Anti-Alzheimer Activity and Structure Activity Relationship of Some Synthesized Terpinoidal Oxaliplatin Analogs

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Abstract: The terpenoidal oxaliplatin derivatives **6** and **12** were synthesized previously using 2β,3α-dihydroxy-11-oxo-18β-olean-12-ene-30-oic acid **(1)** and 2α,2β-dihydroxy-18β-ursan-12-ene-28-oic acid **(7)** as starting materials. Also, some of the previously synthesized compounds exhibited better cytotoxicity and antioxidant activities than Oxaliplatin® and vitamin C as positive controls. Herein, these compounds were evaluated for their anti-alzheimer activities and were compared to Fluriprofen® as positive control. The detailed pharmacological screening and acute toxicity (LD_{50}) for these compounds were reported.
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Keywords: Synthesized terpinoide derivatives, oxaliplatin analogs, anti-alzheimer activity**,** structure activity relationship**.**

1. Introduction

The terpenoidal oxaliplatin analogs were synthesized and screening as anticancer and antioxidant activities [1]. Recently Chao-Mei *et al.* [2] described the isolation of 2α-hydroxyl-ursolic acid **(7)** from the ethyl acetate extract of the peel of apples (Malus pumila Mill). In Alzheimer's disease, brain cells that process, store and retrieve information degenerate and die. Although scientists do not yet know the underlying cause of this destruction, they have identified several possible culprits. One prime suspect is a microscopic protein fragment called beta-amyloid (BAY-tuh AM-uh-loyd). Some researchers believe flaws in processes governing production, accumulation, or disposal of beta-amyloid are the primary cause of Alzheimer's. This theory is called "the amyloid hypothesis" [3,4]. Beta-amyloid is a small piece of a larger protein called "amyloid precursor protein" (APP). Although scientists have not yet determined APP's normal function, they have learned a great deal about how it appears to work. In its complete form, APP extends from the inside to the outside of brain cells by passing through a fatty membrane around the cell. When APP is "activated" to do its normal job, it is cut by other proteins into smaller sections that stay inside and outside cells. There are several different ways APP can be cut. Under some circumstances, one of the pieces produced is beta-amyloid [5-8]. Beta-amyloid is chemically "stickier" than other fragments produced when APP is cut. It accumulates by stages into microscopic amyloid plaques that are considered one hallmark of brains affected by Alzheimer's. The pieces first form small clusters called oligomers (AWL-igg-uh-merz), then chains of clusters called fibrils, then "mats" of fibrils called beta-sheets. The final stage is plaques, which contain clumps of beta-sheets and other substances. According to the amyloid hypothesis, these stages of beta-amyloid aggregation disrupt brain cells by clogging points of cell-to-cell communication, activating immune cells that trigger inflammation and devour disabled cells, and, ultimately, killing cells [9-15]. In view of these reports and in continuation of our previous works in chemistry of natural products [16-20], we have screening some previous compounds [1] containing a triterpenoid ring system for pharmacological evaluation against Alzheimer in comparison to Fluriprofen® as the reference drug.

2. Experimental

2.1. Aβ42 and Aβ40 assay [21]

Aβ42 and Aβ40 were measured in the culture

medium of H4 cells, a human neuroglioma cell line expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw). Cells were seeded onto 24-well plates (2 x 105 cell well-1) and allowed to grow to confluence for 24h, in 5% CO_2/95% air in humidified atmosphere. Increasing concentrations (from 3 to 300-400 μM) of the compounds were added to the cells overnight in a final volume of 0.5 ml. *R*-flurbiprofen was used as positive control (3-1000 μM). DMSO-d_6 (1%) was used as negative control. At the end of the incubation, 100 μl of supernatants were removed and treated with a biotinylated mouse monoclonal antibody (4G8, Signet Laboratories Inc., Dedham, MA, USA), specifically recognizing the 17-24 amino acid region of Aβ and two rabbit polyclonal antibodies (C-term 42 and C-term 40, BioSource International, Camarillo, CA, USA), specifically recognizing the C-terminus of Aβ42and Aβ40, respectively. Antigen–antibodies complexes were recognized by TAG-donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Soham, UK). Streptavidin coated magnetic beads captured the complexes and the signals were read by an electrochemiluminescence instrument (Origen M8 Analyzer, BioVeris Corporation, Gaithersburg, MD, USA). The cytotoxicity potential of test compound was assessed in the same cells of the Aβ assay (H4) with the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazol-iumbromide (MTT) assay. MTT is a soluble pale yellow salt that is reduced by mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product to which the cell membrane is impermeable. The ability of cells to reduce MTT provides an indication of mitochondria integrity and activity and it may be interpreted as a measure of viability and/or cell number. After medium removal for of Aβ42 and Aβ40 determination, cells were incubated for 3hrs with 500μl culture medium containing 0.5 mg ml^{-1} MTT, at 37 ºC, 5% CO_2 and high humidity. After removal of the medium, 500 μl of 100% DMSO-d_6 were added to each well. The amount of formed formazan was determined reading the samples at 570 nm (background 630 nm) using a microplater reader (model 450, Bio-Rad, Hercules, CA, USA).

