2); 1.62–1.42 (m, 4H, 2×CH_2); 0.91–0.77 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.9; 159.8; 136.7; 136.6; 130.3; 129.0; 126.9; 125.6; 49.0 (46.8); 31.2; 21.8 (20.4); 11.2 (11.0). IR cm^{-1}: 1648; 1587; 1344; 1259; 1098; 1023; 801; 712. EI-MS *m*/*z* (%): 286 (M^+, 100). Anal.: calcd for $C_{17}H_{22}N_2O_2$: 71.30%; 7.74%, 9.78%, found: C 70.95%; 8.00%, 9.51%.

*6.2.4.10. N,N-Dibutyl-2-(2-phenyl-1,3-oxazol-4-yl)acetamide (**6c**).* Yield: 18%. Colourless oil by column chromatography (cyclohexane:ethyl acetate = 95:5 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.00 (s, 1H, H-5); 7.97–7.92 (m, 2H, H-Ar); 7.55–7.49 (m, 3H, H-Ar); 3.66 (s, 2H, CH_2); 3.28–3.21 (m, 4H, 2×CH_2); 1.57–1.18 (m, 8H, 4×CH_2); 0.93–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 159.8; 136.7; 136.6; 130.3; 129.0; 126.9; 125.6; 47.3 (44.9); 31.2; 30.8 (29.4); 19.6 (19.5); 13.7. IR cm^{-1}: 1645; 1583; 1094; 1057; 934; 774; 716; 689.EI-MS m/z (%): 314 (M^+, 100).Anal.: calcd for $C_{19}H_{26}N_2O_2$: 72.58%; 8.33%, 8.91%, found: C 71.12%; 8.71%, 8.83%.

*6.2.4.11. N,N-Dipentyl-2-(2-phenyl-1,3-oxazol-4-yl)acetamide (**6d**).* Yield: 23%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 98:2 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.01 (s, 1H, H-5); 7.97–7.92 (m, 2H, H-Ar); 7.56–7.50 (m, 3H, H-Ar); 3.66 (s, 2H, CH_2); 3.25–3.19 (m, 4H, 2×CH_2); 1.53–1.12 (m, 12H, 6×CH_2); 0.88–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 159.8; 136.8; 136.6; 130.3; 128.9; 126.9; 125.6; 47.4 (45.1); 31.2; 28.5 (28.4); 28.3 (26.9); 21.9; 13.8. IR cm^{-1}: 1645; 1587; 1132; 1057; 938; 780; 716; 689. EI-MS *m*/*z* (%):194 (100); 342 (M^+, 64). Anal.: calcd for $C_{21}H_{30}N_2O_2$: 73.65%; 8.83%, 8.18%, found: C 73.32%; 9.13%, 8.06%.

*6.2.4.12. N,N-Dihexyl-2-(2-phenyl-1,3-oxazol-4-yl)acetamide (**6e**).* Yield: 20%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 93:7 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.00 (s, 1H, H-5); 7.96–7.91 (m, 2H, H-Ar); 7.56–7.48 (m, 3H, H-Ar); 3.65 (s, 2H, CH_2); 3.37–3.19 (m, 4H, 2×CH_2); 1.58–1.10 (m, 16H, 8×CH_2); 0.95–0.70 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 159.8; 136.7; 136.6; 130.3; 128.9; 126.9; 125.6; 47.5 (45.1); 31.3 (29.9); 31.0; 28.6 (27.1); 25.5 (25.4); 22.0; 13.38. IR cm^{-1}: 1652; 1590; 1102; 1057; 935; 778; 716; 689. EI-MS *m*/*z* (%):194 (100); 370 (M^+, 43). Anal.: calcd for $C_{23}H_{34}N_2O_2$: 74.55%; 9.25%, 7.56%, found: C 74.15%; 9.49%, 7.28%.

