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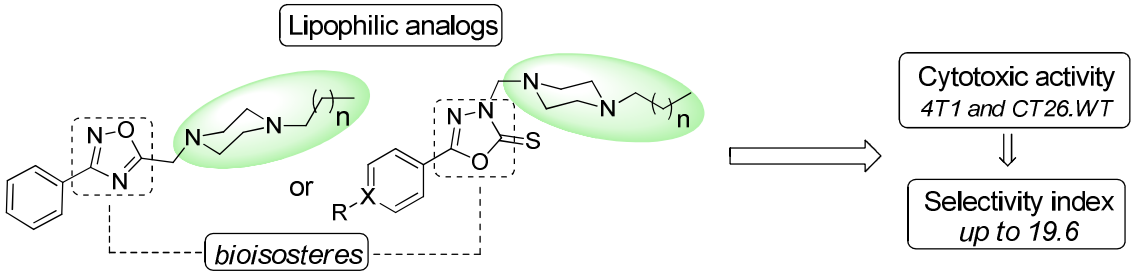

Synthesis and anticancer evaluation of new lipophilic 1,2,4 and 1,3,4-oxadiazoles

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Abstract:A series of1,2,4- and 1,3,4-oxadiazole derivatives were synthesized and evaluated for their anticancer activity. Halogenated 1,2,4-oxadiazoles were obtained from benzonitrile and coupled either lipophilic amines or with aminoalcohols. Lipophilic 1,3,4-oxadiazole derivatives were obtained through the Mannich reactions between 5-(aryl)-1,3,4-oxadiazole-2-thiol and alkylated or acylated amines. The *in vitro* cytotoxic effects were evaluated against 4T1– mammary carcinoma and CT26 – colon cancer cells. The best results were obtained for the 1,3,4-oxadiazole coupled to alkylated piperazine with 10-14 carbon chain moiety, with IC_{50} values ranging from 1.6 to 3.55μM for the 4T1 cell line, and from 1.6 to 3.9 μM for the CT26.WT cell line, and selectivity index up to 19. The most potent compounds were investigated with AnnexinV and PI staining as indicative of apoptosis induction.

1. Introduction

The great challenge for chemists is the discovery of new efficient compounds with minimal side effects and use of small doses, and many studies have been conducted for the development of new selective drugs. Heterocyclicsystems play an

important role in the discovery of new bioactive substances, since they are present in various natural bioactive compounds [1].Due to the high number of deaths [2], cancer treatment has attracted much attention from researchers and industries for the synthesis of new,more selective anticancer agents. Undesirable effects, toxicity, drug resistance and reduced bioavailability are known for current anticancer available agents [3]. Therefore, the discovery of new anticancer agents, more efficient and more selective is urgent.

Over the past years, 1,2,4- and 1,3,4-oxadiazole derivatives have been widely studied for theirantiviral [4], antibacterial [5], antioxidant [6], anti-inflammatory [7], antifungal activity [8] and anticancer activities [9].The biological potential of these heterocycles against cancer cells has been reported with different mechanisms of action, such as inhibition of tubulin, blocking endothelin A receptor involved in apoptosis, mitogenesis, angiogenesis and metastasis in tumors,focal adhesion kinase inhibition, telomerase inhibition, interacting with several receptors involved in proliferation, cell growth and DNA biosynthesis [10-19]. The presence of the azole group in their structures makes them more lipophilic and, therefore, more susceptible to passage through the cell membrane[20].

Moreover, interesting activities,such as antitubercular, immunosuppressive and antiparasitic, associated with lipophilic structureshave been reported in the literature [21-24]. Lipophilic molecules can interact with cell membranes, increasing their permeability and consequently disturbing membrane-embedded proteins, inhibiting respiration, altering the ion transport process or leading to cell lysis [25]. Small lipophilic molecules bearing a moiety with potential anticancer activity can display enhanced anticancer activity, as they are more susceptible to uptake by the cancer cells, where they can interact with a specific binding site.

Considering the importance of oxadiazoles, we report herein the synthesis of 1,2,4 and 1,3,4- oxadiazolederivatives coupled to lipophilic amines and aminoalcohols as promising antitumor agents. The anticancer activity of the compounds was evaluated by colorimetric MTT assay against two different cancer cell lines (4T1 – mammary carcinoma, CT26.WT – colon cancer cells) and one non-tumor cell line (BHK-21 – baby hamster kidney).

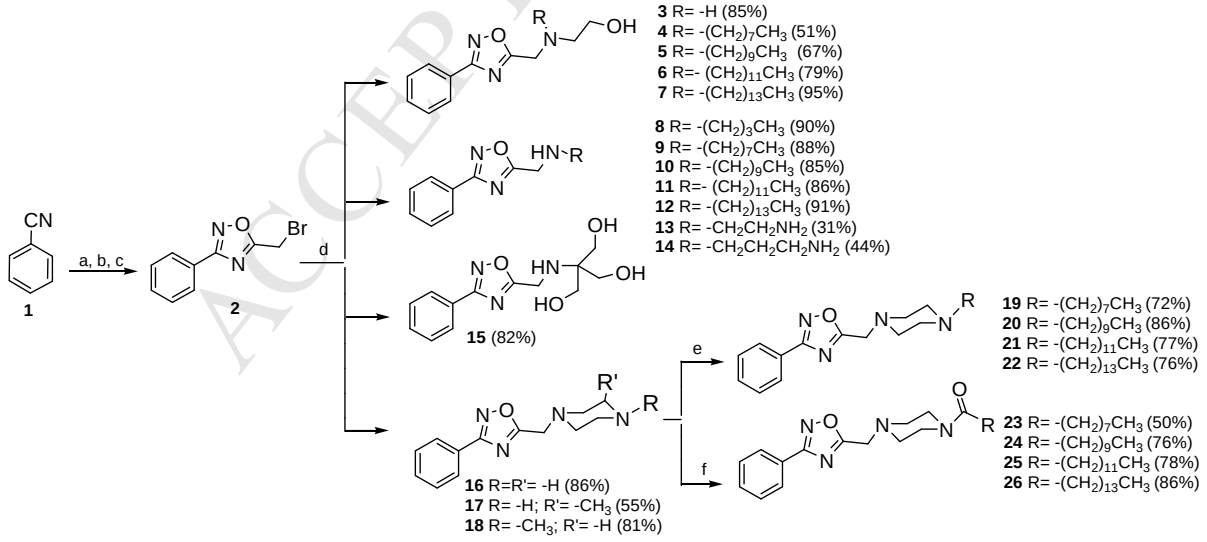
2. Results and Discussion

2.1- Chemistry

The synthesis of the 5-(bromomethyl)-3-phenyl-1,2,4-oxadiazole**2** was initiated by the reaction between benzonitrile **1** and hydroxylamine hydrochloride in basic solution, generating an amidoxime in 87% yield. The latter was treated with bromoacetyl bromide and submitted to heating, leading to the formation of the desired heterocyclic compound **2**(74% yield), which underwent an S_N2 type reaction with 1-methylpiperazine, 2-methylpiperazine and different lipophilic amines and aminoalcohols according to the literature [26], generating the final compounds **3**-**18**. Furthermore, compound **16**,containing a piperazine moiety, was reacted with different long chain alkylandacyl chlorides, leading to compounds **19**-**26** (Scheme 1).

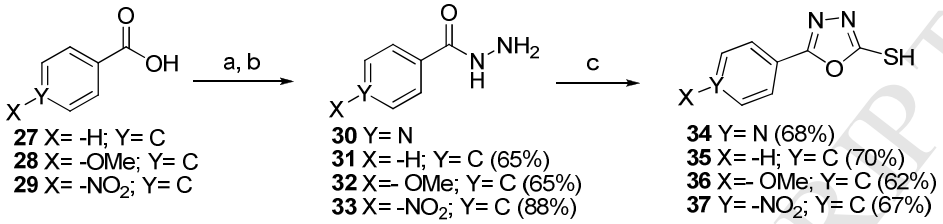
The synthesis of 5-(aryl)-1,3,4-oxadiazole-2-thiol derivatives **34**-**37** was initiated by the esterification reaction [27] of the carboxylic acids **27**-**29** in ethanolic sulfuric acid solution. The aromatic esters were then reacted with hydrazine (68% m/v) [27], generating the corresponding hydrazides **31**-**33**. The synthesized hydrazides and isoniazid **30** were reacted with carbon disulfide in basic solution for the formation of the desired heterocycles (Scheme 2). Finally, the alkylated and acylated piperazine derivatives, previously prepared [28], were reacted separately with 1,3,4-oxadiazoles **34**-**37** and formaldehyde (37% v/v) through a Mannich type reaction [29], giving the desired products **38**-**69** (Scheme 3).

Chemical data of the final compounds are reported in Supporting Information and were characterized by melting point, ^{1}H NMR, ^{13}C NMR and mass spectroscopy (MALDI-TOF) and LC-MS.

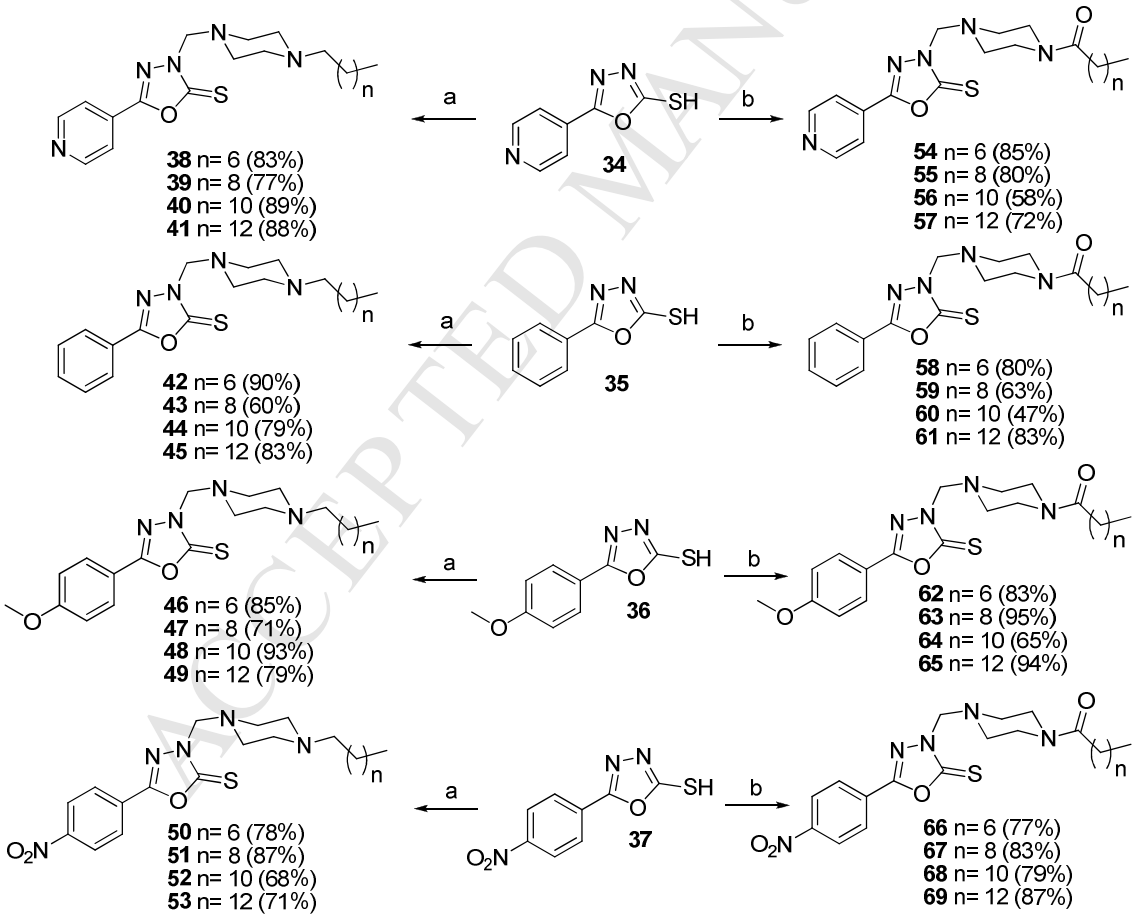


Scheme 1: Synthesis of 1,2,4-oxadiazole derivatives coupled to amines and aminoalcohols. Reactions conditions: a) $H_2NOH.HCl$ (1.5 eq.), $NaHCO_3$(1.6 eq.),

MeOH, reflux, 6h; b) bromoacetyl bromide (1.35 eq.), K_2CO_3(1.35 eq.), $CHCl_3$, r.t., 3h; c) toluene, 110°C, 5h; d) amines (2.0 eq.), K_2CO_3(2.0 eq.), CH_3CN, 70°C, 1-5h; e) alkyl halides (1,5 eq.), K_2CO_3(1.5 eq.), CH_3CN, 70°C, 1-5h; f) acyl chlorides (1.5 eq.), K_2CO_3 (1.5 eq.), CH_3CN, r.t., 1-72h.