2.2. COX-1 and COX-2 assay [22]

The inhibition of the cyclooxygenase activity was estimated measuring PGE2 production from arachidonic acid according to a modified version of the method [22]. Recombinant human prostaglandin H2 synthase-1 (PGHS-1) and synthase-2(PGHS-2) were expressed in transfected spodoptera frugiperda (Sf-9) cells (Invitrogen, San Diego, CA, USA). The microsomal fractions were prepared from the transfected cells and used to assay the enzymatic activities. Briefly, the enzymes (2g) reconstituted in a buffer (100 mMTris-HCl, pH 8.0) containing 2 Mm phenol, were preincubated with vehicle (DMSO-d_6) or test compounds in DMSO-d_6 (1% DMSO-d_6 in the final assay) for 20 min at 22 ºC. The reaction mixture was completed with 1M hematin. The reaction was initiated adding arachidonic acid (4 and 2 μ for COX-1 and COX-2, respectively) and the mixture was incubated for 5 min at 22 ºC for COX-1 assay, or for 10 min at 25 ºC for COX-2 assay. For control measurements, arachidonic acid was omitted from the reaction mixture. The reactions were stopped by the sequential addition of 1 M HCl and IM Tris-HCl (pH 8.0), followed by cooling to 4 ºC. The amount of PGE2 present in the reaction mixture was quantified using an enzyme-immunoassay.

2.3. Studies in Tg2576 transgenic mice

Young male and female transgenic mice (Tg2576) expressing the human *APP* gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter [23] were used for the *in vivo* studies. Male animals were housed singly in an individual cages (to avoid the exaggerating attack of males to each others) while female animals were placed in groups of 3-5 animals per cage. The experiments were performed in accordance with EEC Guidelines (86/609/ECC) for the use of laboratory animals.

2.3.1. Study 1 [24, 25]

Twelve Groups of male mice 4-5 months, each group contain of twenty-one male mice of 4-5 months of age were given by oral *gavage* vehicle (Kool-Aid 7.5 ml Kg^{-1}) or a suspension of each individual compound (100 or 300 mg kg^{-1} day^{-1} in Kool-Acid) once daily for 5 days. This vehicle was selected to replicate that reported with flurbiprofen in similar studies [24, 25]. On day 5, after three hours post drug administration animals were sacrified as described [24,25].

2.3.2. Study 2 [26]

Groups of female mice of 5-7 months of age, each group composed of seventeen female mice were given by oral *gavage* vehicle (Kool-Aid 7.5 ml kg^{-1}) or a suspension of each individual compound (100 or 300 mg k^{-1} day^{-1} in Kool-Aid) once daily for 4 days. On day 4, mice were given a final dose of 100 or 300 mg k^{-1} or vehicle and sacrificed 3 h later, as described below.

2.3.3. Study 3 [27, 28]

Groups of male and female mice of 4-5 months, each group composed of thirty-three male

mice and female mice of 4-5 months were given vehicle or tested compounds or R-flurbiprofen elemented chow *adlibitum* for 4 weeks. There were 11 animals in each treatment group. R-flurbiprofen (Sigma, St, Louis, Mo, USA) and the tested compounds were formulated into standard, color-coded, rodent diety by Charles River (Calco, Italy) at a final drug concentration of 375 ppm. The concentration of the drugs in the diet was the same as that used for flurbiprofen in contenous .Weight and food consumption were monitored every 3-4 days

