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RESULTS OF BACE1-INHIBITOR CLINICAL TRIALS CONFIRM KEY PREDICTIONS FOR APP-INDEPENDENT GENERATION OF BETA-AMYLOID IN SPORADIC AD.

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ABSTRACT.

The present article analyzes the results of recent clinical trials of beta secretase inhibition in sporadic Alzheimer' disease (SAD), considers the striking dichotomy between successes in tests of BACE1 inhibitors in healthy subjects and familial AD (FAD) models versus persistent failures of clinical trials and interprets it as a confirmation of key predictions for a mechanism of APP-independent, beta secretase inhibition-resistant production of beta amyloid in SAD, previously proposed by us. In the light of this concept, FAD and SAD should be regarded as distinctly different diseases as far as beta-amyloid generation mechanisms are concerned, and whereas beta secretase inhibition would be neither applicable nor effective in treatment of SAD, the BACE1 inhibitor(s) deemed failed in SAD trials could be perfectly suitable for treatment of FAD. Moreover, targeting the aspects of AD other than cleavages of the APP by beta and alpha secretases should have analogous impacts in both FAD and SAD.

Keywords: Alzheimer's disease (AD); amyloid precursor protein (APP); familial AD (FAD); sporadic AD (SAD); BACE1 inhibitors; APP-independent generation of beta amyloid.

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Beta amyloid, Aβ, the peptide associated with and widely believed to have a pivotal early role in etiology of Alzheimer's disease, is generated ostensibly by proteolytic cleavages of a much larger molecule, beta amyloid precursor protein, βAPP. In the amyloidogenic proteolytic pathway, two sequential cleavages of βAPP are involved in the production of Aβ. The first is a cleavage of βAPP by the beta secretase enzyme. It occurs between residues 671 and 672 of the βAPP molecule (isoform 770 numbering), generating the N-terminus of Aβ, yielding the 12kDa membrane-bound C-terminal fragment, C99 (residues 672-770), releasing a large ectodomain of βAPP, soluble sAPPβ (residues 1-671), and precluding activity of alpha secretase which cleaves βAPP within its Aβ segment but cannot cut within C99 or Aβ (1–3). The second cleavage, by gamma secretase activity, occurs at one of multiple sites within C99 and generates the C-terminus of Aβ. Thus released, Aβ is secreted from the cell. The size of Aβ ranges from 36 to 43 amino acids, with Aβ40 being the most abundant species normally formed. Studies of the inherited forms of the disease, FAD, strongly indicated that cerebral Aβ accumulation is essential for and underlies the etiology of the disease (4-6). This notion was formalized in a putative theory of AD known as "Amyloid Cascade Hypothesis", ACH (7-12). ACH has become the dominant model of AD pathogenesis and has been guiding the development of potential treatments; most therapeutic strategies attempted to date have been designed within the framework of this theory. Over two hundred autosomal dominant mutations associated with FAD have been identified in genes for βAPP and presenilins, the components of gamma secretase complex (6). In βAPP gene most of the mutations cluster around secretases cleavage sites and increase either the production of total Aβ or the relative proportion of a more neurotoxic 42-residue form of Aβ, Aβ42. In terms of the ACH, there is little doubt that the abnormal processing of βAPP and increased production of total Aβ or its 42-amino acid isoform are pivotal events in the pathogenesis of FAD. Although the number of individuals affected by FAD is substantial, in relative terms this form of the disease is quite rare, representing less than 5%

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(less than 1% by some estimates) of the total Alzheimer's disease burden (5, 13, 14). Since the pathological lesions and symptoms in the non-hereditary form of the disease, SAD, are analogous to those seen in the familial forms, it has been assumed that abnormal amyloidogenic proteolytic processing of βAPP also underlies the pathogenesis of SAD (4, 5).

