AFHELO Report Summary

Project ID: 304900
Funded under: FP7-HEALTH
Country: France

Final Report Summary - AFHELO (Preclinical proof of concept of AF243 potency to prevent and/or treat sensorineural hearing loss)

Executive Summary:
Hearing loss (HL) is a major pathology of the inner ear that affects nearly 600 million people worldwide. Hearing impairment is a growing health concern that is associated with social isolation, depression and reduction of professional capabilities. Hearing disability is also a major limitation for healthy aging since this pathology is associated with numerous deleterious effects including accelerated cognitive decline, increased risk of dementia and early mortality. To date, there is no effective curative or preventive solution for hearing loss. The clinical options are based on the use of prostheses such as cochlear implants. Spiral ganglion neurons (SGN) are major players in hearing conveying electrical signal from cochlear sensory cells (hair cells) to the central auditory pathway. Since SGN degeneration has been extensively described as a cause of hearing loss, neuroactive compounds are potential therapeutic drugs for sensorineural hearing loss (SNHL). Affichem company, in close collaboration with academic researchers, has discovered a new family of original compounds called Dendrogenins that display potent neurotrophic and neuroprotective properties on cellular models of neuronal differentiation. These effects led Affichem to evaluate Dendrogenins potency on an in vivo model of chemo-induced deafness, used for cochlear implant testing, during FP6 Eurohear project. These studies demonstrate the efficacy of Dendrogenins to restore electrical responsiveness of auditory nerve in guinea pig exposed to aminoglycosides. On this basis, Affichem is developing AF243, an effective member of Dendrogenins family, as a drug candidate for the prevention/treatment of hearing loss and to improve cochlear implant efficacy. For this purpose, our objective during AFHELO project is to finalize the preclinical study at the level of determination of the mechanism of action, in vivo efficacy in mice models of noise induced hearing loss, ADME and toxicology to support the clinical evaluation of AF243 in hearing loss.

Intensive studies performed during AFHELO project have lead to major advances for the preclinical development of our drug candidate. First, the chemical synthesis procedure, allowing the preparation of a water soluble AF243 hydrochloride salt at the gram scale, is optimized.

In second step, we have identify that Liver X Receptor (LXR), a nuclear receptor involved in cholesterol homeostasis, inflammation, neuronal differentiation and neuroprotection, is a pharmacological target of AF243 involved in its neurotrophic property.

We also showed that AF243 induced SGN branching in vitro and protect mice from noise induced hearing loss when given orally (a mode of administration suitable in clinic).

Finally, we demonstrate the safety of AF243 at the effective dose given orally on mice.

Data obtained during AFHELO project highlight that AF243 is an innovative drug for the prevention and the treatment of deafness and afford a better knowledge of its mechanism of action. Considering the efficacy and the novelty of our drug candidate, AF243 is prone to afford a therapeutic option for patients suffering from hearing loss that remain actually without satisfactory treatment.

Project Context and Objectives:
Project context
The sense of hearing is dependent on the integrity of the sensory epithelia in the inner ear. Hearing impairment (HI) occurs when this tissue is disrupted. Hearing deficits are generally associated with the loss of the sensory “hair” cells and/or neurons due to genetic defects or to external factors, such as ototoxic damage, noise trauma or ageing. Sensorineural hearing impairment, which affects both the sensory epithelia and the neurons in the cochlea, is a clinical heterogeneous disorder with a high prevalence and currently the only available treatment is limited to hearing aids and cochlear implants. Studies are being conducted to develop alternative treatments combining both preventive and reparative strategies. Lots of preventive strategies were developed around the use of antioxidants to limit cell death induced by oxidative stress. For treatment approaches, the most developed is the use of growth factors (especially brain and glial-cell-line-derived neurotrophic factors and neurotrophin-3). These proteins activate growth, proliferation and differentiation of neuron precursors through binding to membrane receptors, and were showed to be candidates for treatment of HI. Despite of the promising results in animal models, these factors were not very potent in the clinic and showed secondary effects including neuropathic pain in certain contexts. Therefore, new therapeutic approaches must be developed to answer this unmet medical need.