2.4. Plasma and brain Aβ measurements [29]

Twenty-four hours before starting treatment, one blood sample was collected by means of retro-par puncture for measurement of baseline plasma, Aβ40 and Aβ42 concentration. On the last of treatment, mice were sacrificed by decapitation. Blood samples were collected in EDTA-teste tubes and centrifuged at 800 rpm for 20 min. to separate plasma. Plasma samples were divided into two aliquots of approximately 100µl each and stored at -80 ºC until Aβ and drug assay. The brains were quickly removed and placed on an ice-cold plate. Cortex and pocampus were dissected and immediately frozen on dry ice and stored at-80 ºC for Aβ. The remaining brain was immediately frozen on dry ice and stored at -80 ºC for drug level measurements. Plasma was diluted 1:4 for Aβ42 and 1:20 for Aβ40. For measurement of Aβ, in tissue samples were homogenized in 70% formic acid at 1:10 (w/v). Homogenates were measurtated at 4 ºC for 3 hrs and then centrifuged at 15,000xg for 25 min at 4 ºC. the supernatants were collected and neutralized with 1M Tris. PH 11 at 1:20 (w/v) dilution with 3 x protease inhibitor mixtures (Boehringer Mannheim, Mannheim, Germany). Levels of Aβ40 and Aβ42 in plasma and in brain homogenate supernatants were measured with commercial ELISA kits (The Genetics Company, Zurich, Switzerland). The micro-titre plates were coated with capturing purified monoclonal antibodies specifically recognizing the cterminus of human Aβ40 (clone G2-10, reactive to amino acid residues 31-40, isotype IgG2b, kappa). As detection antibody, a monoclonal biotin conjugated antibody recognizing the N-terminus of human Aβ (clone W0-2, reactive to amino acid residues 4-10, isotype IgG2a, kappa) was used. The assay was linear in the range 25-500 pg ml^{-1} and the detection limit was 25 pg ml^{-1}.

2.5. Plasma and brain drug measurements [30]

Drug levels in plasma and in brain samples were measured by liquid chromatography as previously described [30]. Briefly, samples were prepared by adding 300µl acetonitrile and 40µl phosphoric acid 40% to 100µl plasma or brain homogenate and placing the mixture in a vortex for 5 s. plasma and brain samples were then centrifuged at 14,000 rpm for 5 min and the supernatants (15 and 50 µl, respectively) were injected into the HPLC system. Equipment system with fluorescence **(**Water 474, Waters, Guyancourt, France). Fluorescence measurements were made in semimicro quartz cuvettes (Hellmas) witha 1-cm excitation light path containing 1 mL (or 0.5 mL for fluorescamine) of unstirred solution using a Shimadzu RF-5301U fluorimeter (Shimadzu's Windows-based software) equipped with a 150W Xenon lamp. Ozone resolving type lamp housing as light source. Temperature was maintained at *25 °C* by a thermostatted cell holder. Measurements with The flow rate 0.22 ml/min The excitation (ex) (absorption) maximum and enhanced emission (em) for compounds at nm 12 (ex at 445 nm-em at 478 nm), 6 (ex at 434 nm-em at 478 nm), 11 (ex at 432 nm-em at 480 nm), 5 (ex at 422 nm-em at 465 nm), 10 (ex at 386 nm-em at 432 nm), 4 (ex at 345 nm-em at 449 nm), 8 (ex at 401 nm-em at 425 nm), 2 (ex at 421 nm-em at 491 nm), 7 (ex at 398 nm-em at 467 nm), and 1 (ex at 435 nm-em at 485 nm). These settings minimized light scattering contributions to the observed signal, or mass spectrometry (API2000, Applied Biosystems, Foster City, CA, USA with MassLynx Showroom) detectors were used.

The chromatographic conditions were adapted to each compound to obtain good peak separation and detection sensitivity. A mixture of ammonium formate (20 mM) buffer-acetonitrile-methanol was used as mobile phase for the fluorescence detector. For drugs the HPLC assay was linear in the range 20-4000 ng^{-1} in the brain and 5-1000 ng ml^{-1} in plasma with limits of quantitation of 20 ng g^{-1} in the brain and 5 ng ml^{-1} in plasma. For drugs in Mass the assay was liner between 400 and 20,000 ng g^{-1} in the brain and 100-8500 ng ml^{-1} in plasma with limits of quantitation of 400 ng g^{-1} in the brain and 100 ng ml^{-1} in plasma.The detection carried at the following peaks, compounds **12** (M^+ at m/z 767, B^+ at m/z 501), **6** (M^+ at m/z 781 , B^+ at m/z 512), **11** (M^+ at m/z 750, B^+ at m/z 456), **5** (M^+ at m/z 764, B^+ at m/z 342), **10** (M^+ at m/z 484, B^+ at m/z 213), **4** (M^+ at m/z 498, B^+ at m/z 321), **8** (M^+ at m/z 486, B^+ at m/z 112), **2** (M^+ at m/z 500, B^+ at m/z 400), and **7** (M^+ at m/z 781, B^+ at m/z 432)

3. Results and Discussion

3.1. Chemistry

In continuation of our previous work, a series of steroidal cyanopyridone candidates **1-12** (Chart 1) were synthesized in advance and screened as anticancer and antioxidant agents [1]. Herein, we

used these compounds for evaluation as anti-alzheimer agents.