In the framework of ACH, it was understood early on that inhibition of Aβ production might benefit affected individuals. In light of the above discussion, beta secretase activity was viewed, due in part to its temporal position at the top of the cascade sequence, as a strategic target of choice. Therefore, since the identification of BACE1 (Beta-site APP-Cleaving Enzyme) as beta secretase (15-17), it has become the primary therapeutic target for treatment of AD. Designing BACE1-inhibiting agents presented major challenges of cell penetration, oral bioavailability, metabolic clearance, and brain access. Intense efforts, mainly by the pharmaceutical industry, led to development of a number of brain penetrant small molecule BACE1 inhibitors that have been vigorously investigated. The results obtained in the early investigations of BACE1 inhibition, first appearing around 2007 (18-26), are truly striking. As an example, Merck researchers reported in 2012 the discovery of "compound 16", which robustly reduced cortex and CSF levels of Aβ when administered orally to rats (27). Continuous efforts to improve upon "compound 16" culminated in the development of verubecestat (MK-8931). Preclinical tests of this agent achieved dramatic results (28). Levels of Aβ and sAPPβ were reduced by up to 90% in plasma, brain, and CSF after even a single administration of verubecestat to healthy subjects including rats, monkeys, and humans (28). The acute reduction of over 80% in CSF and cortical Aβ and sAPPβ produced by verubecestat was maintained after chronic administration for 9 months in monkeys (28). Because of its favorable initial safety profile and its ability to markedly reduce cerebral and CSF Aβ and sAPPβ concentrations, verubecestat was the first BACE1 inhibitor to progress to phase III clinical trials. Preclinical evaluation of a number of independently developed BACE1 inhibitors, such as BI 1181181,

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LY2811376, LY 2886721, AZD3293 (lanabacestat, LY3314814), CNP520, E2609 (elenbacestat), JNJ-54861911, CTS-21166, HPP854, PF-05297909, RG7129, TAK-070, VTP-37948 yielded similarly impressive results in animals and healthy volunteers and all these agents have entered clinical trials.

With the ability to significantly reduce the production and lower the levels of Aβ thus established, the question remained whether such a reduction would translate into a "treatment" of the disease. This question was answered resolutely and convincingly, at least in the animal models of FAD, in two recent studies using different approaches to inhibit beta secretase activity. One study utilized BACE1 inhibitor NB-360 (29). It was based on a previous study (30) showing NB-360 to be a potent, brain penetrable BACE1 inhibitor capable of completely blocking Aβ deposition in the brains of βAPP transgenic mice, as well as of rats and dogs. Moreover, this inhibitor blocked accumulation of activated inflammatory cells in the brains of βAPP transgenic mice. The more recent study with NB-360 (29) further assessed the notion that suppression of Aβ production can have beneficial downstream effects on the progression of Alzheimer's disease. Using histochemistry, in vivo Ca^{2+} imaging, and behavioral analyses in a mouse model of FAD, the authors demonstrated that along with reducing prefibrillary Aβ surrounding plaques, the inhibition of BACE1 activity rescued neuronal hyperactivity, impaired long-range circuit function and memory defects. That all these effects were due to inhibition of Aβ production was strongly indicated by the observation that functional neuronal impairments reappeared after infusion of soluble Aβ (29). In the second study (31), mimicking BACE1 inhibition in adults, the authors generated BACE1 conditional knockout (BACE1 $^{fl/fl}$) mice and bred them with ubiquitin-Cre mice to induce deletion of BACE1 after passing early developmental stages. Strikingly, sequential and increased deletions of BACE1 in an adult FAD mouse model were capable of reversing amyloid deposition and resulted in significant improvement in gliosis and neuritic dystrophy. Moreover, in correlation with amyloid plaque reversal, it also significantly improved synaptic functions, as was determined by long-term potentiation

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and contextual fear conditioning experiments. These studies offer great hope that sustained inhibition of BACE1 activity can constitute a treatment, or at least be beneficial, for AD patients.

The results of clinical trials of BACE1 inhibitors, however, do not support this hope in the case of sporadic AD. All BACE1 inhibitor clinical trials that ended to date, ended in failure. Some trials, such as that of BI 1181181, LY2811376, LY2886721 and RG7129, were terminated because of technical and safety issues. On the other hand, there were no such issues in the trials of the verubecestat (MK-8931). This agent was shown to be very efficient in suppressing Aβ production in preclinical tests and was proven safe in clinical trials. Yet, its Phase III, 2000 patient-strong "EPOCH" trial in mild to moderate AD patients was terminated prematurely in February 2017 for the lack of efficacy, with an interim analysis by an external data-monitoring committee giving the trial "virtually no chance of finding a positive effect". At that time, a separate large Phase III clinical trial of verubecestat in prodromal patients, the "APECS", set to run until 2019, was continued as investigators found no signs of safety issues. In February 2018, however, this trial, too, was terminated prematurely and for the same reason; lack of efficacy. The clinical trials of several other BACE1 inhibitors are still in progress but the verubecestat results do not inspire confidence in their successful outcome.