Scheme 2: Synthesis of 5-(aryl)-1,3,4-oxadiazole-2-thiol. Reaction conditions: a) H_2SO_4 conc., EtOH, reflux, 20h; b) H_2NNH_2 (68% m/v) (2.0 eq.), EtOH, reflux, 20h; c) CS_2 (4.0 eq.), KOH (3.0 eq.), EtOH, 55°C, 48h.



Scheme3: Synthesis of 1,3,4-oxadiazole derivatives from 1,3,4-oxadiazole. Reactionconditions: a) alkylated amines (1.0 eq.), HCHO (1.5 eq.), MeOH, r.t.; b) acylated amines (1.0 eq.), HCHO (1.5 eq.), MeOH, r.t.

2.2. Cytotoxicity

In order to study the structure activity relationship, fifty-six different lipophilic 1,2,4- and 1,3,4-oxadiazole derivatives, differing from the group attached to the heterocycle, were analyzed *in vitro* by MTT assay for their anticancer activity against tumor cell lines (4T1 – mammary carcinoma and CT26.WT – colon cancer cell line) and non-tumor cell line (BHK-21 – baby hamster kidney), with different embryonic origins (epithelial and fibroblast) used to evaluate the selectivity index. The results are summarized in Table 1. Although similar, the best activities were accessed for those compounds containing an alkylated piperazine moiety. In general, 1,2,4-oxadiazole derivatives did not show significant results for the cell lines tested, with the exception of compounds **20** and **21** containing piperazine bearing a ten or twelve carbon chain with IC_{50} values of 3.6±0.5μM and 8.1±1.6 μM respectively, and a great selectivity index for 4T1 cell (18.4 and 8.8, respectively). Besides, this less lipophilic structures such as **3** and **13-17**, containing primary alcohols, primary orsecondary amines did not show cytotoxicity even for the BHK-21 (non tumor cell line). In 2008, Silva and co-workers [30] investigated the anticancer activity of some lipophilic complexes and found that the most active compounds were those containing a twelve-carbon chain. Our group also reportedthe importance of the lipophilic chain in the anticancer activity, where carbohydrate derivatives with a twelve carbon chain were the most potent for the cells tested [24]. All these results suggest that the alkyl chain linked to the piperazine is important for the anticancer activity of these compounds, mainly for those with twelve carbons.

In the case of 1,3,4-oxadiazoles derivatives, the results showed some relationship between biological activity and lipophilicity. In most cases, the more lipophilic structures showed higher levels of cytotoxicity for the cells used, being optimal for those structures containing a twelve carbon chain (Figure 1). In general, 1,3,4-oxadiazoles derivatives **38-53** containing an alkylated piperazine (Figure 1a and 1b) showed better results than those containing an acylated piperazine **54-69** (Figure 1c and 1d), especially compound **41**, being 65 times more specific for the 4T1 cell line compared to reference drug. Furthermore, comparing the heterocycles studied, 1,3,4-oxadiazolederivatives were more active than the 1,2,4 regioisomers. In addition, for the 1,3,4-oxadiazole regioisomer, the compounds containing phenyl and pyridine ring weremore active against the 4T1 cell line while the compounds containing 4-methoxy

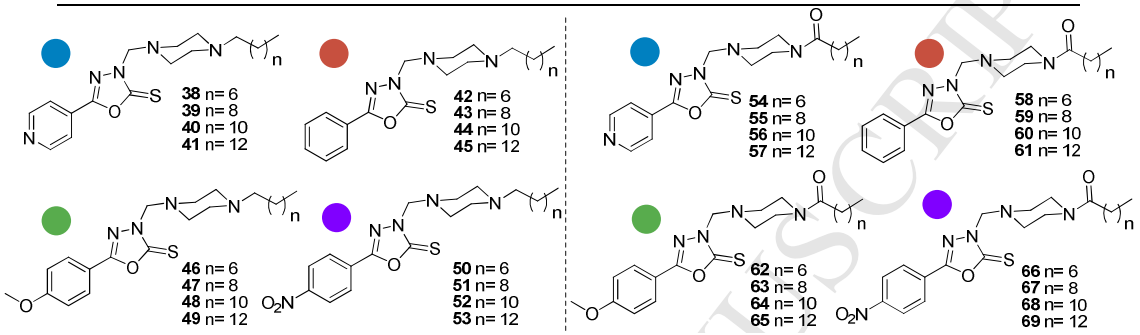
phenyl were the most active against the CT26.WT cell line.For both cell lines tested, optimal results were shown for structures containingtwelve carbonalkyl chain. For the acylated compounds, the best results were obtained for the structures containing fourteen carbons.The alkylated compounds **39-45** showed the most relevant results against the 4T1 cell line, with values ranging from 1.6±0.3μM to 17.5±0.1μM and high selectivity index, varying from 4.3 to 19.6, showing that the presence of an alkyl chain with ten or twelve carbons was optimal for the activity against this cell line. Compound**45** had an interesting IC_{50} (3.1±0.4μM) but poor selectivity index when compared to the normal cell, BHK-21. In the case of the CT26 tumor cell line, compounds **47-49** were the most active (IC_{50} ranging from 1.6±0.7 μM to 3.9±0.1μM) with thehighest specificity of compound **48** bearing a twelve carbons chain. Acylated structures showed a tendency toincrease the anticancer activity with the increase of the alkyl chain highlighting the importance of this moiety for the cytotoxicity. In addition, the analysis of the best results obtained for 1,2,4-oxadiazole derivatives **19-21** and 1,3,4-oxadiazoles **38-41**, demonstrates the importance of the effect of the heterocycle on the cytotoxicity against tumor cells, highlighting the high selectivity index of these compounds exhibited, especially those alkylated structures with ten- carbon atoms. The results show a decrease in the activity of alkylated 1,2,4-oxadiazolic derivatives, while there was an increase in activity for the 1,3,4-oxadiazole regioisomer with concomitant increase in lipophilicity of the same (Figure 2).

Table 1. Cytotoxic activities against cell lines. IC_{50} (μM± SD)

	Tumor Cells IC_{50}				**Non-Tumor Cells**
Compounds	**4T1**	**SI**	**CT26.WT**	**SI**	**BHK-21**
3	>100	-	>100	-	>100
4	>100	-	>100	-	>100
5	82.3 ± 5.0	-	>100	-	>100
6	>100	-	84.3 ± 0.4	1.0	87.3± 3.0
7	42.7 ± 4.0	1.1	30.2 ± 3.0	1.6	48.3 ± 3.0
8	>100	-	>100	-	>100
9	45.1 ± 0.4	2.1	46.5	2.0	94.6 ± 4.0
10	>100	-	>100	-	>100
11	>100	-	>100	-	>100
12	>100	-	>100	-	>100
13	92.3 ± 4.0	-	>100	-	>100

14	>100	-	>100	-	>100
15	>100	-	>100	-	>100
16	>100	-	>100	-	>100
17	>100	-	>100	-	>100
18	>100	-	>100	-	>100
19	12.0 ± 4.1	**4.7**	69.7 ± 2.3	0.8	56.3 ± 1.8
20	3.6 ± 0.5	**18.4**	59.5 ± 1.1	1.1	66.3 ± 1.1
21	8.1 ± 1.6	**8.8**	77.7 ± 4.2	0.9	71.4 ± 2.0
22	26.4 ± 9.1	-	>100	-	>100
23	66.8 ± 1.9	0.5	96.5 ± 2.1	0.3	34.4 ± 4.3
24	62.2 ± 1.4	0.4	54.2 ± 1.2	0.5	28.6 ± 0.6
25	54.6 ± 5.9	0.6	63.8 ± 2.3	0.5	32.5 ± 1.9
26	29.6 ± 3.5	0.8	55.7 ± 7.8	0.4	22.7 ± 0.5
38	17.5 ± 0.1	-	>100	-	>100
39	3.55 ± 0.2	**6.4**	29.0 ± 1.8	0.8	22.9 ± 1.0
40	2.55 ± 0.1	**6.4**	22.1 ± 1.2	0.7	16.4 ± 2.6
41	3.2 ± 2.4	**19.6**	38.2 ± 0.8	1.6	62.7 ± 3.4
42	10.9 ± 2.8	-	15.4 ± 5.0	-	>100
43	1.9± 0.1	**16.7**	15.4 ± 0.4	2.0	31.8 ± 0.2
44	1.6 ± 0.3	**14.5**	6.3 ± 0.1	3.7	23.3 ± 3.7
45	3.1 ± 0.4	**4.3**	7.7 ± 0.2	1.7	13.4 ± 8.4
46	83.7 ± 3.6	0.9	19.4 ± 1.8	3.8	74.0 ± 4.2
47	34.7 ± 1.7	0.6	3.9 ± 0.1	**5.6**	21.9 ± 2.6
48	24.4 ± 2.4	0.9	1.6 ± 0.7	**13.8**	22.1 ± 0.2
49	22.4 ± 2.4	0.9	3.4 ± 0.2	**6.2**	21.1 ± 2.1
50	>100	-	>100	-	69.5 ± 1.9
51	21.1 ± 0.4	1.1	25.1 ± 1.3	1.0	24.0 ± 1.6
52	8.2 ± 0.1	0.7	24.8 ± 1.9	0.2	6.1 ± 0.2
53	6.7 ±0.9	0.1	22.5 ± 1.7	0.0	0.9 ± 0.1
54	>100	-	93.3 ± 1.0	-	>100
55	82.6 ± 4.0	0.9	50.6 ± 1.6	1.5	76.1 ± 3.1
56	21.8 ± 1.6	1.2	21.9 ± 0.5	1.2	27.2 ± 1.1
57	20.1 ± 1.3	1.0	9.5 ± 0.5	2.1	19.8 ± 0.3
58	>100	-	>100	-	>100
59	68.1 ± 0.1	0.9	61.3 ± 4.0	1.0	64.6 ± 2.0
60	17.1 ± 2.0	1.1	12.0 ± 5.0	1.6	18.9 ± 1.7
61	12.6 ± 1.0	0.7	8.4 ± 0.8	1.0	8.6 ± 0.6
62	>100	-	>100	-	>100