3.2. Pharmacological evaluation

The tested compounds **1-12** were previously prepared according to the reported procedures [1]. Amyloid beta (Aβ or Abeta) is a peptide of 36-43 amino acids that appears to be the main constituent of amyloid plaques (deposits found in the brains of patients with Alzheimer's disease). Similar plaques appear in some variants of Lewy body dementia and in inclusion body myositis (a muscle disease), while Aβ can also form the aggregates that coat cerebral blood vessels in cerebral amyloid angiopathy. The plaques are composed of a tangle of regularly ordered fibrillar aggregates called amyloid fibers, a protein fold shared by other peptides such as the prions associated with protein misfolding diseases. Recent research suggests that soluble oligomeric forms of the peptide may be causative agents in the development of Alzheimer's disease.

Aβ is formed after sequential cleavage of the amyloid precursor protein (APP), a transmembrane glycoprotein of undetermined function. APP can be processed by α-, β- and γ-secretases; Aβ protein is generated by successive action of the β and γ secretases. The γ secretase, which produces the C-terminal end of the Aβ peptide, cleaves within the transmembrane region of APP and can generate a number of isoforms of 36-43 amino acid residues in length. The most common isoforms are $A\beta_{40}$ and $A\beta_{42}$; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network. The $A\beta_{40}$ form is the more common of the two, but $A\beta_{42}$ is the more fibrillogenic and is thus associated with disease states. Mutations in APP associated with early-onset Alzheimer's have been noted to increase the relative production of $A\beta_{42}$, and thus one suggested avenue of Alzheimer's therapy involves modulating the activity of β and γ secretases to produce mainly $A\beta_{40}$.

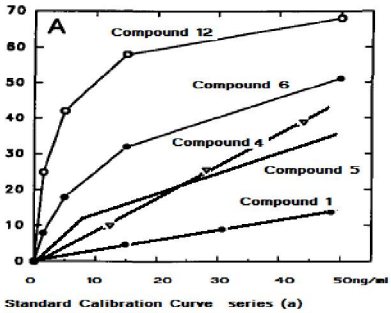
The authors start to examine the anti-Alzheimer activities of two different 2β,3α-dihydroxy pentacyclic triterpinoidal acids, the first is the 28 oic one (Derived from oleanolic acid) and the second is 30 oic acid (derived from Glycyyrrhetinic acid), the rational upon choosen this molecules is their high content of hydroxyls that capable for forming hydrogen bonding with Alzheimer modulator especially Aβ42 and Aβ40 or its percursores so.eventually reach our goal to stop Alzheimer manufastation.

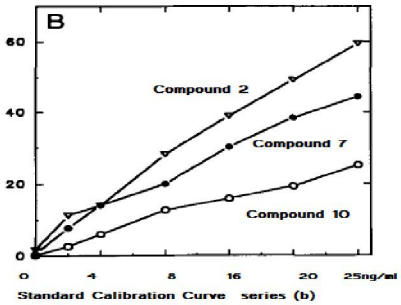
The anti-Alzheimer activities founded for these two compounds proven our hypothesis. The second hypothesis or rational upon our work has built is to introduce multibasic centers as amino that act as proton acceptor and forming hydrogen bonds in the same time with Aβ42 and Aβ40 or its percursores or β and γ secretases leading to interfferre with its biosynthetic pathways so stoping alzehimer, also the authors aimed to introduce chelating platinium atom to study its ability to chelates with Aβ42 and Aβ40 or its percursoresor β and γ secretases.

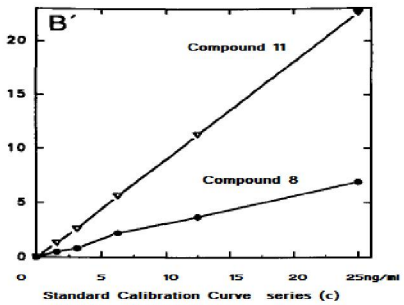
Finally introducing oxalic acid (Poly hydroxy moitey) for combined the triple effect of multi basic,Chelating and hydrogen bonding capabilities that essential for stoping Aβ42 and Aβ40 or its percursores or β and γ secretases in one natural molecules with high therapeutic windows. All the tested compounds showed potent anti-Alzheimer activity due to its Abeta inhibitory effect. The activity order in descending manner is **12, 6, 11, 5, 10, 4, 8, 2, 7, 1** (Table 1).