Why such a dichotomy between highly successful BACE1 inhibition-mediated treatments of the disease in mouse models versus the failure of the same approach, with an efficient and safe agent, in human clinical trials? One possible explanation for such a discrepancy is a physiological difference between mice and humans. Whereas this could be a contributing factor, the main reason may be fundamentally different. To explain the dramatic discordance between the outcomes of animal studies and of human trials, we would like to advance the notion that *two etiologically and mechanistically different, yet symptomatically similar, if not identical, diseases were treated in mouse studies versus human trials*.

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Mouse AD models imitate, by design, FAD. In all such models, multiple copies of human βAPP gene or its fragment containing beta amyloid coding sequences, usually carrying one or more of known AD mutations, are inserted into the genome under the control of a strong promoter. Undisputedly, in FAD, as well as in all of its mouse models and healthy subjects used in BACE1 studies, Aβ is generated solely by the proteolytic cleavage of βAPP by beta- and gamma-secretases. Even if mouse models were constructed by insertion of a large number of wild-type βAPP genes, this would still be the case; such a mouse would not be a model of sporadic AD. *BACE1 inhibitors should be effective in such setting and they are*. On the other hand, all clinical trials of BACE1 inhibitors are, for all practical purposes, those of sporadic AD. This is because FAD cases constitute less than 5% of the AD burden (5, 13, 14). Therefore, assuming that their proportion reflects the natural frequency of familial AD cases, FAD patients would constitute no more than 5% of a cohort and would have little impact on the outcomes of a trial.

One rational explanation for the dichotomy referred to above is that *in the majority of sporadic AD cases, in addition to conventional βAPP/beta secretase-dependent component of Aβ production that operates in FAD (and in healthy subjects), there is another, unconventional, Aβ-generating component in operation*, possibly facilitated or enabled by epigenetic changes associated with the disease (32), *which is both βAPP- and beta secretase-independent*. In these (sporadic) cases, administration of safe and effective BACE1 inhibitors would suppress the βAPP-dependent component, but would have no effects on the second, βAPP- and beta secretase-independent, component. The extent of suppression of total Aβ production by BACE1 inhibitors would depend on the relative input of two components in the generation of Aβ, and if the input of the second significantly exceeds that of the first component, BACE1 inhibitors would be ineffective both in lowering Aβ levels and in the treatment of SAD.

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What could this second component be? Conceivably, several molecular mechanisms might form a basis for such a process; for example shift of transcription start site to a position that generates a short mRNA encoding polypeptides that do not require beta secretase cleavage for generation of Aβ, or an internal initiation of translation of conventional βAPP mRNA that achieves the same result. In fact, a specific mechanism for βAPP- and beta secretase-independent production of C99 and, subsequently, Aβ in sporadic AD was previously proposed (33-35). The central and defined predictions of the concept of βAPP-independent generation of Aβ in SAD but not in FAD are the following: *inhibition of beta secretase activity should be highly effective in suppressing Aβ production in FAD but would have little effect on Aβ generation in sporadic Alzheimer's disease.* These predictions could not be addressed experimentally because animal models of SAD weren't and still aren't available. Therefore, the best and for now the only approach to test it is in large, statistically significant clinical trials of SAD patients. Such trials, with thousands of participants of different symptomatic levels were just carried out and the result is unequivocal: BACE1 inhibitor(s), shown safe and effective in healthy subjects and FAD models, had no effect in SAD. These outcomes constitute an unambiguous confirmation of the predictions for βAPP-independent production of Aβ in SAD.