*6.2.4.13. 2-[2-(4-Chlorophenyl)-1,3-oxazol-4-yl]-N,N-dipropylacetamide (**6h**).* Yield: 45%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.04 (s, 1H, H-5); 7.97–7.93 (AA' part of the system AA'XX', 2H, H-Ar); 7.63–7.58 (XX' part of the system AA'XX', 2H, H-Ar); 3.68 (s, 2H, CH_2); 3.25–3.18 (m, 4H, 2×CH_2); 1.63–1.43 (m, 4H, 2×CH_2); 0.92–0.78 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 168.0; 159.0; 137.2; 136.9; 135.1; 129.3; 127.5; 125.7; 49.1 (46.8); 31.1; 21.8 (20.4); 11.2 (11.0). IR cm^{-1}: 1641; 1427; 1257; 1091; 1014; 839; 729. EI-MS *m*/*z* (%):139 (100); 320 (M^+, 81). Anal.: calcd for $C_{17}H_{21}ClN_2O_2$: 63.64%; 6.60%, 8.73%, found: C 64.01%; 6.96%, 8.73%.

*6.2.4.14. N,N-Dibutyl-2-[2-(4-chlorophenyl)-1,3-oxazol-4-yl]acetamide (**6i**).* Yield: 60%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 93:7 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.05 (s, 1H, H-5); 7.96–7.93 (AA' part of the system AA'XX', 2H, H-Ar); 7.63–7.59 (XX' part of the system AA'XX', 2H, H-Ar); 3.70 (s, 2H, CH_2); 3.28–3.21 (m, 4H, 2×CH_2); 1.53–1.21 (m, 8H, 4×CH_2); 0.94–0.84 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.9; 159.0; 137.2; 136.9; 135.1; 129.3; 127.5; 125.8; 47.2 (44.9); 31.1; 30.8 (29.4); 19.6 (19.5); 13.8 (13.5). IR cm^{-1}: 1655; 1607; 1580; 1092; 1016; 835; 736. EI-MS *m*/*z* (%): 348 (M^+, 100). Anal.: calcd for $C_{19}H_{25}ClN_2O_2$: 65.41%; 7.22%, 8.03%, found: C 65.79%; 7.55%, 8.13%.

*6.2.4.15. 2-[2-(4-Chlorophenyl)-1,3-oxazol-4-yl]-N,N-dipentylacetamide (**6j**).* Yield: 77%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 95:5 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.05 (s, 1H, H-5); 7.97–7.93 (AA' part of the system AA'XX', 2H, H-Ar); 7.62–7.58 (XX' part of the system AA'XX', 2H, H-Ar); 3.66 (s, 2H, CH_2); 3.28–3.21 (m, 4H, 2×CH_2); 1.57–1.22 (m, 12H, 6×CH_2); 0.87–0.81 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.7; 158.9; 137.1; 136.8; 135.0; 129.1; 127.3; 125.6; 47.4 (45.1); 31.1; 28.5 (28.3); 28.2 (26.8); 21.9; 13.8. IR cm^{-1}: 1648; 1423; 1266; 1088; 1013; 839; 723. EI-MS *m*/*z* (%): 139 (100); 376 (M^+, 3). Anal.: calcd for $C_{21}H_{29}ClN_2O_2$: 66.92%; 7.76%, 7.43%, found: C 66.58%; 7.39%, 7.21%.

*6.2.4.16. 2-[2-(4-Chlorophenyl)-1,3-oxazol-4-yl]-N,N-dihexylacetamide (**6k**).* Yield: 48%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.04 (s, 1H, H-5); 7.97–7.93 (AA' part of the system AA'XX', 2H, H-Ar); 7.63–7.58 (XX' part of the system AA'XX', 2H, H-Ar); 3.66 (s, 2H, CH_2); 3.27–3.19 (m, 4H, 2×CH_2); 1.62–1.06 (m, 16H, 8×CH_2); 0.95–0.82 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.7; 158.9; 137.1; 136.8; 135.0; 129.1; 127.3; 125.6; 47.5 (45.1); 31.2 (31.0); 30.9; 28.5 (27.1); 26.0 (25.8); 22.0; 13.8. IR cm^{-1}: 1638; 1423; 1299; 1095; 1013; 835; 736. EI-MS *m*/*z* (%): 139 (100); 404 (M^+, 5). Anal.: calcd for $C_{23}H_{33}ClN_2O_2$: 68.21%; 8.21%, 6.92%, found: C 68.31%; 8.28%, 6.58%.