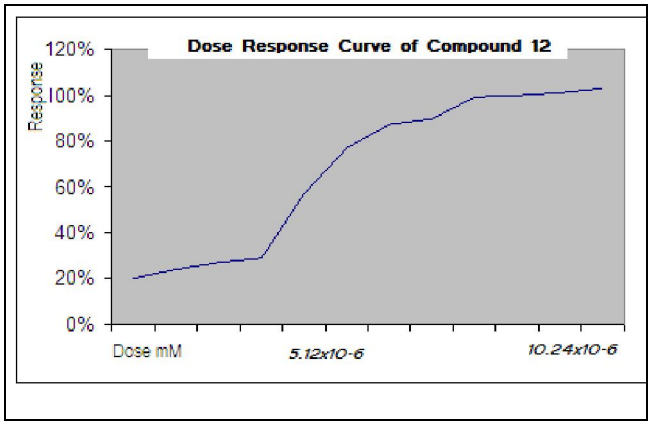
Derivatives **1** and **7** showed resonable anti-alzehimer activity due to their aility to lowering Aβ42 and Aβ40 via hydrogen bond chelating with their percursos or and β and γ secretases. Esterification of both derivatives **1** and **7** into their methyl esters **2** and **8** increases the activity despit that the propability of hydrogen bonding had decreased, but the increasing activity attribuited to the twisting and structural desformation ocurred in the molecule cage due to the remote effect of the introduced methyl group that inable the molecule to interchelate in a such good manner with β and γ secretases leading to suppress it.

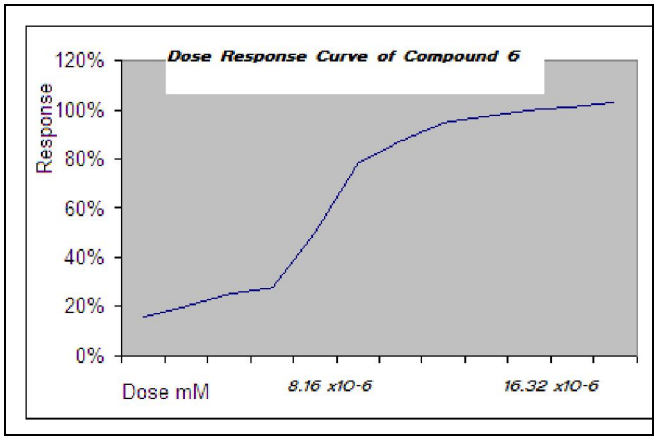
The 2β, 3α configuration manner is selected upon strong biological rational for giving such structure features essential for inter chelating phenomenon. Converting the 2β,3α-diol into 2β,3α-diamino functions (compounds **4 & 9**) increasing the activity due to not the hydrogen forming function of the amino group but due it act as multibasic centers that act as proton acceptor that decativate Aβ42 and Aβ40 percursores or β and γ secretases leading to interfferre with its biosynthetic pathways so stopping alzehimer. Introducing the dichloro platinium moitey into 2β,3α-diamino functions (compounds **5 & 11**) increasing the activity due to yhe high ability of platinium atom to form ligangs and interchelating many molecules in multi central atoms. Replacing the two chlorine atoms in compounds **5 & 11** with oxalo function leads to get the high potent derivatives due to adding moity that hihy able to form hydrogen bond with Aβ42 and Aβ40 percursores or β and γ secretases. It is worth to mention that there is no effect on intracellular domainresponsive genes. The standard calibration curves and dose response curves of compounds **1-12** are illustrated as follows:

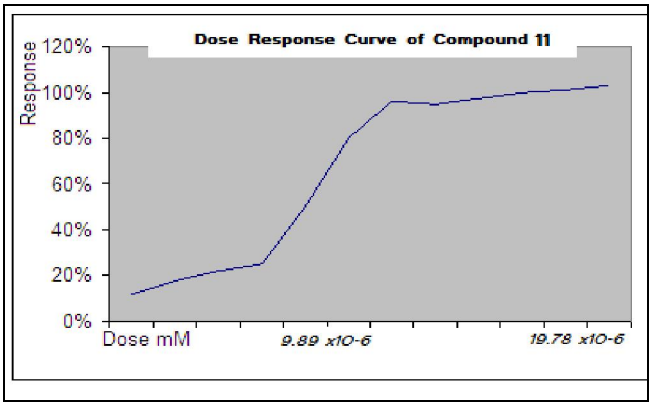

A
Compound 12
Compound 6
Compound 4
Compound 5
Compound 1
Standard Calibration Curve series (a)