Affichem (coordinator - partner 1) is a spin-off of the INSERM, French institute of health, focused on developing small molecules, named Dendrogenins, a class of alkylaminooxysterols, which are strong inducers of cell differentiation (de Medina et al, 2009). Among these, AF243 showed interesting potencies of carcino-embryonic cells differentiation toward neuronal phenotype and neuron survival (de Medina et al, 2009; Khalifa et al, 2014). These effects led Affichem to evaluate AF243 potency on an in vivo model of chemo-induced deafness, used for cochlear implant testing, during FP6 Euroheal project. The results showed that AF243 preserves the electrical responsiveness of the auditory nerve even when the compound was administered two weeks after aminoglycoside exposure. These results are of strong interest leading to a patent (de Medina et al, 2014) and a scientific publication (Fransson et al, 2015). Our results showed that AF243 is an innovative and effective compound that has a strong potential to afford a therapeutic option for patients suffering from hearing loss (Malgrange et al, 2015).

Project objectives

The main objective of AFHELO project is to complete the preclinical development of AF243 development for the prevention and treatment of HI by first extending the therapeutic applications to other types of HI notably noise induced hearing loss and second by completing the preclinical studies (pharmacology, mechanism of action, ADME, safety) supporting the clinical evaluation of AF243. For this purpose, investigations have been focused on two main domains:

- Definition of the pharmacology, the mechanism of action and the target(s) of AF243. These data will support the potential of development of the molecule, i.e. validate the therapeutic/preventive potential, the therapeutic window and the safety profile. All these data are necessary for the definition of the clinical pathway.

- Validation of the potency of the drug in vivo on relevant models. Pre-clinical validation on such robust animal models is a prerequisite for clinical trials.

Moreover, the experimental knowledge and technical know-how accumulated during this project will be efficient tools for future evaluation of the potency of new drug candidates. Finally, partner 2 (GIGA) and partner 3 (CSIC) work in close collaboration with clinical center specialized in sensorineural HI and AFHELO project has received the clinicians point of view to orient this translational research to address actual needs of patients. Therefore, AFHELO includes direct studies of the potency of AF243 use for prevention and/or treatment of sensorineural HI in humans, which is to date an unmet medical need and development of knowledge and know-how that will be suitable for other studies.

The specific objectives of AFHELO project were to:
1) Accumulate pharmacological data to optimize the use of the drug candidate AF243 in human for prevention/treatment of noise-induced HI and presbycusis by studying mode of administration suitable for clinical evaluation (middle-ear topical/trans-tympanic routes, oral administration).

2) Unravel the effect of AF243 on otic cells in culture, to better understand the mechanisms involved in AF243 effects.

3) Study the mechanisms of action of AF243 in vivo and their potential for protection and/or repair of HI caused by excessive exposure to noise.

4) Develop in vivo studies in optimized conditions to evaluate the potency of AF243 as a new tool for prevention/treatment of sensorineural HI related to ageing.

References


Project Results:
Details of the major achievements in each scientific work package are summarised below:

Work package 1: Pharmacology and Safety

The general objective of the Work Package (WP) is to accumulate pharmacokinetic and safety data optimizing the use of the drug candidate AF243 in human for prevention/treatment of noise-induced HI and presbycusis by studying suitable mode of administration for clinical evaluation (middle-ear topical/trans-tympanic and oral routes). Besides this objective, this WP also aims to identify pharmacological target of AF243 that is of first importance to get a better understanding of its mechanism of action related to its neurotrophic/neuroprotective properties.

The work package is divided in two tasks:

- To determine PK/PD profiles and safety of AF243. Setting of pharmacokinetic absorption, distribution, metabolism and elimination (ADME) data is an essential prerequisite to the initiation of a clinical trial for a new chemical entity. Toxicology data is also necessary. Results of this part will help define the conditions under which ADME and regulatory toxicology studies will be performed by subcontractors.

- To complete knowledge of the molecular mechanisms involved in the effects of AF243, validate its target and study the involvement of the target in the mechanism of action of AF243. The understanding of the mechanism of action of AF243 will help to better use this molecule and potentially to propose a rational for combination with other drugs used in the clinic. To allow easier exploratory experiments, transient and/or stable gene overexpression or silencing, and quantifications of gene
and protein-level effects will be performed on immortalized and embryocarcinogenic cell lines. Results will be confirmed in more physiologic conditions in WP2.