*6.2.4.17. 2-[2-(4-Fluorophenyl)-1,3-oxazol-4-yl]-N,N-dipropylacetamide (**6l**).* Yield: 59%. mp: 54.0–57.0 °C (from petroleum ether). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.02–7.95 (m, 3H, H-5 and H-Ar); 7.41–7.32 (m, 2H, H-Ar); 3.67 (s, 2H, CH_2); 3.31–3.17 (m, 4H, 2×CH_2); 1.62–1.41 (m, 4H, 2×CH_2); 0.92–0.78 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 168.1; 163.3 (J = 248.3); 159.2; 136.9; 136.7; 128.2 (J = 8.7); 123.6 (J = 3.4); 116.3 (J = 22.1); 49.1 (46.8); 31.1; 21.8 (20.4); 11.2 (11.0). IR cm^{-1}: 1637; 1460; 1378; 1239; 1094; 852; 736. Anal.: calcd for $C_{17}H_{21}FN_2O_2$: 67.09%; 6.95%, 9.20%, found: C 66.68%; 6.61%, 9.60%.

*6.2.4.18. N,N-Dibutyl-2-[2-(4-fluorophenyl)-1,3-oxazol-4-yl]acetamide (**6m**).* Yield: 46%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.02–7.95 (m, 3H, H-5 and H-Ar); 7.41–7.33 (m, 2H, H-Ar); 3.65 (s, 2H, CH_2); 3.37–3.20 (m, 4H, 2×CH_2); 1.52–1.21 (m, 8H, 4×CH_2); 0.93–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 163.1 (J = 248.1); 158.7; 136.8; 136.6; 128.1 (J = 9.1); 123.5 (J = 3.7); 116.2 (J = 22.9); 47.2 (44.9); 31.1; 30.5 (29.4); 19.7 (19.6); 13.7. IR cm^{-1}: 1648; 1497; 1368; 1214; 1060; 838; 732. Anal.: calcd for $C_{19}H_{25}FN_2O_2$: 68.65%; 7.58%, 8.43%, found: C 69.04%; 7.72%, 8.05%.

*6.2.4.19. 2-[2-(4-Fluorophenyl)-1,3-oxazol-4-yl]-N,N-dipentylacetamide (**6n**).* Yield: 48%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 95:5 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.02–7.95 (m, 3H, H-5 and H-Ar); 7.45–7.32 (m, 2H, H-Ar); 3.64 (s, 2H, CH_2); 3.27–3.16 (m, 4H, 2×CH_2); 1.60–1.15 (m, 12H, 6×CH_2); 0.87–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 163.1 (J = 248.2); 159.0; 136.8; 136.6; 128.1 (J = 9.2); 123.6 (J = 2.7); 116.1 (J = 22.9); 47.4 (45.1); 31.2; 28.5 (28.3); 28.2 (26.9); 21.4; 13.3. IR cm^{-1}: 1648; 1456; 1371; 1235; 1098; 838; 736. Anal.: calcd for $C_{21}H_{29}FN_2O_2$: 69.97%; 8.11%, 7.77%, found: C 69.87%; 8.27%, 7.39%.

*6.2.4.20. 2-[2-(4-Fluorophenyl)-1,3-oxazol-4-yl]-N,N-dihexylacetamide (**6o**).* Yield: 68%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.01–7.94 (m, 3H, H-5 and H-Ar); 7.45–7.32 (m, 2H, H-Ar); 3.64 (s, 2H, CH_2); 3.37–3.19 (m, 4H, 2×CH_2); 1.51–1.22 (m, 16H, 8×CH_2); 0.86–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.7; 163.1 (J = 248.1); 159.0; 136.7; 136.6; 128.0 (J = 9.2); 123.5 (J = 2.7); 116.1 (J = 22.0); 47.5 (45.1); 31.2 (31.1); 31.0; 28.5 (27.1); 26.0 (25.8); 22.0; 13.8. IR cm^{-1}: 1648; 1497; 1371; 1235; 1060; 838; 736. Anal.: calcd for $C_{23}H_{33}FN_2O_2$: 71.10%; 8.56%, 7.21%, found: C 71.29%; 8.42%, 7.00%.