63	82.6 ± 2.6	0.7	>100	-	60.3 ± 1.3
64	24.0 ± 0.8	0.9	28.1 ± 0.3	0.8	22.6 ± 0.5
65	22.3 ± 1.1	0.5	23.9 ± 4.6	0.5	11.6 ± 2.9
66	>100	-	>100	-	83.4 ± 3.4
67	59.6 ± 1.1	1.0	69.5 ± 1.9	0.8	58.6 ± 1.5
68	8.9 ± 0.4	1.4	24.6 ± 0.1	0.5	12.7 ± 0.8
69	5.2 ± 0.3	0.4	11.7 ± 1.3	0.2	2.2 ± 0.6
Daunorubicin	0.3 ± 0.01	0.3	0.42 ± 0.04	0.2	0.09± 0.01



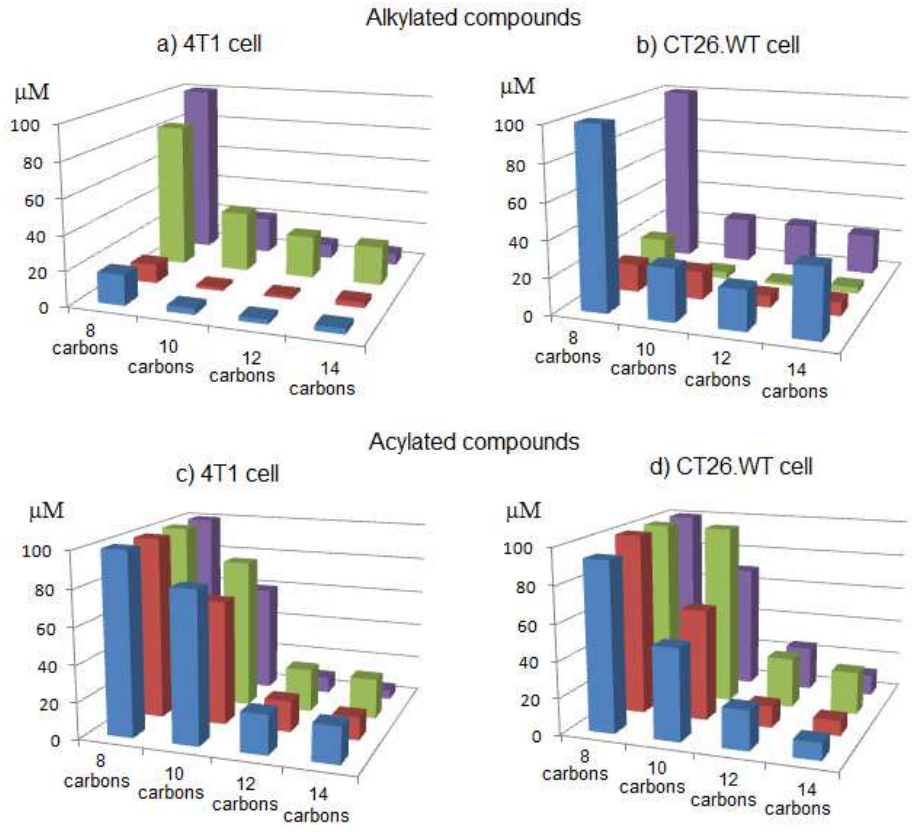


Figure 1: Anticancer activity of 1,3,4-oxadiazolesversus lipophilicity: a and b) alkylated structures; c and d) acylated structures; Blue: 5-(pyridine-4-yl)-1,3,4-oxadiazole-3*H*-thione derivatives; Red:5-(4-phenyl)-1,3,4-oxadiazole-3*H*-thione

derivatives;Green:5-(4-methoxyphenyl)-1,3,4-oxadiazole-3*H*-thione derivatives; Purple: 5-(4-nitrophenyl)-1,3,4-oxadiazole-3*H*-thione derivatives.

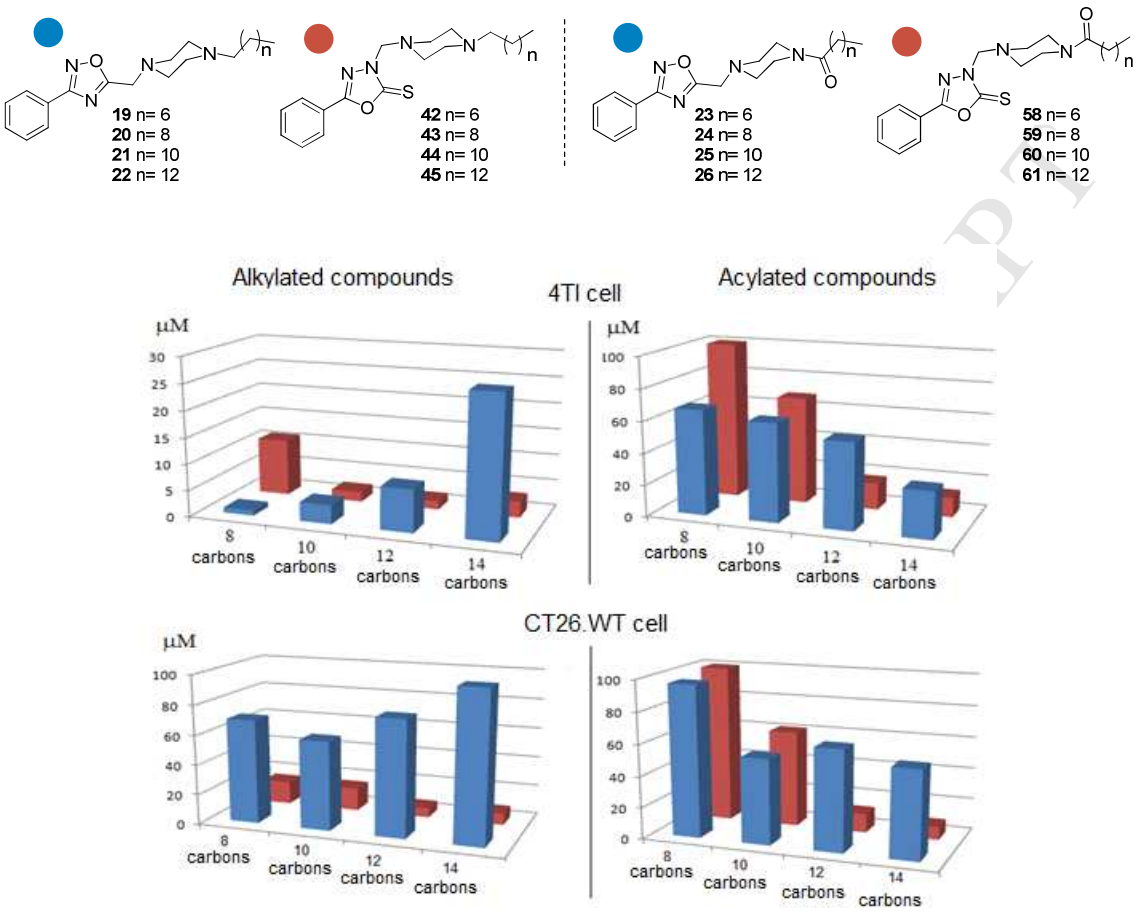


Figure 2: Comparison of the cytotoxic effect between 1,2,4 and 1,3,4-oxadiazole heterocycles for 4T1 and CT26.WT cells

2.3. Pro-apoptotic activity

Considering that some chemotherapeutic agents promote apoptosis we investigated this event by AnnexinV and PI staining analysis. According to the IC-50 values, compounds **44** and **48** were chosen to assess their pro-apoptotic activity in 4T1 and CT26.WT cell lines, respectively. Firstly, subdiploid DNA (sub-G1 phase cells) was analyzed as indicator of apoptosis as described in the literature [31]. Compound **44** increased DNA fragmentation around 2 and 4 times at 5 µM and 10 µM, respectively in 4T1 cells, compared to control. In CT26.WT, compound **48** increased 66 times subdiploid DNA at 50 µM, compared to control (Figure 3A). These increases in DNA fragmentation indicates a potential pro-apoptotic activity which was confirmed analyzing Annexin V positive cells. As shown in Figure 3B, 83.6% of 4T1 cells had apoptosis induced by

compound **44** 10 µM (49.1% early apoptosis and 34.5% late apoptosis). On the other hand, compound **48** 50 µM induced apoptosis in 99% of CT26.WT cells, most of them (98%) in late stage. Even the experiment was carried out in the same time exposure of MTT assay, the Annexin V/PI staining assay can show the difference of cell response on the course of apoptosis. Both compounds belong to alkyl derivatives with the same carbon chain length and differ only on *para*-benzyl substituent metoxyl. Although with some differences in chain length and substitutes moieties, Khanam et al (2017) also described an induction of apoptosis by potent antitumor thioalkyl-aryl-oxadiazole derivatives [32].

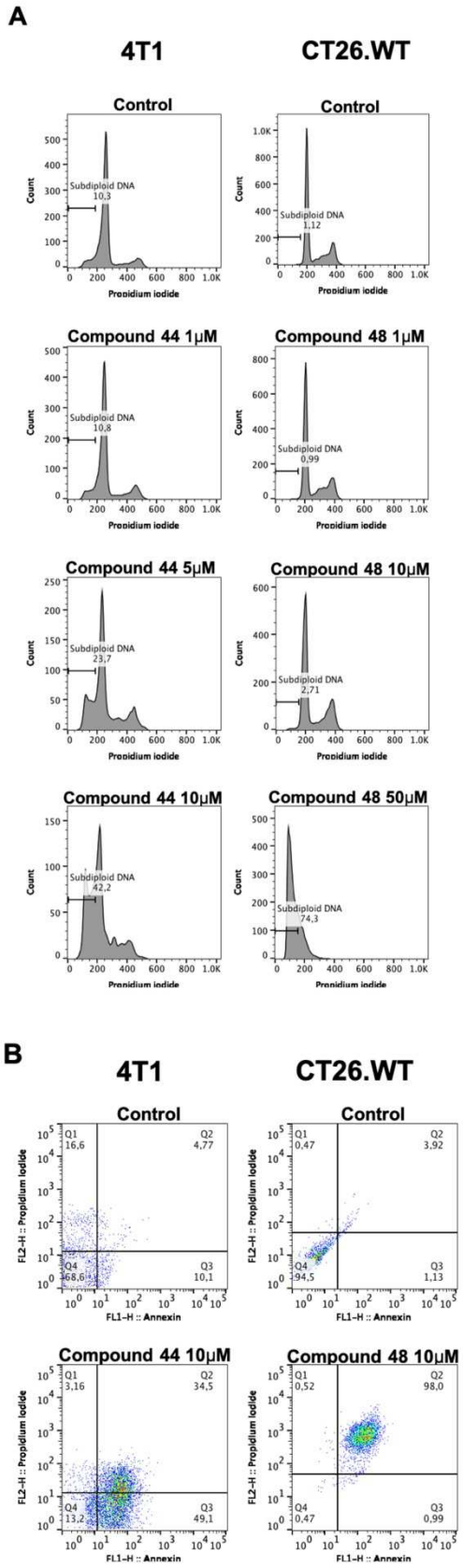


Figure 3: Pro-apoptotic activity of compounds **44** and **48** in 4T1 and CT26.WT cells. (A) Representative histograms of cell cycle profile with subdiploid DNA indicated. (B) Representative dotplot of Annexin V and Propidium iodide stained cells.

3. Conclusion:

In conclusion,novel series of 1,2,4 and 1,3,4-oxadiazole derivatives containing a lipophilic moiety were synthesized and evaluated for their antitumor activity. The results showed that alkylated structures were more active thanthe acylated ones. Although the compounds were less active when compared to the reference drug, they displayed a higher SI. For the 1,2,4- oxadiazole derivatives, compounds**20** and **21**were highly selective for the 4T1 cell line.In the case of 1,3,4-oxadiazole derivatives, compounds containingphenyl and pyridine rings were themost active in the cytotoxic assay, highlighting compounds **38-45**. Less lipophilic structures did not show cytotoxicity, suggesting that this feature is important forthe anticancer activity.The two most active alkylated compounds **44** and **48** showed to be capable to induce apoptosis in 4T1 and CT26, a crucial cellular process for effective chemotherapeutic agents.