Preliminary PK/PD & toxicology studies

Safety study

Preliminary safety study aims to evaluate whether our drug candidate is well tolerated in mice before the initiation of costly regulatory test. It is of first importance to establish the safety of AF243 using an experimental setting (mode of administration and dose) that lead to a beneficial effect on hearing loss in mice. As described in work package 3, we have characterized that daily oral administration of AF243 (50 mg/Kg) lead to a robust and reproducible prevention of noise induced hearing loss whereas local delivery has failed to demonstrate a beneficial impact of our drug candidate. Safety study through local administration of AF243 (bullostomy and transtympanic approaches) have been tested and are presented in WP3. The safety evaluation of an effective oral dose of AF243 is presented below.

We have investigated the safety profile of an effective dose of AF243 (per os; 50 mg/Kg/day; 14 days treatment) in vivo. Treatment duration was chosen on the basis of classical regulatory safety studies that often use a 14 days toxicology study on animals. Besides in vivo safety evaluation, the cytotoxicity of our drug candidate has also been tested on normal cells in vitro.

in vitro

Normal mice melanocytes (Melan-A) have been exposed to increased doses of AF243 or solvent vehicle for three days. Cells viability was measured using trypan blue exclusion test. We observed no cytotoxicity at the highest dose tested (10 µM). It is worth noting that AF243 displays neurotrophic and neuroprotective activity from 10 nM highlighting that this compound is devoid of cytotoxicity at 1000 fold higher concentration compared to neuroactive one.

in vivo

To evaluate the safety of our drug candidate, we treated male and female C57Bl/6JRj mice with an effective dose of AF243 (50mg/Kg/d; per os) for 14 days. Body weight was measured daily. At the end of the experiment, blood and organs have been collected and analyzed. We do not observed significant modification of body weight in AF243 treated mice compared to control. No observable adverse effects were observed during the treatment (no sign of lethargy, behaviour change or other physical indicators of sickness). Organ weight was not affected by AF243 treatment. Histological analysis of organs (brain, liver, kidney, thymus, bowel, spleen, pancreas, heart, muscle) was performed. No alterations were observed since fibrosis, immune cells infiltration and other modifications were not detected. Peripheral blood count and biochemical blood analysis are also indicative of the safety of AF243. Indeed, blood markers indicative of kidney, liver or brain function are not significantly modified by AF243 treatment in both male and female mice. Preliminary safety evaluation showed that our drug candidate is well tolerated in mice treated orally by an effective dose of AF243.

PK/PD studies

Characterization of pharmacokinetic and biodistribution parameters of a drug candidate is a prerequisite for clinical evaluation in patients. Before the initiation of costly regulatory test that include PK/PD parameters determination, “homemade” studies are needed notably to identify and overcome potential problems and limit the risk of increased cost and unsatisfactory results arising from the use of methodologies unadapted to a given compound. These preliminary studies are particulary important for original compounds such as AF243 for which no previous data concerning method of quantification and metabolism are available. Consequently, the measurement of pharmacokinetic parameters and tissues biodistribution requires a robust and reproducible method of quantification.
Quantitative analytic method for AF243

We have analyzed AF243 by LC-MS using a binary solvent gradient with solvent A (water/formic acid 1%/pH 2.3) and solvent B (MeOH/ formic acid 1%/pH 2.3) and a reverse phase LC column (C18). LC-MS profile revealed major limitations with enlarged peak and a strong and persistent carry-over (e.g. re-appearance of peaks corresponding to the analyte in later runs which do not contain the analyte).

Despite intensive washing procedures with different solvents, changes in the elution program and in the nature of LC column (phenyl hexyl, C18 from different suppliers), carry over remains a strong limitation. We postulate that AF243 directly stick with inoxydable steel of LC-MS apparatus since complexation with inoxydable steel was encountered for other compounds in particular amine bearing substance. Interestingly, LC-MS system devoid of ferrous metals and steels (called bioinert materials) has been developed to resolve carry over arising from direct interaction between inoxydable steel and the analyte. Affichem has recently initiate experiments aiming to develop an AF243 quantitative method using a bioinert LC-MS system. Preliminary results are very encouraging. Indeed, we obtained chromatogram with satisfactory peak for AF243, well separated compared to structural analogues belonging to Dendrogenin family. The method is also sensitive (limit of quantification below nM concentration) allowing the dosage on biological samples.

The carry over is also acceptable with less than 1% after injection of a high dose of AF243 (1µg/ml) and strongly decrease after washing. Consequently, these results showed that the encountered limitations are solved.