*6.2.4.21. 2-[2-(4-Methylphenyl)-1,3-oxazol-4-yl]-N,N-dipropylacetamide (**6p**).* Yield: 58%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 97:3 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 7.93 (s, 1H, H-5); 7.84–7.80 (AA' part of the system AA'XX', 2H, H-Ar); 7.34–7.30 (XX' part of the system AA'XX', 2H, H-Ar); 3.63 (s, 2H, CH_2); 3.35–3.16 (m, 4H, 2×CH_2); 2.36 (s, 3H, CH_3); 1.62–1.42 (m, 4H, 2×CH_2); 0.91–0.77 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 168.0; 160.0; 140.1; 136.4; 136.3; 129.5; 125.6; 124.2; 49.1 (46.8); 31.2; 21.8 (20.9); 20.4; 11.2 (10.9). IR cm^{-1}: 1648; 1583; 1497; 1371; 1344; 1060; 825; 729. Anal.: calcd for $C_{18}H_{24}N_2O_2$: 71.97%; 8.05%, 9.33%, found: C 71.57%; 8.42%, 8.98%.

*6.2.4.22. N,N-Dibutyl-2-[2-(4-methylphenyl)-1,3-oxazol-4-yl]acetamide (**6q**).* Yield: 60%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 90:10 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 7.95 (s, 1H, H-5); 7.84–7.80 (AA' part of the system AA'XX', 2H, H-Ar); 7.35–7.31 (XX' part of the system AA'XX', 2H, H-Ar); 3.64 (s, 2H, CH_2); 3.37–3.21 (m, 4H, 2×CH_2); 2.36 (s, 3H, CH_3); 1.56–1.18 (m, 8H, 4×CH_2); 0.93–0.83 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 160.0; 140.1; 136.4; 136.3; 129.5;

125.6; 124.2; 47.2 (44.8); 31.2; 30.8 (29.4); 20.9 (19.5); 13.7. IR cm^{-1}: 1641; 1586, 1497; 1207; 1098; 1064; 937; 729. Anal.: calcd for $C_{20}H_{28}N_2O_2$: 73.14%; 8.59%, 8.53%, found: C 72.76%; 8.72%, 8.05%.

*6.2.4.23. 2-[2-(4-Methylphenyl)-1,3-oxazol-4-yl]-N,N-dipentylacetamide (**6r**).* Yield: 68%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 95:5 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 7.95 (s, 1H, H-5); 7.84–7.80 (AA' part of the system AA'XX', 2H, H-Ar); 7.34–7.30 (XX' part of the system AA'XX', 2H, H-Ar); 3.63 (s, 2H, CH_2); 3.37–3.19 (m, 4H, 2×CH_2); 2.36 (s, 3H, CH_3); 1.56–1.20 (m, 12H, 6×CH_2); 0.89–0.80 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 160.0; 140.1; 136.4; 136.3; 129.5; 125.6; 124.2; 47.4 (45.1); 31.2; 28.5 (28.4); 28.3 (26.9); 21.9 (20.9); 13.8. IR cm^{-1}: 1651; 1587; 1497; 1375; 1180; 1101; 821; 732. Anal.: calcd for $C_{22}H_{32}N_2O_2$: 74.12%; 9.05%, 7.86%, found: C 74.48%; 9.33%, 7.50%.

*6.2.4.24. N,N-Dihexyl-2-[2-(4-methylphenyl)-1,3-oxazol-4-yl]acetamide (**6s**).* Yield: 54%. Colourless oil by column chromatography (petroleum ether:ethyl acetate = 93:7 v/v as eluant). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 7.95 (s, 1H, H-5); 7.84–7.80 (AA' part of the system AA'XX', 2H, H-Ar); 7.34–7.30 (XX' part of the system AA'XX', 2H, H-Ar); 3.63 (s, 2H, CH_2); 3.37–3.19 (m, 4H, 2×CH_2); 2.36 (s, 3H, CH_3); 1.60–1.12 (m, 16H, 8×CH_2); 0.90–0.78 (m, 6H, 2×CH_3). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 167.8; 160.0; 140.1; 136.4; 136.3; 129.5; 125.6; 124.2; 47.5 (45.1); 31.3 (31.0); 31.0; 28.5 (27.1); 26.0 (25.9); 22.0 (20.9); 13.8. IR cm^{-1}: 1651; 1590; 1497; 1183; 1101; 1064; 821; 736. Anal.: calcd for $C_{24}H_{36}N_2O_2$: 74.96%; 9.44%, 7.28%, found: C 75.18%; 9.75%, 7.06%.