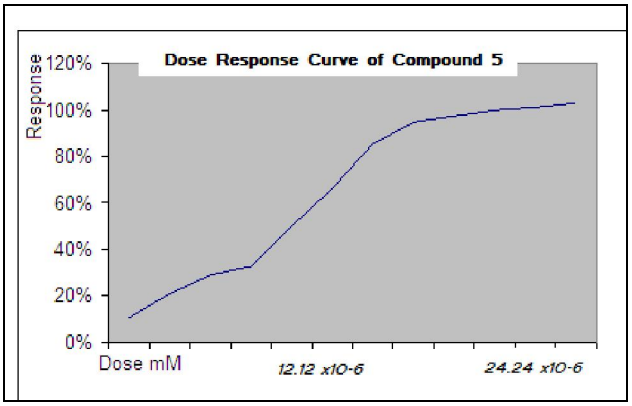

B
Compound 2
Compound 7
Compound 10
Standard Calibration Curve series (b)

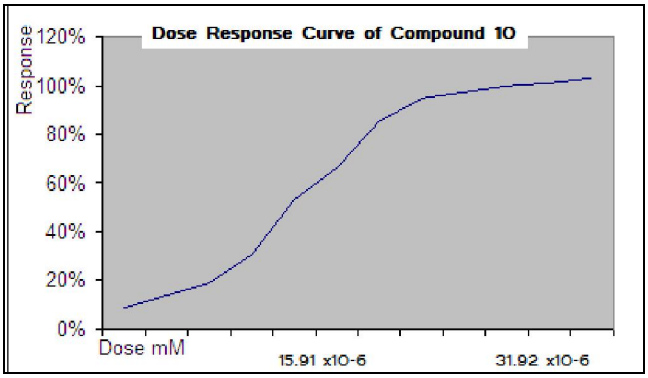

B´
Compound 11
Compound 8
Standard Calibration Curve series (c)