The unexpected limitations related to AF243 quantification has been time consuming hampering the preliminary PK/PD investigation and regulatory test to be performed during this project.

The identification of these limitations demonstrates that preliminary studies are needed before the initiation of the regulatory test. In our case, it is obvious that direct initiation of regulatory test have resulted to the waste of lot of money without any exploitable results.

Mechanism of action of AF243

The elucidation of the mechanism of action of AF243 constitutes the main objectives of this task. For this study, we used human neuroblastoma SH-SY5Y cell lines because human and rodent pharmacological targets are not similar. In addition, SH-SY5Y cells are easily transfectable compared to normal otic cells. Consequently, this cell line is a suitable model for this task.

We mainly investigated whether LXR receptors are pharmacological targets of AF243 for the following reasons: first, LXR is a nuclear receptor involved in numerous biological processes including neuronal differentiation and neuroprotection; second, LXR binds cholesterol derivates and AF243 belongs to this structural family; third, our previous study allow the identification of LXR as a pharmacological target for Dendrogenin A, a neuroactive structural analogue of AF243 developed by Affichem for the treatment of cancer.

We tested the capacity of AF243 to directly bind to LXR receptors. For this purpose, we measured using surface Plasmon resonance methodology (Biacore) the physical interaction between AF243 and recombinant human LXR alpha and LXR beta ligand binding domain (hLXR-LBD). We showed that AF243 binds to LXR alpha and beta with respectively higher affinity for LXR beta isoform. It is worth noting that AF243 is more potent to bind LXRs compared to the canonical LXR ligand GW3965 in this experimental setting.

We also evaluated the effect of AF243 (5µM, 5h) on endogenous LXR responsive genes (ABCA1 and ABCG1) in cultured mouse embryonic fibroblasts (MEF) isolated from WT mice or KO mice for LXR beta. A natural LXR ligand 22(R)-HC (10 µM, 5h) was used as a positive control. Both AF243 and 22(R)-HC stimulated the transcription of ABCA1 and ABCG1 and the induction was completely abolished in LXR beta-KO MEF. This result showed that AF243 induces LXR-responsive genes expression through an LXR beta dependant mechanism. Altogether, this study demonstrates that LXRs in particular beta isoform are
pharmacological targets of AF243. The role of LXR beta on the neurotrophic activity of AF243 was evaluated on human neuroblastoma SH-SY5Y. For this purpose, LXR beta was knocked down in SH-SY5Y cells by using a shRNA approach. Two clones (shLXRb1 and shLXRb2) were chosen that had a strong reduction in LXR beta mRNA and protein expression. We showed that AF243 failed to trigger neurite outgrowth on shLXRb1 and shLXRb2 as opposed to wild type (WT) or shControl (shC1 and ShC2) cells. In addition, LXR beta invalidation also prevents the increase of betall-tubulin and Map2a expression induced by our drug candidate. These results demonstrate that LXR beta mediates the neurotrophic activity of AF243.

We then compared the effect of AF243 with well known LXR ligands (TO901317, GW3965 and 22(R)- hydroxycholesterol) for their neurotrophic potency. We found that TO901317 and GW3965 had no significant effect whereas 22(R)-hydroxycholesterol slightly increase neurite outgrowth but was less potent than AF243. This data showed that AF243 has unique properties compared to LXR ligands tested. Differences between AF243 and other LXR ligands should be associated with differential recruitment of coactivators/corepressors and subsequent regulation of gene expression patterns, which strongly depend on the structure of LXR/ligand complex.

Work package 2: Action of AF243 on otic cell lines in vitro

The objectives of this WP are to unravel the effect of AF243 on otic cells in culture, in view to get a better understanding related to the mode of action of AF243 in conditions close to physiology, assessing the possible benefits and risks of effects on the different cell type of the inner cell and the interest of combination, for instance with growth factors. Each task explores important parts to fulfil the overall purpose to provide relevant information to unravel the mode of action of AF243 on normal otic cells.