*6.2.5. N,N-Dipentyl-2-(3-phenylisoxazol-5-yl)acetamide (**7d**)*

Potassium permanganate (0.253 g, 1.6 mmol) was added portion-wise to compound **28** in 3 mL of H_20 and 0.3 mL of concentrated H_2SO_4. The mixture was stirred at room temperature overnight and then was added $NaHSO_3$ (0.308 g, 3.0 mmol). The aqueous suspension was extracted with ethyl acetate (3×10 mL). The organic phases collected were dried (Na_2SO_4) and evaporated to dryness to give the crude product, purified by flash-chromatography (petroleum ether/ethyl acetate 4:1 v/v) to give the desired amide **7d** (8%) as a white wax after treatment with petroleum ether. ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.80–7.77 (m, 2H, H-Ar); 7.45–7.42 (m, 3H, H-Ar); 6.62 (s, 1H, H-4); 3.90 (s, 2H, CH_2); 3.37–3.33 (m, 2H, NCH_2); 3.32–3.29 (m, 2H, NCH_2); 1.64–1.53 (m, 4H, CH_2); 1.40–1.22 (m, 8H, 4×CH_2); 0.95–0.86 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.3; 166.4; 162.7; 130.0; 129.0; 128.4; 126.8; 101.3; 48.7; 46.5; 32.2; 29.1; 28.9; 28.8; 27.2; 22.4; 22.4; 14.0; 13.9. Anal.: calcd for $C_{21}H_{30}N_2O_2$: C 73.65%; 8.83%, 8.18%, found: C 73.33%; 9.11%, 8.08%.

*6.2.6. N,N-Dipentyl-2-(5-phenylisoxazol-3-yl)acetamide (**8d**)*

A solution of 3-nitro-*N*,*N*-dipentylpropanamide (**30**) (0.520 g, 2.0 mmol) and triethylamine (2 drops) in toluene anhydrous (3 mL) was added dropwise to a solution of phenyl isocyanate (0.4 mL, 4 mmol) and phenylacetylene (0.2 mL, 2 mmol) in toluene anhydrous (5 mL). After stirring for 1 h, the reaction mixture was warmed for an additional hour at 80 °C, cooled and checked (TLC, petroleum ether/ethyl acetate 4:1 v/v). The solid was filtered off, and the filtrate was evaporated to dryness to give the crude product, purified by flash-chromatography (petroleum ether/ethyl acetate 4:1 v/v) to give the desired amide **8d** (6%) as a colourless oil. ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.80–7.72 (m, 2H, H-Ar); 7.46–7.43 (m, 3H, H-Ar); 6.67 (s, 1H, H-4); 3.80 (s, 2H, CH_2); 3.35–3.29 (m, 2H, NCH_2); 3.27–3.23 (m, 2H, NCH_2); 1.67–1.47 (m, 4H, CH_2); 1.40–1.20 (m, 8H, 4×CH_2); 0.95–0.86 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 169.9; 167.7; 159.7; 130.1; 129.0; 128.9; 125.8; 100.3; 70.4; 48.4; 46.2; 31.7; 29.1; 28.9; 27.2; 22.4; 22.3; 14.0; 13.9. Anal.: calcd for $C_{21}H_{30}N_2O_2$: C 73.65%; 8.83%, 8.18%, found: C 73.35%; 9.10%, 8.05%.