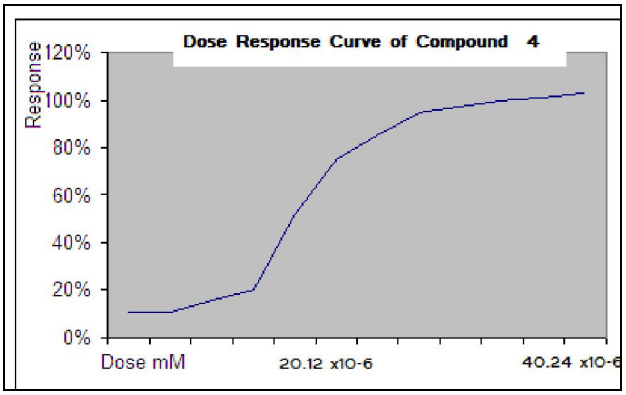

Dose Response Curve of Compound 12
Response
Dose mM
5.12x10-6
10.24x10-6

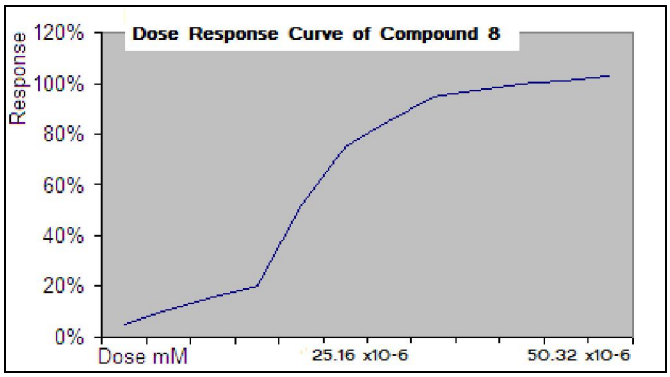

Dose Response Curve of Compound 6
Response
Dose mM
8.16 x10-6
16.32 x10-6

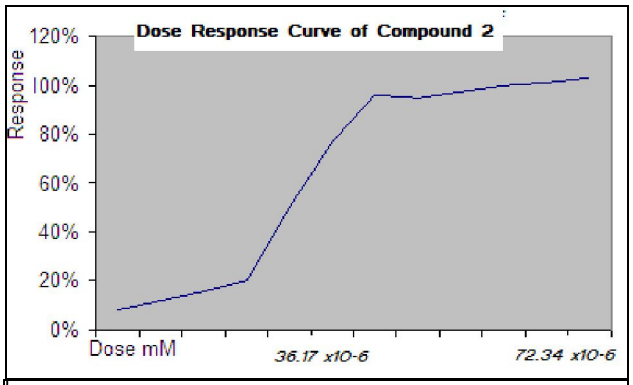

Dose Response Curve of Compound 11
Response
Dose mM
9.89 x10-6
19.78 x10-6

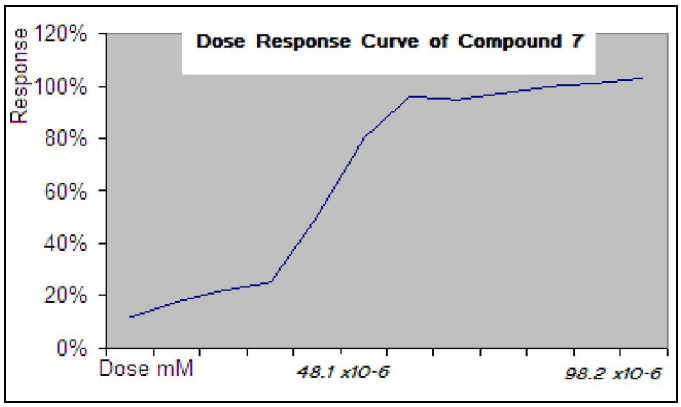

Dose Response Curve of Compound 5
Response
Dose mM
12.12 x10-6
24.24 x10-6


Dose Response Curve of Compound 10
Response
Dose mM
15.91 x10-6
31.92 x10-6


Dose Response Curve of Compound 4
Response
Dose mM
20.12 x10-6
40.24 x10-6







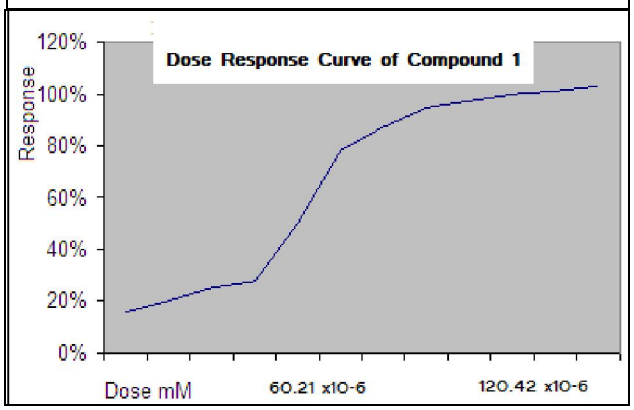


Table 1. IC_{50} activity of some tested compounds

Compound No	$(IC(50)^s$ {µM}	Effect On Notch intracellular domain responsive genes
12	$5.12x10^{-6}$±0.000012	Nil
6	$8.16 x10^{-6}$±0.000023	Nil
11	$9.89 x10^{-6}$±0.000024	Nil
5	$12.12 x10^{-6}$±0.000034	Nil
10	$15.91 x10^{-6}$±0.000032	Nil
4	$20.12 x10^{-6}$±0.000012	Nil
8	$25.16 x10^{-6}$±0.000012	Nil
2	$36.17 x10^{-6}$±0.000034	Nil
7	$48.1 x10^{-6}$ ±0.000067	Nil
1	$60.21 x10^{-6}$±0.000089	Nil
Fluriprofen	$76.89 x 10^{-3}$±0.000012	Nil