4. Experimental Protocols

4.1. Materials and experiments

Reagents and solvents were purchased as reagent grade and used without purification. Thin layer chromatography (TLC) was performed on glass plates and silica gel sheets (silica gel F254; Merck) visualized with iodine vapor, and /or ultraviolet light 264 nm. Column chromatography was carried out on silica-gel 60G 0.063-0.200mm (70-230 mesh). Melting points were determined on a MQAPF-Microquimica apparatus. IR spectra were acquired using a Bruker Alpha-E ATR (Attenuated total reflection) spectrometer. NMR data were recorded on a Bruker 500 Advance spectrometer. Chemical shifts of ^{1}H NMR and ^{13}C NMR are given in ppm and constant coupling (*J*) are given in Hz. TMS signal was used as internal reference. Mass spectra of the compounds **3-13**; **15-16**; **19-26**; **38-49**; **54-56** and **58-65** were performed on Maldi-TOF Shimadzu-Biotech Axima Performance spectrometer.The mass-to-charge (m/z) characterization of the compounds **14**; **17-18**; **50-53**; **57** and **66-69** was made with 6120Quadrupole LC/MS (Agilent Technologies, Singapore) equipped with anAPI-ES ion source, coupled to the Capillary Electrophoresis instrument, Agilent CE 7100 (Agilent Technologies, Singapore), used to compounds infusion into the MS by flush with methanol (MeOH). The sheathliquid (0.1% offormic aciddissolved in 1:1 (v/v) methanol/water) was delivered at 0.01 mL/min flow using an LC isocratic pump (1260 Infinity, Agilent Technologies, Waldbronn, Germany).The MS was operated in the positive ionization mode applying electrospray voltage of 3.0 kV. Nitrogen was used as

drying gas at 250ºC, with a flowrate of 6 L/min; the pressure of the nebulizer gas was set at 15 psig. The m/z characterization of the compounds was made byselected ion monitoring (SIM) mode.In order to perform compounds infusion into the quadrupoleMS by flush with MeOH, the fused-silica capillary (Polymicro Technologies, Phoenix, USA) of 100 cm length and internal diameter of 50μm was used. The new capillary was conditioned by flushing with 1.0mol L-1NaOH (10 min), water (10 min), and MeOH (15 min). Samples were injected hydrodinamically at 50 mbar for 2.0 sec and cassette temperature was maintained at 25º C. Among sample injections, a short preconditioning in the capillary was performed by flushing with 1 M NaOH (1 min), water (1 min) and MeOH (43 min).The data processing was performed with the Agilent ChemStation for CE-MS version software.

4.2. General procedure for the synthesis of compound **2***:*

To a solution of benzonitrile (80 mmol) in MeOH (100 mL), $H_2NOH.HCl$ (1.5 eq.) and $NaHCO_3$ (1.6 eq.) were added at room temperature. The resulting mixture was allowed to reflux for 5h. The reaction was cooled to room temperature, the solvent was removed under reduced pressure and the resulting mixture was extracted with EtOAc and H_2O. The organic phase was washed with brine solution and concentrated under vacuum, affording the amidoxime as a colorless oil in 83% yield. Then, bromoacetyl bromide (1.35 eq.) was added dropwise to a solution of the amidoxime and K_2CO_3 (1.35 eq.) in $CHCl_3$ (60 mL). The reaction was stirred at room temperature for 3h. The solvent was removed, the mixture was extracted with EtOAc and H_2O and the organic phase was washed with a brine solution, dried with Na_2SO_4, filtrated and concentrated under reduced pressure. The resulting mixture was dissolved in toluene (50 mL) and heated at 100°C for 2h. The reaction was cooled and the solvent removed. The resulting mixture was purified by chromatography on silica gel (eluent: EtOAc/hexane) which afforded the desired product as white solid.

4.2.1- 5-(bromomethyl)-3-phenyl-1,2,4-oxadiazole **2**

White solid; Yield: 74%; m.p.: 48-50°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.08 (2H; dd; ^3J= 8.0 Hz; ^4J= 1.5 Hz), 7.51-7.46 (3H; m), 4.56(2H; s)[33].^{13}C RMN ($CDCl_3$; 125 MHz) δ ppm:174.7, 169.0, 131.6, 129.0, 127.6, 126.3, 16.6.

4.3. General procedure for the synthesis of compounds **3-18**:

To a solution of **2** (1.0 mmol) and K_2CO_3 (2.0 eq.) in CH_3CN, amine (2.0 eq.) was added at room temperature. The resulting mixture was allowed to reflux for 1 to 5h. The solvent was removed under reduced pressure and the mixture was extracted with CH_2Cl_2 and H_2O and washed with brine solution. The organic layer was dried with Na_2SO_4, filtrated and concentrated under reduced pressure. Then, the crude mixture was purified by chromatography on silica gel (eluent: CH_2Cl_2/ MeOH) affording the desired compounds.

4.3.1)5-[(2-hydroxyethyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **3**

Oil colorless; Yield: 85%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09-8.07 (2H, m); 7.52-7.47 (3H, m), 4.16 (2H, s), 3.71 (2H, t, *J*= 5.0 Hz), 2.93 (2H, t, *J*= 5.0 Hz), 2.32 (2H, bs). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.6, 168.4, 131.5, 129.0, 127.6, 126.6, 61.1, 51.0, 44.7.MALDI-TOF m/z: [M+H] calculated 220.1088; [M+H] found 220.1080.

4.3.2) 5-[(2-hydroxyethyl)(N-octyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **4**

Brown oil, Yield: 51%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09-8.07 (2H; m), 7.51-7.47 (3H; m), 4.07 (2H; s), 3.66 (2H; d; *J*= 5.0 Hz), 3.03 (1H; bs), 2.85 (2H; d; *J*= 5.0 Hz), 2.65 (2H; t; *J*= 7.0 Hz), 1.54 (2H; m), 1.29-1.26 (10H; sl), 0.87 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 177.6, 168.3, 131.4, 129.0, 127.6, 126.7, 59.1, 56.5, 54.4, 48.8, 32.0-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 332.2340; [M+H] found 332.2300.

4.3.3) 5-[(2-hydroxyethyl)(N-decyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **5**

Colorless oil, Yield: 67%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09 (2H; m), 7.52-7.48 (3H; m), 4.07 (2H; s), 3.66 (2H; d; *J*= 5.0 Hz), 3.03 (1H; bs), 2.85 (2H; d; *J*= 5.0 Hz), 2.65 (2H; t; *J*= 7.0 Hz), 1.54 (2H; m), 1.29-1.25 (14H; bs), 0.88 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 177.6, 168.3, 131.5, 129.1, 127.7, 126.8, 59.1, 56.5, 54.5, 48.8, 32.1-22.9, 14.3. MALDI-TOF m/z: [M+H] calculated 360.2653; [M+H] found 360.2650.

4.3.4) 5-[(2-hydroxyethyl)(N-dodecyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **6**

Colorless oil, Yield: 79%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09 (2H; d; *J*= 7.5 Hz), 7.52-7.48 (3H; m), 4.07 (2H; s), 3.66 (2H; d; *J*= 5.0 Hz), 3.04 (1H; bs), 2.85 (2H; d; *J*= 5.0 Hz), 2.65 (2H; t; *J*= 7.0 Hz), 1.54 (2H; m), 1.29-1.25 (18H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 177.6, 168.4, 131.5, 129.1, 127.7, 126.8,

59.2, 56.6, 54.5, 48.8, 32.1-22.9, 14.3. MALDI-TOF m/z: [M+H] calculated 388.2966; [M+H] found 388.2970.

4.3.5) 5-[(2-hydroxyethyl)(N-tetradecyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **7**

Colorless oil, Yield: 95%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09-8.07 (2H; m), 7.50-7.45 (3H; m), 4.06 (2H; s), 3,65 (2H; d; *J*= 5.0 Hz), 3.10 (1H; bs), 2.83 (2H; d; *J*= 5.0 Hz), 2.64 (2H; t; *J*= 7.0 Hz), 1.52 (2H; m), 1.29-1.24 (22H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 177.6, 168.3, 131.4, 129.0, 127.6, 126.7, 59.1, 56.5, 54.4, 48.8, 32.0-22.8, 14.3. MALDI-TOF m/z: [M+H] calculated 416.3279; [M+H] found 416.3286.

4.3.6) 5-[(N-butyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **8**

Colorless oil, Yield: 84%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.10-8.08 (2H; m), 7.50-7.46 (3H; m), 4.13 (2H; s), 2.73 (2H; t; *J*= 7.0 Hz), 2.19 (1H, s), 1.53 (2H; m), 1.37 (2H; m), 0.92 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.4, 168.5, 131.4, 129.0, 127.6, 126.9, 49.2, 44.9, 32.0, 20.4, 14.0. MALDI-TOF m/z: [M+H] calculated 232.1452; [M+H] found 232.1446.

4.3.7) 5-[(N-octyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **9**

Colorless oil; Yield: 88%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09-8.07 (2H; m), 7.50-7.46 (3H; m), 4.11 (2H; s), 2.70 (2H; t; *J*= 7.0 Hz), 1.52 (3H; m), 1.28-1.26 (10H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.7, 168.4, 131.3, 129.0, 127.6, 126.9, 49.6, 45.1, 31.9-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 288.2078; [M+H] found 288.2083.

4.3.8)5-[(N-decyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **10**

Colorless oil; Yield: 85%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.10-8.08 (2H; m), 7.50-7.46 (3H; m), 4.11 (2H; s), 2.70 (2H; t; *J*= 7.0 Hz), 1.71 (1H; s), 1.53 (2H; m), 1.32-1.25 (22H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.7, 168.4, 131.3, 129.0, 127.6, 126.9, 49.6, 45.1, 32.0-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 316.2391; [M+H] found 316.2397.

4.3.9)5-[(N-dodecyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **11**

Colorless oil; Yield: 86%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.10-8.08 (2H; m), 7.50-7.45 (3H; m), 4.11 (2H; s), 2.70 (2H; t; *J*= 7.0 Hz), 1.51 (3H; m), 1.29-1.23 (18H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.7, 168.4, 131.3, 129.0, 127.6, 126.9, 49.6, 45.1, 32.1-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 344.2704; [M+H] found 344.2702.

4.3.10)5-[(N-tetradecyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **12**

White solid; Yield: 91%; m.p.: 29-31°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.09 (2H; m), 7.49 (3H; m), 4.11 (2H; s), 2.71 (2H; t; *J*= 7.0 Hz), 1.68 (1H; s), 1.53 (2H; m), 1.25 (22H; bs), 0.88 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.7, 168.4, 131.3, 129.0, 127.6, 126.9, 49.6, 45.1, 32.1-22.9, 14.3. MALDI-TOF m/z: [M+H] calculated 372.3017; [M+H] found 372.3013.

4.3.11)5-[N-(2-aminoethyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **13**

Brown oil; Yield: 33%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.06 (2H; d; *J*= 8.0 Hz), 7.49-7.43 (3H; m), 4.11 (2H; s), 2.82 (2H; t; *J*= 5.0 Hz), 2.76 (2H; t; *J*= 5.0 Hz), 1.65 (3H, bs). ^{13}C NMR ($CDCl_3$; 125 MHz) δppm:178.5, 168.4, 131.3, 128.9, 127.5, 126.7, 51.9, 44.9, 41.6. MALDI-TOF m/z: [M+H] calculated 219.1248; [M+H] found 219.1240; m/z [M+Na] calculated 241.1068; [M+Na] found 241.6152.