*6.2.7. 3-(4-Methylphenyl)isoxazole-5-carboxylic acid (**16**)*

Potassium dichromate (5.0 g, 17.0 mmol) was added to a stirred solution of H_2SO_4 20% (130 ml). [3-(4-Methylphenyl)isoxazol-5-il] methanol **19** (2.1 g, 11.2 mmol) was added to the resulting solution and the mixture was stirred and heated at reflux for 1 h, after cooling at room temperature, the solid mass was filtered and washed with water until pH = 7. The crude product was crystallized by H_2O/MeOH to give white needles of acid **16** (1.38 g; 57%); m.p. 204.1–204.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 14.4 (bs, 1H, COOH); 7.84 (pd, 2H, H-Ar); 7.74 (s, 1H, H-4); 7.33 (pd, 2H, H-Ar); 2.36 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.6; 161.6; 157.7; 140.4; 129.7; 126.6; 124.9; 107.4; 20.9. Anal.: calcd for $C_{11}H_9NO_3$: C 65.02%; 4.46%, 6.89%, found: C 65.21%; 4.22%, 6.78%.

*6.2.8. 4-Methylbenzaldehyde oxime (**18**)*

Hydroxylamine hydrochloride (3.5 g, 50.0 mmol) was added in small portions at room temperature to a solution of NaOH (3 g, 75 mmol) and 4-methylbenzaldehyde (**17**) (6.0 g, 50 mmol) in water (8.5 mL). After the addition was complete, the mixture was diluted in 100 mL of water and saturated with CO_2 gas. The reaction mixture was stirred at room temperature for 1 h and then the solid obtained was filtered, washed with water and anhydrified (94%); m.p. 74.8–75.5 °C (lit [36]. m.p. 76–78 °C).

*6.2.9. [3-(4-Methylphenyl)isoxazol-5-yl]methanol (**19**)*

Prop-2-yn-1-ol (0.96 mL, 16.5 mmol) was added dropwise to a cooled solution (−5 °C) of triethylamine (0.16 mL, 1.1 mmol) in $CHCl_3$ (10 mL), maintaining the temperature in the range −5 ÷ 0 °C. At this temperature a solution of 4-methylbenzaldehyde oxime (**18**) (1.5 g, 11 mmol) in $CHCl_3$ (15 mL) was added in 15 min and then also 35 mL of NaOCl (5% of chloro active solution) in 35 min. The biphasic mixture was energetically stirred at room temperature for 2 h. Then the organic phase was separated and the aqueous phase was extracted with $CHCl_3$ (3×10 mL). The organic phases collected were treated with water (3×20 mL) and then anhydrified on Na_2SO_4. The solvent was removed to reduced pressure to give pure compound **19** (86%); m.p. 54.0–55.0 °C. ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.69 (AA' part of the system AA'XX', 2H, H-Ar); 7.27 (XX' part of the system AA'XX', 2H, H-Ar); 6.55 (s, 1H, H-4); 4.82 (s, 2H, CH_2); 2.41 (s, 3H, CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.7; 162.5; 140.2; 129.6; 126.8; 126.2; 99.9; 56.7; 21.3. Anal.: calcd for $C_{11}H_{11}NO_2$: C 69.83%; 5.86%, 7.40%, found: C 69.70%; 5.75%, 7.35%.

*6.2.10. N,N-Dipentylbut-3-enamide (**27**)*

Thionyl chloride (1.2 mL) was added at 0 °C to the vinylacetic acid **26** (1.0 g, 11.6 mmol). The obtained suspension was stirred and heated at reflux for 30 min and then cooled at −5 °C by an ice-salt bath. At this temperature, was added dropwise a solution of dipentylamine (4.7 mL, 23.2 mmol) in dry THF (5 mL). The reaction mixture was stirred at room temperature for 2 h (TLC, petroleum ether/ethyl acetate 4:1 v/v) and then the solid mass was filtered off and washed with THF. The filtrate was evaporated to dryness; the residue was treated with saturated sodium bicarbonate solution (100 mL) and extracted with dichloromethane (5×50 mL). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness to give the crude product, purified by flash-chromatography (petroleum ether/ethyl acetate 4:1 v/v) to give the desired amide **27** (40%) pure by NMR spectra. ^{1}H NMR (400 MHz; $CDCl_3$): 6.02–5.94 (m, 1H, CH); 5.17–5.07 (m, 2H, CH_2 =); 3.31–3.25 (m, 2H, NCH_2); 3.20–3.15 (m, 2H, NCH_2); 3.10 (d, 2H, J = 6.8, CH_2CO); 1.60–1.45 (m, 4H, 2×CH_2); 1.40–1.21 (m, 8H,

4×CH_2); 0.93–0.85 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 170.3; 132.2; 117.3; 48.0; 45.8; 38.6; 29.2; 29.0; 28.7; 27.4; 22.4; 22.3; 14.0; 13.9. Anal.: calcd for $C_{14}H_{27}NO$: C 74.61%; 12.08%, 6.21%, found: C 74.20%; 11.60%, 5.98%.