The objectives of WP2 are:

- to evaluate the neuritogenic potency of AF243 using cell-culture based assays
- to evaluate the differentiation potency of AF243 using cell-culture based assays
- to identify targets and unravel mechanisms of action of AF243

Characterization of the neuritogenic potency of AF243 using cell-culture based assays

To address the neuritogenic effect AF243, we used first: dissociated culture of spiral ganglion neurons in which we analysed the length and the complexity of neuritic arborisation. Spiral ganglion neurons (SGN) were isolated from mouse P0 NMRI pups. Cells were allowed to recover for one day prior to AF treatment. Neurons were treated with 1 or 10nM AF243, H2O was used as control, for 7 days in vitro (DIV), half of the medium was changed every 2 days. At the end point of 7 DIV, neurons were fixed and stained for beta III Tubulin and Neurofilament (NF). The neurites length was measured and the neuritic complexity was assessed. A distinction between type I and type II neurons was made by looking at NF staining, since type I neurons do not present NF staining in their cell soma unlike type II neurons (Hafidi et al, 1996).

Using NeuronJ for measuring the length of the neurites, they were classified in primary (for neurites arising from the cell soma), in secondary (for neurites arising from a primary neurite), and so on and so forth, until for some conditions to an order 5.

AF243 tends to increase neurite length from order 2 to 5 on both types of neurons although not significantly. While assessing the neuritic complexity, it appears that AF243 tends to increases the number of higher complexity neurites.

Characterization of the differentiation potency of AF243 using cell based assays

AF243 could trigger the differentiation of neuroblastoma cells, we wonder whether this effect could also be achieved using neural stem cells generated from human iPSCs.
Human neural stem cells (hNSC) were treated with different concentrations of AF243 (1 and 10nM or H2O as the vehicle) during 3, 5 and 7 days. To assess differentiation cells were stained for beta III Tubulin and DAPI, then, the ratio of beta III Tubulin positive cells on the total number of DAPI positive nuclei was calculated. Increasing concentration of AF243 induce an increased number of beta III Tubulin positive cells after 3 days in vitro (DIV). At 5 DIV of treatment, AF243 induces an increased differentiation of hNSC but the dose effect is less pronounced. Later at 7 DIV of treatment, the effect of AF243 is no longer observable. During the time course studied, hNSC showed a spontaneous differentiation, however at early time point AF243 increases the amount of differentiated cells. Consequently, our drug candidate increases the kinetic of differentiation of iPSCs toward neuronal phenotype.

Validation of targets and unravelling mechanisms of action of AF243 in normal cells

As described in WP1, AF243 appears to activate LXR receptor signalling pathway. We wonder whether AF243 could stimulate these signalling cascades in the inner ear. We address first the expression of LXR receptor, and second their potential activation by AF243.

- The effect of AF243 could be mediated by the activation of LXR receptors. To address whether these receptors were expressed in the inner ear, RT-qPCR was performed to see the expression of LXR receptors. Inner ear was dissected and separated in organ of Corti, stria vascularis and spiral ganglion. Both LXR alpha and beta are expressed in the spiral ganglion, but no transcripts were detected in the organ of Corti or the stria vascularis. In order to characterise the expression of these receptor in the spiral ganglion neurons, LXR beta staining was performed on dissociated culture of SGN. Nuclear LXR beta is present in both type I (cell soma negative for neurofilament) and type II neurons (cell soma positive for neurofilament and peripherin).

- We notably investigated the impact of AF243 on NR4A transcription factors (Nur77 and Nurr1) since these latter are involved in neuronal differentiation and neuroprotection and are activated by DDA (a member of Dendrogenin family) through an LXR dependant mechanism (Segala et al, Cancer Cell, in revision). Spiral ganglia were dissected from P2 NMRI mice and cultured in presence of either H2O (vehicle) or AF243 1µM for 24h. LXR response genes (Nur77 and Nurr1) were monitored by RT-qPCR. Upon AF243 treatment Nur77 was slightly induced and Nurr1 more markedly induced.

In conclusion, AF243 promotes differentiation of hNSC at early time points, showing a faster differentiation event. Concerning the neuritogenesis of SGN, the effect of AF243 is not very striking on neurites length, but the molecule increases neuritic complexity of SGN, as they harbour more branches. The mechanism of action of this molecule could be mediated through LXR receptors present in SGN, and activation of LXR response genes.

Work package 3 : Action of AF243 in in vivo models

The main objectives of WP3 are:

- To improve knowledge of etiology of hearing impairment by evaluating the role and mechanism of action of IGF-I and homocysteine metabolism in sensorineural hearing loss.

- To study the potential of AF243 for protection and/or repair of HI in vivo caused by excessive exposure to noise.