4.3.12)5-[N-(3-aminopropyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **14**

Brown oil; Yield: 44%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.10-8.07 (2H; m); 7.52-7.46 (3H; m); 5.04 (1H; s); 4.12 (2H; s); 2.85 (2H; t; *J*= 5.0 Hz); 2.81 (2H; t; *J*= 5.0 Hz); 2.67 (2H; s); 1.72 (2H; q; *J*= 5.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.5, 168.3, 131.2, 128.8, 127.4, 126.5, 47.2, 44.8, 40.0, 32.5. ESI-MS m/z: [M+H] calculated 233.1; [M+H] found 233.0; m/z [M+Na] calculated 255.1; [M+Na] found 255.0.

4.3.13)5-[N-(tris hydroxymethyl)aminomethyl]-3-phenyl-1,2,4-oxadiazole **15**

White solid; Yield: 82%; m.p.: 97-99°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.99-7.97 (2H; m), 7.57-7.52 (3H; m), 4.48 (2H; bs), 4.20 (2H; d; *J*= 7.0 Hz), 3.39 (6H; s), 2.27 (1H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 180.5, 167.6, 131.8, 129.5, 127.2, 126.4, 61.4, 60.3, 38.6. MALDI-TOF m/z: [M+H] calculated 280.1299; [M+H] found 280.1307; [M+Na] calculated 302.1119; [M+Na] found 302.2585; [M+K] calculated 318.2202; [M+K] found 318.0740.

4.3.14) 3-phenyl-5-(piperazin-1-ylmethyl)-1,2,4-oxadiazole **16**

Brown oil; Yield: 86%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.08 (2H; m), 7.48-7.43 (3H; m), 3.88 (2H; s), 2.92 (4H; t; *J*= 5.0 Hz), 2.61-2.60 (4H; bs), 1.79 (1H; bs). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 176.3, 168.4, 131.3, 128.9, 127.6, 126.7, 54.2, 53.6, 45.9. MALDI-TOF m/z: [M+H] calculated 245.1404; [M+H] found 245.1410.

4.3.15) (R)-5-[(3-methylpiperazin-1-yl)methyl]-3-phenyl-1,2,4-oxadiazole **17**

Yellow oil; Yield: 55%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.02-8.01 (2H; m), 7.61-7.55 (3H; m), 3.92 (2H; s), 2.81-2.65 (6H; m), 2.14-2.10 (1H; dt; ^3J= 11.0 Hz; ^4J= 3.5 Hz), 1,78 (1H; t; *J*= 10.0 Hz), 0.89 (2H; d; *J*=5.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:177.0, 167.4, 131.6, 129.3, 127.0, 126.1, 60.1, 52.5, 50.0, 45.2. ESI-MS m/z: [M+H] calculated 259.1; [M+H] found 259.0; [M+Na] calculated 281.1; [M+Na] found 281.0.

4.3.16) 5-[(4-methylpiperazin-1-yl)methy]-3-phenyl-1,2,4-oxadiazole **18**

Yellowoil; Yield: 81%; ^{1}H NMR (DMSO-$_{d6}$; 500 MHz) δ ppm: 8.03-8.02 (2H; m), 7.61-7.56 (3H; m), 3.96 (2H; s), 2.56 (4H; s), 2.34 (4H; s), 2.15 (3H, s). ^{13}C NMR (DMSO-$_{d6}$; 125 MHz) δ ppm:177.4, 167.8, 131.9, 129.6, 127.4, 126.5, 54.9, 52.5, 46.1. ESI-MS m/z: [M+Na] calculated 281.1; [M+Na] found 281.0.

4.4. General procedure for the synthesis of compounds **19**-**22**:

To a solution of oxadiazole **16** in CH_3CN and K_2CO_3 (1.5 eq.), alkyl chloride (1.5 eq.) was added in ether solution. The reaction mixture was stirred at 70°C for 20h and concentrated under reduced pressure. The crude mixture was extracted with CH_2Cl_2 and H_2O, the organic layer was washed with brine solution, dried with Na_2SO_4, filtrated and concentrated under reduced pressure. Then, the mixture was purified by chromatography on silica gel (eluent: CH_2Cl_2/MeOH) affording the desired compounds.

4.4.1) 5-[(4-octylpiperazin-1-yl)methy)]-3-phenyl-1,2,4-oxadiazole **19**

Yellow oil; Yield: 72%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.08-8.06 (2H; m), 7.47-7.42 (3H; m), 3.89 (2H; s), 2.70-2.32 (8H; m), 2.33 (2H; t; *J*= 8.0 Hz), 1.47 (2H; m); 1.25 (10H; bs); 0.85 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 500 MHz) δ ppm: 176.2, 168.4, 131.3, 128.9, 127.6, 126.8, 58.7, 53.1, 53.0, 52.8, 31.9-22.7, 14.2. MALDI-TOF m/z: [M+H] calculated 357.2656; [M+H] found 357.2649.

4.4.2) 5[(4-decylpiperazin-1-yl)methyl]-3-phenyl-1,2,4-oxadiazole **20**

Colorless oil; Yield: 86%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:8.06-8.05 (2H; m), 7.46-7.41 (3H; m), 3.88 (2H; s), 2.68-2.51 (8H; m), 2.32 (2H; t; *J*= 7.0 Hz), 1.45 (2H; bs), 1.24-1.22 (14H; bs), 0.84 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 176.2, 168.4, 131.3, 128.9, 127.6, 126.7, 58.7, 53.0, 52.9, 52.8, 31.9-22.7, 14.2. MALDI-TOF m/z: [M+H] calculated 385.2969; [M+H] found 385.2973.

*4.4.3) 5-[(4-dodecylpiperazin-1-yl)methyl]-3-phenyl-1,2,4-oxadiazole***21**

White solid; Yield: 77%; m.p.: 47-49°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.10-8.08 (2H; m), 7.50-7.45 (3H; m), 3.93 (2H; s), 2.78-2.64 (8H; m), 2.42 (2H; t; *J*= 8.0 Hz), 1.53 (2H; m), 1.28-1.25 (18H; bs), 0.87 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:176.2, 168.5, 131.4, 129.0, 127.7, 126.8, 58.6, 53.0, 52.9, 52.5, 32.0-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 413.3282; [M+H] found 413.3287.

4.4.4) 5[(4-tetradecylpiperazin-1-yl)methyl]-3-phenyl-1,2,4-oxadiazole **22**

Brown solid; Yield: 76%; m.p.: 55-56°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.04-8.02 (2H; m), 7.42-7.83 (3H; m), 3.84 (2H; s), 2.64-2.47 (8H; m), 2.27 (2H; m), 1.41 (2H; m), 1.21-1.19 (22H; sl), 0.82 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 176.1, 168.3, 131.1, 128.8, 127.5, 126.7, 58.6, 52.9, 52.8, 31.9-22.7, 14.1. MALDI-TOF m/z: [M+H] calculated 441.3595; [M+H] found 441.3603.

4.5. General procedure for the synthesis of compounds **23**-**26:**

Carboxylic acids (caprylic acid, capric acid, lauric acid or myristic acid) were reacted with $SOCl_2$ (5 eq.) and heated at 75°C for 2h. The excess of $SOCl_2$ was removed under reduced pressure and the mixture was solubilized in dried CH_2Cl_2. Then, the acyl chlorides (1.5 eq.) were added dropwise to a solution of the oxadiazole derivative **16** in CH_3CN and K_2CO_3 (1.5 eq.). The reaction mixture was stirred at room temperature for 30h. The solvent was removed under reduced pressure and the crude mixture was extracted with CH_2Cl_2 and H_2O and washed with brine solution. The organic layer was dried with Na_2SO_4, filtrated and concentrated under vacuum. The mixture was purified by chromatography on silica gel (eluent: CH_2Cl_2/ MeOH) affording the desired compounds.

4.5.1) 5-{[4-(piperazin-1-yl)octan-1-one]methyl}-3-phenyl-1,2,4-oxadiazole **23**

Brown oil; Yield: 50%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.05-8.03 (2H; m), 7.46-7.41 (3H; m), 3.91 (2H; s), 3.67-3.49 (4H; m), 2.63-2.60 (4H; m), 2.26 (2H; t; *J*= 7.0 Hz); 1.57 (2H; m), 1.27-1.22 (8H; m), 0.82 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 175.6, 171.7, 168.4, 131.3, 128.9, 127.7, 126.5, 52.9, 52.5, 45.3, 41.3, 33.2-22.6, 14.1. MALDI-TOF m/z: [M+H] calculated 371.2449; [M+H] found 371.2455; [M+K] calculated 409.3352; [M+K] found 409.6732.

4.5.2) 5-{[4-(piperazin-1-yl)decan-1-one]methyl}-3-phenyl-1,2,4-oxadiazole **24**

Brown solid; Yield: 76%; m.p.: 25-26°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.99-7.97 (2H; m), 7.38-7.34 (3H; m), 3.83 (2H; s), 3.58-3.40 (4H; m), 2.55-2.51 (4H; m), 2.18

(2H; t; *J*= 7.0 Hz), 1.50 (2H; m), 1.19-1.14 (12H; m), 0.86 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 175.6, 171.4, 168.1, 131.1, 128.7, 127.3, 126.4, 52.6, 52.4, 45.2, 41.1, 33.0-22.5, 13.9. MALDI-TOF m/z: [M+H] calculated 399.2702; [M+H] found 399.2765; [M+Na] calculated 421.2582; [M+Na] found 421.2617.

4.5.3) 5{[4-(piperazin-1-yl)dodecan-1-one]methyl}-3-phenyl-1,2,4-oxadiazole **25**

White solid; Yield: 78%; m.p.: 35-37°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.03-8.01 (2H; m), 7.44-7.39 (3H; m), 3.89 (2H; s), 3.63-3.46 (4H; m), 2.60-2.57 (4H; m), 2.24 (2H; t; *J*= 7.5 Hz), 1.54 (2H; m), 1.23-1.19 (16H; m); 0.81 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 175.6, 171.7, 168.3, 131.3, 128.8, 127.4, 126.5, 52.8, 52.4, 45.3, 41.2, 33.2-22.6, 14.1. MALDI-TOF m/z: [M+H] calculated 427.3075; [M+H] found 427.3069.

*4.5.4) 5{[4-(piperazin-1-yl)tetradecan-1-one]methyl}-3-phenyl-1,2,4-oxadiazole***26**

White solid; Yield: 86%; m.p.: 41-43°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.04-8.01 (2H; m), 7.44-7.39 (3H; m), 3.89 (2H; s), 3.64-3.46 (4H; m), 2.60-2.59 (4H; bs), 2.24 (2H; t; *J*= 7.5 Hz), 1.55 (2H; m), 1.23-1.19 (20H; m), 0.81 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 175.6, 171.6, 168.3, 131.3, 128.8, 127.4, 126.5, 52.8, 52.5, 45.3, 41.2, 33.2-22.6, 14.1. MALDI-TOF m/z: [M+H] calculated 455.3388; [M+H] found 455.3394; [M+Na] calculated 477.3208; [M+Na] found 477.3342; [M+K] calculated 493.4291; [M+K] found 493.4710.

4.6. General procedure for the synthesis of N-acylhydrazides **31**-**33**:

Benzoic acid **27**, *p*-methoxybenzoic acid **28** and *p*-nitrobenzoic acid **29** (40 mmol) were treated with a sulfuric acid-ethanol solution. The resulting mixture was allowed to reflux with stirring for 20h. Ethanol was removed under reduced pressure and the mixture was extracted with Et_2O and H_2O and washed with saturated $NaHCO_3$ solution. The organic layer was dried with Na_2SO_4, filtrated and concentrated, furnishing the esters derivatives. Then, a mixture of the appropriated aromatic esters and 2.0 equivalents of hydrazine hydrated (69% m/v) were reacted in an ethanol solution. The resulting mixture was allowed under reflux with stirring for 20h. The reaction was cooled and the precipitated formed was washed with ethanol and filtrated. The solid obtained was recrystallized under ethanol solution affording the desired products.