6.2.11. N,N-Dipentyl-2-(3-phenyl-4,5-dihydroisoxazol-5-yl) acetamide (**28**)

N,*N*-Dipentylbut-3-enamide (**27**) (4.1 g, 18.0 mmol) was added dropwise to a cooled solution (−5 °C) of triethylamine (0.16 mL, 1.1 mmol) in $CHCl_3$ (10 mL), maintaining the temperature in the range −5÷0 °C. At this temperature a solution of benzaldehyde oxime (1.5 g, 12 mmol) in $CHCl_3$ (15 mL) was added in 15 min and then also 35 mL of NaOCl (5% of chloro active solution) in 35 min. The biphasic mixture was energetically stirred at room temperature for 3 h. Then the organic phase is separated and the aqueous phase was extracted with $CHCl_3$ (3×10 mL). The organic phases collected were treated with water (3×20 mL) and then anhydrified on Na_2SO_4. The solvent was removed to reduced pressure to give compound **28** that was purified by flash-chromatography (petroleum ether/ethyl acetate 4:1 v/v) to give the desired amide **28** (66%). An analytical sample was obtained by crystallization from hexane; m.p. 60.0–61.0 °C. ^{1}H NMR (400 MHz; $CDCl_3$) δ: 7.68–7.65 (m, 2H, H-Ar); 7.40–7.36 (m, 3H, H-Ar); 5.20–5.07 (m, 1H, H-5); 3.69–3.60 (m, 1H, 4–CH_2); 3.37–3.09 (m, 5H, 2×NCH_2 and 4–CH_2); 2.99–2.90 (m, 1H, CH_2); 2.66–2.57 (m, 1H, CH_2); 1.60–1.45 (m, 4H, 2×CH_2); 1.38–1.21 (m, 8H, 4×CH_2); 0.92–0.86 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 169.0; 157.1; 130.0; 129.6; 128.7; 126.7; 78.5; 48.0; 45.8; 40.9; 38.5; 29.2; 29.0; 28.6; 27.4; 22.4; 22.3; 14.0; 13.9. Anal.: calcd for $C_{21}H_{32}N_2O_2$: C 73.22%; 9.36%, 8.13%, found: C 73.53%; 9.04, 8.28%.

6.2.12. 3-Nitro-N,N-dipentylpropanamide (**30**)

Thionyl chloride (1.2 mL) was added at 0 °C to the 3-nitropropionic acid **26** (1.38 g, 11.6 mmol). The obtained suspension was stirred and heated at reflux for 30 min and then cooled at −5 °C by an ice-salt bath. At this temperature, was added dropwise a solution of dipentylamine (4.7 mL, 23.2 mmol) in dry THF (5 mL). The reaction mixture was stirred at room temperature for 2 h (TLC, petroleum ether/ethyl acetate 4:1 v/v) and then the solid mass was filtered off and washed with THF. The filtrate was evaporated to dryness; the residue was treated with saturated sodium bicarbonate solution (100 mL) and extracted with dichloromethane (5×40 mL). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness to give the crude product, purified by flash-chromatography (petroleum ether/ethyl acetate 4:1 v/v) to give the desired amide **30** (45%) pure by NMR spectra. ^{1}H NMR (400 MHz; $CDCl_3$) δ: 4.72 (t, 2H, J = 6.1, CH_2NO_2); 3.33–3.27 (m, 2H, NCH_2); 3.27–3.21 (m, 2H, NCH_2); 2.97 (t, 2H, J = 6.1, CH_2CO); 1.65–1.56 (m, 2H, CH_2); 1.55–1.46 (m, 2H, CH_2); 1.40–1.23 (m, 8H, 4×CH_2); 0.95–0.89 (m, 6H, 2×CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 167.5; 70.45; 47.6; 46.2; 29.9; 29.1; 29.0; 28.6; 27.3; 22.4; 22.3; 14.0; 13.9. Anal.: calcd for $C_{13}H_{26}N_2O_3$: C 60.44%; 10.14%, 10.84%, found: C 60.05%; 10.15%, 10.20%.