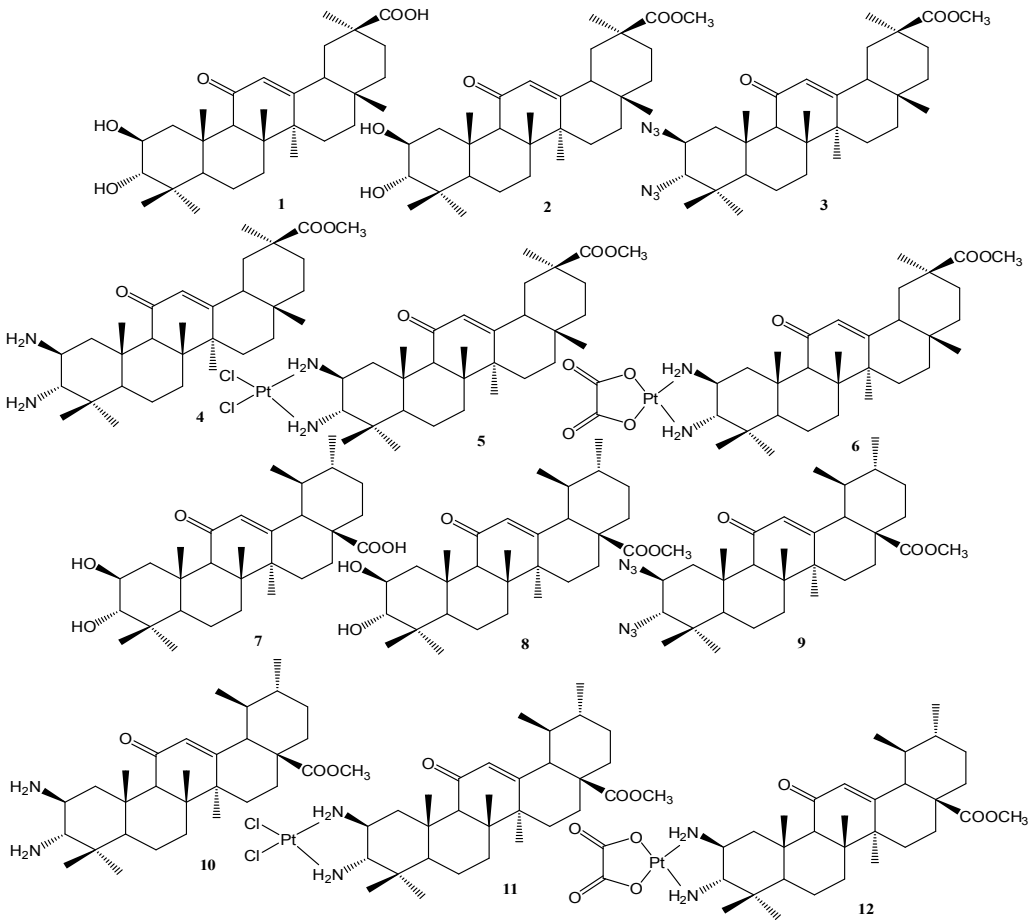


Chart 1

3.3. Pharmacokinetics

From Table 2, the studied compounds showed excellent pharmacokinetics and dynamics profiles, where both the plasma and brain concentration is high after 4 days. The high plasma concentration of the tested compounds may attributed to their reasonable plasma protein binding, while the high brain levels of this compounds attributed to their phospholipids binding in the brain. Both the characters of plasma protein and phospholipids bindings are due to the ability of these compounds for forming hydrogen bonding.

Table 2. In *vivo* pharmacokinetic and pharmacodynamic profiles of the of some newly synthesized agents were evaluated in young APPsw transgenic mice (Tg2576) after oral gavage (100 or 300 mg kg(-1) day(-1) for 4-5 days

Compound No	Plasma Drug Conc after 4 days {microM}	Brain Drug Conc after 4 days{microM}	% alteration in Plasma Abet 42	% alteration Brain Abeta level 2
12	15.56 x10^{-14}	19.11x10^{-16}	91.78	80.13
6	30.22 x10^{-14}	35.67 x10^{-16}	88.23	78.59
11	35.26 x10^{-14}	40.34 x10^{-16}	70.45	77.43
5	39.36 x10^{-14}	45.67 x10^{-16}	65.38	75.34
10	40.23 x10^{-14}	48.67 x10^{-16}	61.12	72.29
4	60.13 x10^{-14}	50.11 x10^{-16}	60.14	71.29
8	65.34 x10^{-14}	52.67 x10^{-16}	57.45	70.11
2	68.12 x10^{-14}	53.67 x10^{-16}	55.32	70.01
7	69.76 x10^{-14}	60.12 x10^{-16}	54.36	69.78
1	75.43 x10^{-14}	63.14 x10^{-16}	50.12	60.12
Fluriprofen	25.41 x10^{-14}	32.04 x10^{-16}	55.12	61.123

3.4. Structure Activity Relationship (SAR)

1. Platinium atom increases the activity sharply
2. Chlorine atom decreases the activity than the oxalofuction due to it not form hydrogen molecule with the target biological molecules.
3. Oxaloplatin provide the most potent agent
4. Olean-28-oic acids analogue is more active than the 30 analouges due to the cagr and the remote effect of the groups after chemical alterations on the terpinoidal skeleton.
5. The 2,3-diamino ring A substitution provide more activity than the diol analogue.
6. Esterifications of the acid increasing the activity due to the same reason as in assumption 4

3.5. Determination of acute toxicity (LD_{50})

The LD_{50} were determined by injection of increasing doses of the tested compounds to adult male albino rats, and then the dose causing 50% animals' death was calculated according to Austen *et al.* [31] (Table 3).

Table 3. Acute toxicity (LD_{50}) of the tested compounds

Compound	LD_{50} (mg/kg)
1	656 ± 0.62
2	793 ± 0.78
4	893 ± 0.88
5	864 ± 0.89
6	871 ± 0.85
7	816 ± 0.88
8	818 ± 0.82
10	836 ± 0.85
11	846 ± 0.89
12	822 ± 0.99
Oxaliplatin	223 ± 0.28
Fluriprofen	1338 ± 0.27

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