Mechanism of action of IGF-I in sensorineural hearing loss

The physiological age-related decrease in circulating IGF-I levels has been related to cognitive and brain alterations. Therefore, IGF-I is considered a neuroprotective agent. Human IGF-I deficiency is a rare disease associated with poor growth rates, mental retardation and syndromic hearing loss (OMIM608747). Igf1-/- mice are dwarfs with poor survival rates and congenital profound deafness, which worsens with ageing. Our objective was to compare the susceptibility of Igf1+/+ and Igf1+/+ mice to damage by using exposure to excessive noise at different ages. Igf1+/+ and Igf1+/+ mice show an age-dependent decrease in IGF-I serum levels, especially from 6 months of age on, which correlates with the increase in ABR thresholds. Noise exposure experiments with 1 and 3 months-old mice did not reveal
differences between genotypes, both genotypes were equally sensible to noise induced hearing loss (NIHL). However, 6 month-old Igf1+/-mice presented greater susceptibility to noise damage, with higher threshold shifts and a poorer recovery compared to noise exposed Igf1+/+ mice.

The cellular and molecular mechanisms underlying susceptibility to damage underly an increased inflammatory signals with a reduction in antiinflammatory response. Our data indicate that age-associated IGF-I deficit enhances otic sensibility to damage.

Mechanism of action of homocysteine metabolism in sensorineural hearing loss

Genetic and environmental factors contribute to age-related hearing loss. High homocysteine (Hcy) in plasma is a well-known risk factor in several human pathological conditions and its levels increase with ageing. Furthermore, different alterations in Hcy metabolism and the methionine cycle have been associated to hearing loss. We have studied the potential impact of diet-induced hyperhomocysteinemia on hearing.

Folic acid deficiency increments plasmatic and cochlear Hcy levels, impairs methionine cochlear metabolism causing oxidative stress, major cellular alterations, cell death and premature hearing loss (Martinez-Vega, Pajares and Varela-Nieto, 2015) showing that bad eating habits can have deep impact on hearing.

Evaluation of AF243 on noise induced hearing loss in mice

The main objective was to investigate whether AF243 protects mice from noise induced hearing loss. For this purpose, both local and oral administration of our drug candidate have been evaluated:

- Local administration

Two different microsurgical approaches for drug delivery to the middle ear (and from there, to the inner ear) were developed. Bullostomy consist in a perforation in the bony wall of the tympanic bulla, allowing the direct visualization of round window niche, where the drug could be precisely located by a 34G catheter connected to a Hamilton micro syringe. Transtympanic (TT) injection is a very simple procedure; the product is administered through a small incision in the tympanic membrane, filling the middle ear. Compared to bullostomy, TT injection is simpler and allows bilateral administrations.

Before evaluating the effect of AF243 administered by bullostomy and transtympanic injection, we studied the impact of these procedures on hearing performance and cochlear morphology. We showed that both bullostomy and transtympanic surgeries maintained hearing, with ABR parameters similar to those determined before the microsurgery, and cochlear morphology. In addition, transtympanic injection is faster and easier than bullostomy, and allows bilateral administrations. It seems to have a better control of the inflammatory response and cellular survival mechanisms.

We have then tested the toxicity of increasing concentrations of AF243 administered by bullostomy and transtympanic injection. Local administration in the middle ear by bullostomy of AF243 at 8, 11, 23 and 46 mM did not modify auditory function during the 28 days follow up as compared to baseline situation, although at 76 mM, AF243 induced a notable ototoxic effect with statistically significant threshold shifts. At this toxic concentration, severe morphological alterations were observed in the cochlea, including a labyrinthitis ossificans and atrophy of the stria vascularis.

Local administration of AF243 at 11 and 30 mM by transtympanic injection induced a rapid increase in ABR thresholds in response to click and tone burst stimuli, although at 11 mM it was observed a notable recovery in the first two weeks. In addition, 11 mM AF243 shows higher anti-inflammatory and anti-apoptotic effects and a better redox balance.

Finally, we have evaluated the impact of local administration of AF243 by bullostomy and transtympanic on hearing performance on mice exposed to noise. We observed no beneficial impact on the evolution of hearing thresholds after noise exposure on mice treated by AF243 delivered by bullostomy approach. Transtympanic injection of AF243 triggers a transient
increase of ABR but promoted a stronger recovery of ABR thresholds between post-noise days 2 and 14, with improved ABR thresholds compared to control mice. In this context, AF243 provoked an increase in both pro-inflammatory and anti-inflammatory markers 1 day post-noise, and upregulation of the LXR responsive gene Scd1 28 days post-noise.