4.6.1) Benzohydrazide **31**

Yield: 65%; ^{1}H NMR (DMSO-d_6; 500 MHz) δ ppm: 9.77 (1H; s), 7.82 (2H; dd; ^3J= 7.0 Hz; ^4J= 1.5 Hz), 7.49 (1H; m), 7.43 (2H; t; J= 7.0 Hz), 4.41 (2H; bs). ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm: 165.9, 133.3, 131.0, 128.3, 126.9.

4.6.2) 4-Methoxybenzohydrazide **32**

Yield: 65%; ^{1}H NMR (DMSO-d_6; 500 MHz) δ ppm: 9.63 (1H; s), 7.80 (2H; d; J= 9.0 Hz), 6.97 (2H; d; J= 9.0 Hz), 4.24 (2H; bs), 3.78 (3H; s). ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm: 165.6, 161.4, 128.7, 125.5, 113.5, 55.3.

4.6.3) 4-Nitrobenzohydrazide **33**

Yield: 88%; ^{1}H NMR (DMSO-d_6; 500 MHz) δ ppm: 10.12 (1H; s); 8.27 (2H; d; J= 9.0 Hz); 8.03 (2H; d; J= 9.0 Hz); 4.63 (2H; bs). ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm: 164.0, 148.9, 139.0, 128.5, 123.6.

4.7. General procedure for the synthesis of 1,3,4-oxadiazoles **34**-**37**:

A mixture of isoniazid **30** and *N*-acylhydrazines**31**-**33**, CS_2 (4.0 eq.) and KOH (3.0 equivalents) was reacted in ethanol. The resulting mixture were stirred at 55°C for 48h. The excess of CS_2 and ethanol was removed under reduced pressure and the crude product was solubilized in a minimal quantity of H_2O. The mixture was acidified with HCl 4M until pH~1 and the resulting precipitated was filtered and washed with cold water, affording the desired heterocycles.

4.7.1)5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thiol **34**

Yield: 68%; ^{1}H NMR (DMSO-d_6; 500 MHz) δ ppm: 8.92 (2H; dd; ^3J= 4.5 Hz, ^4J= 1.5 Hz),7.83 (2H, dd, ^3J= 4.5 Hz; ^4J= 1.5 Hz) [34]. ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm: 177.9, 158.6, 150.6, 129.7, 119.5.

4.7.2) 5-Phenyl-1,3,4-oxadiazole-2-thiol **35**

Yield: 70%; ^{1}H NMR (DMSO-d_6; 500 MHz) δppm: 13.53 (1H; bs), 7.87 (2H; dd; ^3J= 8.5 Hz; ^4J= 1.5 Hz), 7.64-7.56 (3H; m)[35]. ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm:177.4, 160.4, 132.1, 129.4, 126.0, 122.5.

4.7.3) 5-(4-Methoxyphenyl)-1,3,4-oxadiazole-2-thiol **36**

Yield: 62%; ^{1}H NMR (DMSO-d_6; 500 MHz) δppm: 14.64 (1H; bs), 7.80 (2H; d; J= 8.5 Hz), 7.11 (2H; d; J= 8.5 Hz), 3.83 (3H; s) [34]. ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm:177.1, 162.2, 160.5, 127.9, 114.8, 114.7, 55.5.

4.7.4) 5-(4-Nitrophenyl)-1,3,4-oxadiazole-2-thiol **37**

Yield: 67%; ^{1}H NMR (DMSO-d_6; 500 MHz) δ ppm: 10.12 (1H; s); 8.28 (2H; dd; ^{3}J= 7.0 Hz; ^{4}J= 2.0 Hz); 8.04 (2H; dd; ^{3}J= 7.0 Hz; ^{4}J= 2.0 Hz) [36]. ^{13}C NMR (DMSO-d_6; 125 MHz) δ ppm: 163.9, 161.1, 148.9, 139.0, 128.4, 123.5.

4.8. General procedure for the synthesis of Mannich bases **38**-**69**:

To a suspension of 1.0 mmol of 5-(aryl)-3*H*-1,3,4-oxadiazole-2-thione **1** in 5 mL of absolute ethanol, piperazine derivatives (1.0 eq.).Formaldehyde 37% v/v (1.5 eq) was added dropwise to the stirred suspensionand the reaction mixture was stirred at room temperature for 16 h. After concentration under reduced pressure, the residue was extracted with CH_2Cl_2 and H_2O. The organic layer was dried with Na_2SO_4 and concentrated, affording the Mannich bases.

4.8.1) 3-[(4-octylpiperazin-1-yl)methyl]-5-(piridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **38**

Yellow solid; Yield: 83%; m.p.: 79-81 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:8.65 (2H; d; *J*= 5.5 Hz), 7.60 (2H; d; *J*= 5.5 Hz), 4.97 (2H; s), 2.85-2.47 (8H; bs), 2.30 (2H; t; *J*= 8.0 Hz), 1.36 (2H; m), 1.11 (10H; bs), 0.70 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.1, 156.5, 150.7, 129.3, 119.4, 70.2, 58.2, 52.6, 49.3, 31.5-22.4, 13.9. MALDI-TOF m/z: [M+H] calculated 390.2329; [M+H] found 390.2321.

4.8.2) 3-[(4-decylpiperazin-1-yl)methyl]-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **39**

Orange solid; Yield: 77%; m.p.: 72-74 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:8.57 (2H; d; *J*= 5.0 Hz), 7.52 (2H; d; *J*= 5.0 Hz), 4.91 (2H; s), 2.80-2.45 (8H; bs), 2.28 (2H; t; *J*= 7.0 Hz), 1.32 (2H; m), 1.06-1.01 (14H; bs), 0.64 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:177.9, 156.3, 150.5, 129.1, 119.2, 69.9, 58.0, 52.3, 49.1, 31.5-22.3, 13.8. MALDI-TOF m/z: [M+H] calculated 418.6192; [M+H] found 418.6183.

4.8.3) 3-[(4-dodecylpiperazin-1-yl)methyl]-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **40**

Brown solid; Yield: 89%; m.p.: 75-77 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.65 (2H; d; *J*= 6.0 Hz), 7.59 (2H; dd; ^{3}J= 6.0 Hz; ^{4}J= 1.5 Hz), 4.96 (2H; s), 2.77 (4H; bs), 2.36 (4H; bs), 2.19 (2H; t; *J*= 7.5 Hz), 1.30 (2H; bs), 1.13-1.10 (18H; bs), 0.70 (3H; t; *J*= 7.5 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.1, 156.4, 150.7, 129.3, 119.4, 70.3,

58.4, 52.7, 49.7, 31.6-22.4, 13.9. MALDI-TOF m/z: [M+H] calculated 446.2955; [M+H] found 446.2948.

4.8.4) 3-[(4-tetradecylpiperazin-1-yl)methyl]-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **41**

Brown solid; Yield: 88%; m.p.: 83-85 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:8.71 (2H; d; *J*= 6.0 Hz), 7.66 (2H; d; *J*= 6.0 Hz), 5.02 (2H; s), 2.84-2.43 (8H; bs), 2.26 (2H; t; *J*= 7.0 Hz), 1.37 (2H; m), 1.16-1.14 (22H; bs), 0.77 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.3, 156.6, 150.8, 129.5, 119.5, 70.4, 58.5, 52.8, 49.8, 31.8-22.6, 14.1. MALDI-TOF m/z: [M+H] calculated 474.3268; [M+H] found 474.3266.

4.8.5) 3-[(4-octylpiperazin-1-yl)methyl]5-phenyl-1,3,4-oxadiazole-2(3H)-thione **42**

Brown solid; Yield: 90%; m.p.: 56-58 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.87 (2H; d; *J*=7.0 Hz), 7.54 (1H; tt; ^{3}J= 7.5 Hz; ^{4}J= 1.0 Hz), 7.47 (2H; m), 5.07 (2H; s), 2.91-2.47 (8H; bs), 2.30 (2H; m), 1.43 (2H; m), 1.27-1.24 (12H; bs), 0.84 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.3, 159.0, 132.5, 129.2, 126.6, 122.5, 70.3, 58.8, 53.1, 50.2,31.9-22.7, 14.2.MALDI-TOF m/z: [M+H] calculated 389.2377; [M+H] found 389.2380.

4.8.6) 3-[(4-decylpiperazin-1-yl)methyl]5-phenyl-1,3,4-oxadiazole-2(3H)-thione **43**

White solid; Yield: 60%; m.p.: 65-67 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.87 (2H; d; *J*=7.0 Hz), 7.50 (1H; bs), 7.44 (2H; bs), 5.04 (2H; s), 2.89-2.47 (8H; bs), 2.30 (2H; t; *J*= 7.0 Hz), 1.42 (2H; bs), 1.19 (12H; bs), 0.82 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.2, 158.9, 132.3, 129.1, 126.5, 122.4, 70.2, 58.6, 53.0, 49.9,31.9-22.7, 14.1.MALDI-TOF m/z: [M+H] calculated 417.2690; [M+H] found 417.2686.

4.8.7) 3-[(4-dodecylpiperazin-1-yl)methyl]5-phenyl-1,3,4-oxadiazole-2(3H)-thione **44**

Brown solid; Yield: 79%; m.p.: 69-71 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.87 (2H; d; *J*=7.5 Hz), 7.50 (1H; t; *J*= 7.5 Hz), 7.44 (2H; t; *J*= 7.5 Hz), 5.05 (2H; s), 2.89-2.45 (8H; bs), 2.83 (2H; t; *J*= 7.5 Hz), 1.41 (2H; m), 1.21-1.19 (18H; bs), 0.82 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.2, 158.9, 132.4, 129.1, 126.5, 122.4, 70.2, 58.7, 53.0, 50.1, 31.9-22.7, 14.2.MALDI-TOF m/z: [M+H] calculated 445.3003; [M+H] found 445.3008.

4.8.8) 3-[(4-tetradecylpiperazin-1-yl)methyl]-5-phenyl-1,3,4-oxadiazole-2(3H)-thione **45**

Brown solid; Yield: 83%; m.p.: 72-74 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.87 (2H; d; ^{3}J= 7.0 Hz; ^{4}J= 1.5 Hz), 7.50 (1H; t; *J*= 7.5 Hz), 7.44 (2H; t; *J*= 7.5 Hz), 5.04

(2H; s), 2.88-2.44 (8H; bs), 2.27 (2H; t; *J*= 7.0 Hz), 1.40 (2H; bs), 1.19 (22H; bs), 0.83 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.2,158.9, 132.3, 129.1, 126.5, 122.4, 70.2, 58.7, 53.1, 50.1, 31.9-22.7, 14.2.MALDI-TOF m/z: [M+H] calculated 473.3316; [M+H] found 473.3322.

4.8.9) 3-[(4-octylpiperazin-1-yl)methyl]-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **46**

White solid; Yield: 85%; m.p.: 89-90 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.73 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 6.87 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 4.96 (2H; s), 3.76 (3H; s), 2.83-2.40 (8H; bs), 2.23 (2H; t; *J*= 7.0 Hz), 1.36 (2H; m), 1.19-1.15 (14H; bs), 0.76 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:177.8, 162.7, 158.8, 128.2, 114.6, 114.5, 69.9, 58.5, 55.4, 52.9, 49.9, 31.7-22.5, 14.0.MALDI-TOF m/z: [M+H] calculated 419.2482; [M+H] found 419.2483.