6.3. Biological methods

6.3.1. Materials

[^{3}H]PK 11195 (S.A. 85.5 Ci/mmol) and [^{3}H]Ro15-1788 (S.A. 83.4 Ci/mmol) were purchased from Perkin–Elmer Life Sciences. PK 11195 and Ro5-4864 were obtained from Sigma–Aldrich. All reagents were obtained from commercial suppliers.

6.3.2. [^{3}H]PK11195 binding to rat kidney mitochondrial membranes

For binding studies, crude mitochondrial membranes were incubated with 0.6 nM [^{3}H]PK 11195 in the presence of a compound concentration range (0.1 nM–10 μM) in 50 mM Tris–HCl, pH 7.4, as previously described [15]. For the active compounds, the IC_{50} values were determined and K_i values were derived in accordance with the equation of Cheng and Prusoff [37].

6.3.3. [^{3}H]Ro15-1788 Binding to rat cerebral cortex membranes

Rat cerebral cortex membranes were prepared as previously described [38]. After differential centrifugation, the crude membrane fraction obtained was subjected to washing procedures to remove endogenous GABA [39]. The washed membranes were incubated with 0.4 nM [^{3}H]Ro15-1788 for 90 min at 0 °C in 500 μL of 50 mM Tris-citrate buffer, pH 7.4, as previously described [40].

6.3.4. Cell culture and treatments

The human glioblastoma multiforme (GBM) cell line U87MG was obtained from the National Institute for Cancer Research (ICLC) of Genoa. The U87MG cells (1×10^6) were seeded onto T-75 flasks and cultured in RPMI medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% non-essential amino acids, at 37 °C in humidified atmosphere composed of 5% CO_2 and 95% O_2. For U87MG cell treatments, the cells were seeded at appropriate densities depending on the experimental test in complete medium at 37 °C and 5% CO_2. After 24 h the medium was replaced either with complete culture medium containing DMSO (untreated control cells) or with complete medium supplemented with the compounds for the time period indicated. The compounds were dissolved in DMSO (<1% v/v of medium).

6.3.5. Cell proliferation/viability analysis

Cell proliferation/viability was estimated by the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) conversion assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega) and by trypan blue (TB) exclusion assay, as previously reported [41]. Concerning the dose-dependent treatment cells were exposed to a single incubation time (24 h) and increasing **6d** concentrations, corresponding to 15, 30 and 150 times the K_i value. Concerning the time-dependent treatments cells were exposed to a single concentration of compound **6d**, 30 times the K_i value, for 24 h, 48 h and 72 h. Each drug concentration was tested in triplicate, and the experiments were repeated at least three times.

6.3.6. Data analyses

Data were analyzed by use of the GraphPad Prism software (GraphPad Software, version 5.0; San Diego, CA). Statistical analyses were performed by one-way ANOVA (with post hoc Bonferroni test).

6.3.7. Mitochondrial membrane potential (ΔΨm) Cytofluorimetric analysis

Changes in ΔΨm were analized by use of the fluorescent dye JC-1, as previously described [6,42]. J-aggregate and J-monomer were recorded with a FACScalibur flow cytometer (Becton Dickinson, USA) in fluorescence channel 2 (FL2) and in fluorescence channel 1 (FL1), respectively. The cells were selected electronically and chosen on the basis of morphological characteristics observed on the forward *vs* side scatter dot plot. In brief, the cells were seeded in 24-well plates and after treatments with DMSO (control sample) or with tested compound for 12, 24 and 48 h, cells were collected by centrifugation. Pellets were suspended in JC-1 solution and incubated for 30 min at room temperature in the dark. After incubation the pellets were suspended in PBS 1X and their fluorescence was analyzed by FACS. As a positive control, cells were incubated in the presence of the uncoupling agent CCCP (carbonylcyanide *m*-chlorophenylhydrazone) in each experiment.

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