Several experiments related to local administration of AF243 have been done during the project giving interesting information but without evidence of robust efficacy. Local delivery is highly challenging since repeated treatment is difficult. Considering this statement, oral administration of AF243 has been investigated since this mode of administration is suitable for clinical application and allows repeated treatment.

- Oral administration

Three independent experiments in two mouse strains (inbred C57BL/6J and mixed 129Sv/MF1) were performed. Briefly, mice were treated with AF243 diluted in Ringer’s lactate solution and administered by oral gavage at 50 mg/kg/day for three weeks, one week before noise exposure and two weeks after. Auditory function was evaluated with ABR before the beginning of the treatment (baseline) and 2, 14 and 28 days after noise exposure. Cochlear samples were taken one day after exposure (to see a potential protective effect on acute noise damage) or at the end of the experiment (28 days after noise) to see effect on permanent cochlear changes.

First experiment was developed in C57BL/6J mice. Our results show that, in C57BL/6J mice, the oral administration of AF243 induces a protective effect, and mice receiving the treatment present lower temporal and permanent threshold shifts after noise exposure, in response to click and also to pure tone stimuli, compared to control mice. Accordingly, C57BL/6J mice treated with AF243 present a better conservation of the organ of Corti in the basal turn (higher frequencies) at the end of the experiment.

Similarly, two independent experiments in 129Sv/MF1 were performed. Mice treated with AF243 by oral gavage during three weeks show better thresholds after noise exposure, although statistical differences were evident two weeks after damage.

In conclusion, Pre-treatment with AF243 (50 mg/kg/24h) reduced the impact of noise exposure and improved hearing thresholds along the study when compared to control and vehicle, both in C57BL/6J and MF1/129Sv mouse strains.

Oral administration of AF243 induced among other actions on gene expression: i) downregulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory genes; ii) downregulation of the cellular damage factors Kim1 and Apaf1; iii) and upregulation of anti-apoptotic S1pr5 gene, of the nuclear receptor LXR alpha and of LXR responsive genes ApoE and Scd1, when compared to vehicle oral administration.

Altogether, this study highlight that AF243 is an innovative drug candidate that displays potent protective property on noise induced hearing loss.

Work package 4 : Chemistry

The general objective of this Work Package is to synthesize AF243 and derivatives needed in the project. The control of yields, costs and use of methods is a key stage towards industrial production. Refined methods have been developed in order to get an optimal and transposable synthesis protocol for production of AF243 and all its derivatives presented hereafter.

The following tasks are included in this work package:

- Synthesis of a water soluble AF243
- Synthesis of 2H-labelled AF243 used as an internal standard for quantification of AF243.
- Synthesis of AF243 analogues and potential metabolites.
Synthesis of AF243
The main objective of this task is to produce AF243 batch in sufficient amount and high purity to perform the tasks of Afhelo project. Before Afhelo project AF243 was produced as a trifluoroacetate salt at milligram scale (de Medina et al, J Med Chem, 2009) or at gram scale as a free base (Poirot M et al, patent number EP20110306548). For this purpose, the optimization of chemical synthesis and purification methods have been required to produce AF243 as a water soluble hydrochloride salt at the gram scale.

AF243 is an aminosterol derivate that bear a spermidine grafted at Carbon 6 (C6) of the cholesterol skeleton by primary amine N1 of spermidine.
A regioselective addition of spermidine by amine N1 is required to produce AF243. Consequently, the first step involves the chemical synthesis of a spermidine bearing protecting group at N4 and N8. The second step is the aminolysis of 5,6alpha epoxycholestan-3beta-ol by protected spermidine (N4,N8-Di-tert-butyloxycarbonylspermidine). 5,6alpha epoxycholestan-3beta-ol is produced from epoxidation of cholesterol with meta Chloroperbenzoic acid. Step 2 affords 3,4-CSPD-BOC (the protected spermidine grafted at the carbon 6 of the cholesterol skeleton). This latter is hydrolyzed under acidic condition (MeOH/HCl) to generate AF243 as a water soluble chlorhydrate salt at the gram scale. It is worth noting that several aspects of chemical synthesis have been optimized during Afhelo project leading to a strong improvement compared to the previous procedure. Indeed, the yield of step 1 (production of N4,N8-Di-tert-butyloxycarbonylspermidine) has been improved from 47% to 91%. A major improvement of chemical production is related to step 3. Deprotection of 3,4-CSPD-BOC using methanol/hydrochloric acid followed by precipitation of the expected product in methanol/diethyl ether lead to the obtention of AF243 chlorhydrate salt with high yield (83%).