4.8.10) 3-[(4-decylpiperazin-1-yl)methyl]-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **47**

White solid; Yield: 71%; m.p.: 85-88 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.81 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 6.94 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 5.04 (2H; s), 3.84 (3H; s), 2.99-2.61 (8H; bs), 2.43 (2H; t; *J*= 7.0 Hz), 1.50 (2H; bs); 1.24-1.21 (14H; bs); 0.84 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.0, 162.9, 159.2, 128.5, 114.7, 114.7, 69.9, 58.5, 55.6, 52.8, 49.5, 31.9-22.7, 14.2.MALDI-TOF m/z: [M+H] calculated 447.2795; [M+H] found 447.2791.

4.8.11) 3-[(4-dodecylpiperazin-1-yl)methyl]-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **48**

White solid; Yield: 93%; m.p.: 88-90°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.84 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 6.97 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 5.05 (2H; s), 3.87 (3H; s), 3.00-2.64 (8H; bs), 2.45 (2H; t; *J*= 7.0 Hz), 1.53 (2H; bs), 1.26-1.23 (18H; bs), 0.86 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.0, 163.0, 159.3, 128.6, 114.8, 114.7, 70.0, 58.6, 55.6, 52.9, 49.5,32.0-22.8, 14.3.MALDI-TOF m/z: [M+H] calculated 475.3108; [M+H] found 475.3107.

4.8.12)3-[(4-tetradecylpiperazin-1-yl)methyl]-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **49**

White solid; Yield: 79%; m.p.: 91-93 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.75 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 6.88 (2H; dd; ^3J= 7.0 Hz; ^4J= 2.0 Hz), 4.97 (2H; s), 3.77 (3H; s), 2.85-2.42 (8H; bs), 2.25 (2H; t; *J*= 7.0 Hz), 1.38 (2H; m), 1.15 (22H; bs), 0.79

(3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 177.8, 162.7, 158.8, 128.2, 114.6, 114.5, 69.9, 58.6, 55.4, 52.9, 49.9, 31.8-22.6, 14.1.MALDI-TOF m/z: [M+H] calculated 503.3421; [M+H] found 503.3433.

4.8.13) 3-[(4-octylpiperazin-1-yl)methyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **50**

Yellow solid; Yield: 78%; m.p.: 86-87 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.34 (2H; d; *J*= 8.5 Hz); 8.08 (2H; d; *J*= 8.5 Hz); 5.12 (2H; s); 2.98 (4H; t; *J*= 4.5 Hz); 2.58 (4H; m); 2.41 (2H; t; *J*= 8.0 Hz); 1.48 (2H; bs); 1.25-1.23 (10H; bs); 0.84 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.5, 156.9, 149.9, 127.9, 127.5, 124.6, 70.6, 58.6, 52.9, 49.8, 31.9, 29.5, 29.3, 27.5, 26.6, 22.7, 14.2.ESI-MS: m/z433.2 calculated; m/z 433.0 found; [M+H] calculated 434.2; [M+H] found 434.0; [M+Na] calculated 456.2; [M+Na] found 456.0.

.4.8.14) 3-[(4-decylpiperazin-1-yl)methyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **51**

Yellow solid; Yield: 87%; m.p.: 84-86 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.31 (2H; d; *J*= 8.5 Hz); 8.06 (2H; d; *J*= 8.5 Hz); 5.11 (2H; s); 3.02 (4H; t; *J*= 4.5 Hz); 2.67 (4H; bs); 2.49 (2H; t; *J*= 8.0 Hz); 1.53-1.52 (2H; bs); 1.25-1.21 (12H; bs); 0.83 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.4, 157.0, 127.8, 127.5, 124.5, 70.4, 58.5, 52.8, 49.4, 31.9, 29.6, 29.5, 29.3, 27.4, 26.2, 22.7, 14.2..ESI-MS: m/z461.2 calculated; m/z 461.0 found; [M+H] calculated 462.2; [M+H] found 462.0; [M+Na] calculated 484.2; [M+Na] found 484.0.

4.8.15) 3-[(4-dodecylpiperazin-1-yl)methyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **52**

Yellow solid; Yield: 68%; m.p.: 91-93 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.32 (2H; d; *J*= 8.5 Hz); 8.07 (2H; d; *J*= 8.5 Hz); 5.09 (2H; s); 2.96 (4H; t; *J*= 4.5 Hz); 2.94 (4H; bs); 2.49 (2H; t; *J*= 7.0 Hz); 1.46 (2H; bs); 1.23-1.20 (18H; bs); 0.83 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.4, 156.9, 149.8, 127.9, 127.5, 124.5, 70.5, 58.6, 52.9, 49.7, 31.9, 29.7, 29.6, 29.5, 29.4, 27.5, 26.5, 22.7, 14.2.ESI-MS: m/z489.3calculated; m/z found489.0; [M+H] calculated 490.3; [M+H] found 490.0; [M+Na] calculated 512.3; [M+Na] found 512.0.

4.8.16) 3-[(4-tetradecylpiperazin-1-yl)methyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **53**

Yellow solid; Yield: 71%; m.p.: 93-94 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.31 (2H; d; *J*= 9.0 Hz); 8.06 (2H; d; *J*= 9.0 Hz); 5.09 (2H; s); 2.92 (4H; bs); 2.52 (4H; bs); 2.34 (2H; t; *J*= 7.5 Hz); 1.44 (2H; bs); 1.22-1.19 (22H; bs); 0.83 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.4, 156.8, 149.8, 127.8, 127.4, 124.5, 70.5, 58.6, 52.9, 49.9, 31.9, 29.7, 29.6, 29.4, 27.5, 26.6, 22.7, 14.2.ESI-MS: m/z 517.3 calculated; m/z found 517.0; [M+H] calculated 518.3; [M+H] found 518.0; [M+Na] calculated 540.3; [M+Na] found 540.0.

4.8.17) 3-{[4-(piperazin-1-yl)octan-1-one]methyl}-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **54**

White solid; Yield: 85%; m.p.: 74-76 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:8.75 (2H; dd; ^{3}J= 4.5 Hz; ^{4}J= 2.0 Hz), 7.70 (2H; ^{3}J= 4.5 Hz; ^{4}J= 2.0 Hz), 5.06 (2H; s), 3.58-3.41 (4H; bs), 2.77 (4H; m), 2.20 (2H; t; *J*= 7.0 Hz), 1.50 (2H; m), 1.21-1.15 (8H; m), 0.77 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.3, 171.6, 156.9, 150.9, 129.4, 119.6, 70.5, 50.5, 50.3, 45.4, 41.3, 33.2-22.5, 14.0. MALDI-TOF m/z: calculated [M-H] 402.1962; [M-H] found 402.1948.

4.8.18) 3-{[4-(piperazin-1-yl)decan-1-one] methyl}- 5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **55**

White solid; Yield: 80%; m.p.: 79-81 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.77 (2H; dd; ^{3}J= 7.0 Hz; ^{4}J= 2.0 Hz), 7.72 (2H; ^{3}J= 7.0 Hz; ^{4}J= 2.0 Hz), 5.07 (2H; s), 3.60-3.43 (4H; bs), 2.78 (4H; m), 2.22 (2H; t; *J*= 7.0 Hz), 1.50 (2H; m); 1.21-1.17 (12H; m), 0.80 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.4, 171.7, 157.0, 151.0, 129.5, 119.7, 70.6, 50.5, 50.1, 45.3, 41.4, 33.2-22.6, 14.1. MALDI-TOF m/z: [M+K] calculated 470.3323; [M+K] found 470.2569.

4.8.19) 3-{[4-(piperazin-1-yl)dodecan-1-one]methyl}-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **56**

Yellow solid; Yield: 58%; m.p.: 81-83°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.80 (2H; dd; ^{3}J= 4.5 Hz; ^{4}J= 2.0 Hz), 7.75 (2H; ^{3}J= 7.0 Hz; ^{4}J= 2.0 Hz), 5.10 (2H; s), 3.62-3.47 (4H; bs), 2.81 (4H; bs), 2.24 (2H; t; *J*= 7.0 Hz), 1.54 (2H; m), 1.24-1.20 (16H; m), 0.84 (3H; t; *J*= 7.0 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.5, 171.8, 157.1, 151.1, 129.6, 119.8, 70.6, 50.6, 50.1, 45.5, 41.5, 33.3-22.8, 14.2. MALDI-TOF m/z: [M-H] calculated 458.2588; [M-H] found 458.2592.

4.8.20) 3-{[4-(piperazin-1-yl)tetradecan-1-one]methyl}-5-(pyridin-4-yl)-1,3,4-oxadiazole-2(3H)-thione **57**

White solid; Yield: 72%; m.p.: 86-88 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.80 (2H; d; *J*= 6.0 Hz), 7.76 (2H; *J*= 6.0 Hz), 5.11 (2H; s), 3.63-3.46 (4H; bs), 2.82 (4H; bs), 2.25 (2H; t; *J*= 7.5 Hz), 1.55 (2H; m), 1.25-1.21 (20H; m), 0.85 (3H; t; *J*= 7.5 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm: 178.5, 171.8, 157.1, 151.1, 129.6, 119.8, 70.7, 50.7, 50.2, 45.6, 41.5, 33.9-22.8, 14.2. ESI-MS: m/z 487.7 calculated; m/z 487.0 found; [M+H] calculated 488.7; [M+H] found 488.0; [M+Na] calculated 510.0; [M+Na] found 510.0.

4.8.21)3-{[4-(piperazin-1-yl)octan-1-one]methyl}-5-phenyl-1,3,4-oxadiazole-2(3H)-thione **58**

Brown solid; Yield: 80%; m.p.: 78-80°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.91 (2H; m), 7.55 (2H; m), 7.49 (2H; m), 5.09 (2H; s), 3.62-3.46 (4H; bs), 2.83 (4H; bs), 2.25 (2H; t; *J*= 7.0 Hz), 1.56 (2H; m), 1.26-1.22 (8H; bs), 0.83 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.3, 171.8, 159.2, 132.6, 129.3, 126.6, 122.3, 70.3, 50.6-50.2, 45.6-41.5, 33.3-22.7, 14.2. MALDI-TOF m/z: [M+H] calculated 403.2169; [M+H] found 403.2163.

4.8.22)3-{[4-(piperazin-1-yl) decan-1-one]methyl}-5-phenyl-1,3,4-oxadiazole-2(3H)-thione **59**

Brown oil; Yield: 63%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.89 (2H; m), 7.56 (2H; m), 7.50 (2H; m), 5.07 (2H; s), 3.62-3.44 (4H; bs), 2.82 (4H; bs), 2.24 (2H; t; *J*= 7.0 Hz), 1.55 (2H; m); 1.25-1.19 (12H; bs), 0.82 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.3, 171.7, 159.1, 132.6, 129.3, 126.6, 122.3, 70.2, 50.6, 50.2, 45.5, 41.5,33.3-22.7, 14.1. MALDI-TOF m/z: [M+H] calculated 431.2482; [M+H] found 431.2483.

4.8.23) 3-{[4-(piperazin-1-yl)dodecan-1-one]methyl}-5-phenyl-1,3,4-oxadiazole-2(3H)-thione **60**

Colorless oil; Yield: 47%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.92 (2H; dd; ^{3}J= 8.0 Hz; ^{4}J= 1.5 Hz), 7.56 (2H; tt; ^{3}J= 7.5 Hz; ^{4}J= 1.0 Hz), 7.51-7.48 (2H; m), 5.10 (2H; s), 3.65-3.47 (4H; bs), 2.84 (4H; m), 2.26 (2H; t; *J*= 7.0 Hz), 1.57 (2H; m), 1.26-1.22 (16H; bs), 0.86 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.4, 171.8, 159.2, 132.7, 129.3, 126.7, 122.4, 70.3, 50.7, 50.2, 45.6, 41.5, 33.4-22.8, 14.3. MALDI-TOF m/z: [M+H] calculated 459.2795; [M+H] found 459.2786.