It should be emphasized that the notion of "second component" of Aβ generation in sporadic AD does not challenge the presumption of amyloid hypothesis that SAD is mainly driven by the increased production of Aβ, just as FAD (or rather its subset associated with increased beta secretase cleavage) is. It posits, however, that the mechanism of the Aβ production in SAD, or, more specifically, its major component, is different from that in FAD and independent from both βAPP and the activity of beta secretase. Conceptually, it introduces two pathways ("components") of Aβ production. One, which

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occurs in all systems –healthy subjects, FAD, and SAD, is the excision of Aβ from βAPP requiring cleavages by beta and gamma secretases; *this pathway can be suppressed by beta secretase inhibition*. Another pathway, that occurs only in SAD, generates, as its *primary* protein product, C99, which is subsequently cleaved by gamma secretase, yielding Aβ; *this pathway is resistant to beta secretase inhibition*. Considering such dynamics of Aβ production, it could be stated that the difference between FAD and healthy subjects is quantitative, i.e. different rates of βAPP cleavage at the beta site and different relative rates of cleavage at gamma sites, whereas the difference between SAD and FAD (and healthy subjects) is qualitative. Conceivably, some cases of SAD can be due to epigenetically mediated changes, for example an increase in beta secretase expression in response to loss of certain microRNA species (36), in the first, βAPP-based, pathway alone. Increase in βAPP gene expression or epigenetic changes resulting in the reduced efficiency of alpha secretase or of amyloid plaques clearance or in increased use of Aβ position 42 as gamma secretase cleavage site may also elevate the production and/or accumulation of Aβ without contribution of the second pathway and could result in SAD. *Such cases should respond to beta secretase inhibition.* However, considering the inefficiency of BACE1 inhibitors in clinical trials, such cases appear to be in the minority.

The notion of the "second component" of Aβ production in SAD but not in FAD suggests a decisive and crucial yet feasible and verifiable prediction, namely *effective and safe BACE1 inhibitors would suppress production of Aβ and provide significant benefits, and possibly constitute a "treatment", in familial AD*. If proven, even if it could not be applied to the AD in general, it could provide a relief to the quarter of a million of current FAD mutation carriers and their decedents in the USA alone. To implement verification of this prediction, verubecestat or any other effective and safe BACE1 inhibitor should be tested with a cohort of FAD patients or children of FAD mutations carriers. *In fact, if trials of BACE1 inhibitors that have been concluded or are being evaluated included FAD patients, and if the*

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data for these patients can be extracted and analyzed separately, the trend could become evident. There are currently two FAD-centered trials in progress, DIAN (dominantly inherited Alzheimer network) NCT01760005, and one with the extended Colombian family of FAD carriers aimed at the prevention of the disease, but in both only the immunotherapy approaches are tested.

By the same logic, *addressing the aspects of AD other than cleavages of the βAPP by beta and alpha secretases should have similar impacts in both FAD and SAD* (activation of alpha secretase could potentially be effective in FAD but not in SAD because the primary product of the second pathway, C99, is not susceptible to alpha secretase cleavage). Thus, immunotherapy targeting Aβ, or modulation of gamma secretase activity toward production of less harmful Aβ species would both be independent of the nature of C99 generation and each approach should be equally effective in FAD and SAD.

The results of the BACE1 inhibitor trials, thus far persistently negative, should not be dismissed; they contain an important message. While they are being interpreted by some as a verdict on the validity of the amyloid cascade hypothesis, such a judgment appears to be premature; the ACH remains the more versatile putative theory when compared with alternative interpretations of Alzheimer's disease. In the framework of the "second component" model for Aβ overproduction in SAD, the lessons from the trials can be formulated as follows: (a)-Familial Alzheimer's disease and sporadic Alzheimer's disease should be considered two distinctly different diseases as far as the mechanisms of beta amyloid generation are concerned. (b)-Human trials of BACE1 inhibitors should be conducted separately, with discrete familial AD and sporadic AD cohorts. (c)-It could be expected that BACE1 inhibitors effective in healthy individuals and in animal studies would also be effective in treatment of FAD. (d)- In sporadic AD, two components of Aβ production may be in operation and a substantive portion of Aβ may be generated in βAPP/beta secretase cleavage-independent, BACE1 inhibition-insensitive manner. Therefore, BACE1

inhibitors would be neither applicable nor effective as a treatment of sporadic AD. (e)-Mechanisms of βAPP-independent generation of Aβ in sporadic AD should be further studied; their elucidation could offer additional therapeutic targets. (f)-Targeting the aspects of Alzheimer's disease other than beta secretase (inhibition) and alpha secretase (activation) cleavages of the beta amyloid precursor protein would have analogous impacts in familial and sporadic AD cases.

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