Optimization of the chemical synthesis procedure was successful to prepare water soluble AF243 chlorhydrate salt at the gram scale allowing to perform all the tasks of this project. Purity of the compound has been confirmed by combined methodologies (mass spectrometry, 1H and 13C-NMR, elemental analysis, infrared, melting point). Interestingly, we succeeded to set up a suitable method of AF243 chlorhydrate salt cristallization allowing to obtain a x-ray structure of our drug candidate.

X-ray structure is of great interest since it constitutes an indisputable proof of regio and stereochemistry of a compound. In addition AF243 three dimensional structure elucidated by x-ray will be used to study by molecular modelling the interaction of AF243 with LXR receptors in order to obtain information related to difference between AF243 and other LXR ligands, selectivity of AF243 for LXR beta isoform and others.

We have also produced 2H-labelled AF243 chlorhydrate by the same procedure described above using commercially available 2H-labelled cholesterol as a reactant. 2H-labelled AF243 has been used as an internal standard for quantification of AF243 by liquid chromatography coupled to mass spectrometry (LC-MS).

AF243 derivates batches
The determination of structural parameters associated with the efficacy of AF243 on models physiologically relevant in hearing loss (spiral ganglion neurons, cochlear explants) is of interest. Consequently, during the Afhelo project, we perform the chemical synthesis of AF243 structural analogues using a previously described chemical procedure (de Medina et al, J Med Chem, 2009).

The AF243 structural analogues have been produced at the milligram scale that is sufficient for in vitro studies. These derivates will be evaluated on in vitro models physiologically relevant in hearing loss to get more information regarding the structural parameters associated with the efficacy of AF243.

Potential Impact:
Hearing impairment is a growing health concern that is associated with social isolation, depression and reduction of...
professional capabilities.
Hearing disability is also a major limitation for healthy aging since this pathology is associated with numerous deleterious effects including accelerated cognitive decline, increased risk of dementia and early mortality.

To date, there is no effective curative or preventive solution for hearing loss. Therefore, prevention and treatment of hearing loss is an unmet medical need underlying the strong expectation toward innovative and therapeutic options.
The expected final results of this project were to finalize the preclinical study of AF243 as an original and effective drug candidate for the prevention and the treatment of hearing loss. Thanks to European funding, collaborative studies between Affichem and auditory research experts (CSIC and GIGA) have generated strong results highlighting that AF243 is an innovative and effective drug candidate for patients suffering from hearing loss. Reaching this objective should afford a new therapeutic option for patients suffering from hearing loss.
Considering the strong socio-economic impact of deafness (i.e. social isolation, depression reduction of professional capabilities, increased risk of dementia and early mortality), the emergence of AF243 as new effective drug is prone to give rise to a significant beneficial socio-economic impact.

The Cooperation Theme 1 Health is aligned with the fundamental objectives of EU research policies: improving the healthy lifetime of European citizen and increasing competitiveness of European health-related industries and services.
For this purpose, the present project will help to set up a policy of prevention of hearing loss such as the one existing for cardiovascular diseases or diabetes.
In addition, a therapeutic option will be developed, aimed at helping patients with hearing impairment to improve their quality of life by a new treatment that will prevent hearing impairment and/or repair the affected inner ear. Indeed, hearing impairment is a significant health-care problem with tremendous socio-economic impact.
The project is also SME-relevant since its success will benefit in particular to the SME with the access to a fruitful niche market.

List of Websites:
www.afhelo.eu

Related information

<table>
<thead>
<tr>
<th>Result In Brief</th>
<th>Pre-clinical study success for hearing loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documents and Publications</td>
<td>final1-logo-afhelo-eu.pdf</td>
</tr>
<tr>
<td></td>
<td>final1-logo-afhelo.pdf</td>
</tr>
</tbody>
</table>

Reported by
AFFICHEM SA
France

Subjects
Scientific Research

Last updated on 2017-01-11
Retrieved on 2019-05-23