4.8.24)3-{[4-(piperazin-1-yl)tetradecan-1-one]methyl}-5-phenyl-1,3,4-oxadiazole-2(3H)-thione **61**

Brown oil; Yield: 83%; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.92 (2H; dd; ^3J= 8.0 Hz; ^4J= 1.5 Hz), 7.56 (2H; tt; ^3J= 8.0 Hz; ^4J= 1.5 Hz), 7.51-7.48 (2H; m), 5.10 (2H; s), 3.65-3.48 (4H; bs), 2.84 (4H; m), 2.26 (2H; t; *J*= 7.5 Hz), 1.57 (2H; m), 1.24-1.22 (20H; bs), 0.86 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.4, 171.9, 159.3, 132.7, 129.3, 126.7, 122.4, 70,3, 50.7, 50.2, 45.6, 41.5, 33.4-22.8, 14.3. MALDI-TOF m/z: [M+H] calculated 487.3108; [M+H] found 487.3109.

4.8.25)3-{[4-(piperazin-1-yl)octan-1-one]methyl}-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **62**

White solid; Yield: 83%; m.p.: 80-82 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.86 (2H; dd; ^3J= 9.0 Hz; ^4J= 1.5 Hz), 6.99 (2H; d; ^3J= 9.0 Hz; ^4J= 1.5 Hz), 5.07 (2H; s), 3.87 (3H; s), 3.64-3.47 (4H; bs), 2.84 (4H; bs), 2.27 (2H; t; *J*= 7.0 Hz), 1.57 (2H; m), 1.27-1.24 (8H; bs), 0.84 (3H; t; *J*= 7,0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.1, 171.8, 163.1, 159.4, 128.6, 114.8, 114.7, 70.2, 55.7, 50.7, 50.3, 45.6, 41.5, 33.4-22.7, 14.2.MALDI-TOF m/z: [M+H] calculated 433.2275; [M+H] found 433.2270.

4.8.26) 3-{[4-(piperazin-1-yl)decan-1-one]methyl}-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **63**

White solid; Yield: 95%; m.p.: 83-85 °C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm:7.85 (2H; d; *J*= 9.0 Hz), 6.98 (2H; d; *J*= 9.0 Hz), 5.07 (2H; s), 3.87 (3H; s), 3.64-3.48 (4H; bs), 2.84 (4H; bs), 2.27 (2H; t; *J*= 8.0 Hz), 1.57 (2H; m), 1.26-1.23 (12H; bs), 0.85 (3H; t; *J*= 8.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:181.9, 171.9, 163.1, 159.4, 128.6, 114.8, 114.7, 70.2, 55.7, 50.7-50.3, 45.6, 41.6, 33.4-22.8, 14.2. MALDI-TOF m/z: [M+H] calculated 461.2588; [M+H] found 461.2586.

4.8.27)3-{[4-(piperazin-1-yl)dodecan-1-one]methyl}-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **64**

White solid; Yield: 65%; m.p.: 38-40°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 7.85 (2H; m), 6.98 (2H; m), 5.05 (2H; s), 3.85 (3H; s), 3.62-3.46 (4H; bs), 2.82 (4H; bs), 2.25 (2H; t; *J*= 8.0 Hz), 1.57 (2H; bs), 1.26-1.23 (16H; bs); 0.85 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.0, 171.8, 163.0, 159.3, 128.5, 114.7, 114.6, 70.2, 55.6, 50.6, 50.2, 45.6, 41.5, 29.6-22.7, 14.2. MALDI-TOF m/z: [M+H] calculated 489.2901; [M+H] found 489.2897.

4.8.28)3-{[4-(piperazin-1-yl)tetradecan-1-one]methyl}-5-(4-methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione **65**

White solid; Yield: 65%; m.p.: 38-40°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δppm: 7.85 (2H; m), 6.98 (2H; m), 5.05 (2H; s), 3.85 (3H; s), 3.62-3.46 (4H; bs), 2.82 (4H; bs), 2.25 (2H; t; *J*= 8.0 Hz), 1.57 (2H; bs), 1.26-1.23 (16H; bs); 0.85 (3H; t; *J*= 7.0 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.0, 171.8, 163.0, 159.3, 128.5, 114.7, 114.6, 70.2, 55.6, 50.6, 50.2, 45.6, 41.5, 29.6-22.7, 14.2. MALDI-TOF m/z: [M+H] calculated 517.3214; [M+H] found 517.3217.

4.8.29) 3-{[4-(piperazin-1-yl)octan-1-one]methyl}-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **66**

Orange solid; Yield: 77%; m.p.: 60-61°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.34 (2H; dd; ^{3}J= 9.0 Hz; ^{4}J= 2.0 Hz); 8.09 (2H; d; *J*= 9.0 Hz); 5.10 (2H; s); 3.61 (2H; t; *J*= 5.0 Hz); 3.46 (2H; t; *J*= 5.0 Hz); 2.82 (4H; m); 2.22 (2H; t; *J*= 7.5 Hz); 1.50 (2H; m); 1.23-1.19 (8H; m); 0.79 (3H; t; *J*= 7.5 Hz).^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.4, 171.9, 157.0, 149.8, 127.7, 127.5, 124.5, 70.6, 50.6, 50.0, 45.5, 41.4, 33.2, 31.7, 29.4, 29.0, 25.3, 22.6, 14.1. ESI-MS: m/z calculated 447.2; m/z found 447.0; [M+H] calculated 448.2; [M+H] found 448.0; [M+Na] calculated 470.3; [M+Na] found 470.0.

4.8.30) 3-{[4-(piperazin-1-yl)decan-1-one]methyl}-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **67**

Orange solid; Yield: 83%; m.p.: 62-63°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.37 (2H; d; *J*= 9.0 Hz); 8.12 (2H; d; *J*= 9.0 Hz); 5.13 (2H; s); 3.66 (2H; t; *J*= 5.0 Hz); 3.49 (2H; t; *J*= 5.0 Hz); 2.85 (4H; m); 2.27 (2H; t; *J*= 7.5 Hz); 1.56 (2H; m); 1.26-1.22 (12H; m); 0.85 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.5, 171.9, 157., 150.0, 127.8, 127.6, 124.6, 70.7, 50.7, 50.2, 45.6, 41.5, 33.4, 32.0, 29.6, 29.5, 29.4, 25.4, 22.8, 14.2.ESI-MS: m/z calculated 475.2; m/z found 475.0; [M+H] calculated 476.2; [M+H] found 476.0; [M+Na] calculated 498.3; [M+Na] found 498.0.

4.8.31) 3-{[4-(piperazin-1-yl)dodecan-1-one]methyl}-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **68**

Orange solid; Yield: 79%; m.p.: 65-67°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δppm: 8.34 (2H; d; *J*= 8.5 Hz); 8.12 (2H; d; *J*= 8.5 Hz); 5.11 (2H; s); 3.62 (2H; bs); 3.48 (2H; bs; H); 2.83-2.81 (4H; m); 2.25 (2H; t; *J*= 7.5 Hz); 1.53 (2H; m); 1.21-1.19 (12H; m); 0.83 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:178.4, 171.9, 157.0, 149.9, 127.7, 127.5, 124.5, 70.6, 50.6, 50.1, 45.5, 41.5, 33.3, 31.9, 29.6, 29.5, 29.4, 29.3, 25.3, 22.7, 14.2. ESI-MS: m/z calculated 503.2; m/z found 503.0; [M+H] calculated 504.2; [M+H] found 504.0; [M+Na] calculated 526.2; [M+Na] found 526.0.

4.8.32) 3-{[4-(piperazin-1-yl)tetradecan-1-one]methyl}-5-(4-nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione **69**

Yellow solid; Yield: 87%; m.p.: 70-72°C; ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 8.33 (2H; d; *J*= 9.0 Hz); 8.09 (2H; d; *J*= 9.0 Hz); 5.11 (2H; s); 3.62 (2H; bs); 3.47 (2H; bs); 2.82 (4H; bs); 2.24 (2H; t; *J*= 7.5 Hz); 1.53 (2H; bs); 1.19 (16H; m); 0.83 (3H; t; *J*= 7.5 Hz). ^{13}C NMR ($CDCl_3$; 125 MHz) δ ppm:171.8, 157.0, 149.8, 127.7, 127.5, 124.5, 70.6, 50.6, 50.1, 45.5, 41.4, 33.3, 31.9, 29.7, 29.6, 29.5, 29.3, 29.4, 25.3, 22.7, 14.2.ESI-MS: m/z calculated 531.3; m/z found 531.0; [M+H] calculated 532.3; [M+H] found 532.0; ; [M+Na] calculated 554.3; [M+Na] found 554.0.

4.9. Cytotoxic assay

Cytotoxic activity was investigated against tumor cell lines (4T1 – mammary carcinoma and CT26.WT – colon cancer cells) and non-tumor cell line(BHK-21 – baby hamster kidney). All the cell lines were propagated in culture medium RPMI 1640 pH 7.4, supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS),Hepes (4.0 $mmolL^{-1}$), $NaHCO_3$ (14.0 $mmol.L^{-1}$), ampicillin (0.27 $mmolL^{-1}$), and streptomycin (0.06 $mmol.L^{-1}$).

Cells were harvested by trypsinization and seeded in 96-well tissue culture plates (100 µL/well) at defined density (1 x 10^3 viable cells/well) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 hours. Stock solutions of the tested compounds in DMSO were serially diluted in cell culture medium (< 1% DMSO). After drug exposure for 72 hours, the cells were incubated with MTT (0.01 $mol.L^{-1}$ in water solution - 10 µL/well) for 4 hours. MTT is metabolized by viable cells resulting in a violet complex product that, after being solubilized in 100 µL of DMSO, can be quantified through colorimetry (absorbance at 570 nm)[37].

The negative control (100 % value of viability) was obtained with cells exposure by RPMI 1640 medium supplemented with 10% FBS.

The raw data were normalized to the untreated control cells and set into relation to the metabolic activity of the viable treated cells.

4.10. Subdiploid DNA and Apoptosis assay

4T1 or CT26.WT cells were seeded in 96-well tissue culture plates (1 x 10^4 viable cells/well) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for

24 hours. 4T1 and CT26.WT were exposed to Compound 44 (1 – 10 μM) and Compound 48 (1 – 50 μM), respectively, for 72 hours.

DNA fragmentation was analyzed as described by (RICCARDI; NICOLETTI, 2006), with slight modifications. After time exposure, cells were washed two times with PBS 1x and suspended in 0.3 mL hypotonic fluorochrome solution containing 50 μg/mL propidium iodide and 0.1% Triton X-100 in 0.1% sodium citrate. After 2 – 4 h incubation at 4 °C in the dark, nuclei fluorescence (10,000 events) was measured in a FACSCalibur™ flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

In order to analyze pro-apoptotic activity, treated cells were stained with 0.5 ml PI and Annexin V-FITC according to the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit I – 556547 – BD). Subsequent to staining, the percentages of viable, apoptotic and necrotic cells were analyzed by flow cytometry using the FACSCalibur™ flow cytometer.

Data both from DNA fragmentation and apoptosis assay were analyzed in FlowJo v10 software (FlowJo LCC, OR, USA).

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6. Conflict of Interest

The authors declare no conflict of interests.

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Highligths

- A serie of fifty-six 1,2,4- and 1,3,4-oxadiazoles coupled to lipophilic aminoalcohols and amines were synthesized.
- The compounds were evaluated for their antitumor activity.
- 1,3,4-oxadiazoles coupled to alkyllated piperazine with twelve carbon chain were best active.
- High selectivity index were achieved.
- Alkylated compounds showed to be capable to induce apoptosis in 4T1 and CT26, a crucial cellular process for effective chemotherapeutic agents.