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The antineoplastic drug flavopiridol reverses memory impairment induced by Amyloid-ß$_{1-42}$ oligomers in mice

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A B S T R A C T

The ectopic re-activation of cell cycle in neurons is an early event in the pathogenesis of Alzheimer's disease (AD), which could lead to synaptic failure and ensuing cognitive deficits before frank neuronal death. Cytostatic drugs that act as cyclin-dependent kinase (CDK) inhibitors have been poorly investigated in animal models of AD. In the present study, we examined the effects of flavopiridol, an inhibitor of CDKs currently used as antineoplastic drug, against cell cycle reactivation and memory loss induced by intracerebroventricular injection of Aß$_{1-42}$ oligomers in CD1 mice. Cycling neurons, scored as NeuN-positive cells expressing cyclin A, were found both in the frontal cortex and in the hippocampus of Aβ-injected mice, paralleling memory deficits. Starting from three days after Aβ injection, flavopiridol (0.5, 1 and 3 mg/kg) was intraperitoneally injected daily, for eleven days. Here we show that a treatment with flavopiridol (0.5 and 1 mg/kg) was able to rescue the loss of memory induced by Aβ$_{1-42}$, and to prevent the occurrence of ectopic cell-cycle events in the mouse frontal cortex and hippocampus. This is the first evidence that a cytostatic drug can prevent cognitive deficits in a non-transgenic animal model of AD.

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by memory loss and cognitive decline leading to dementia. Senile plaques, containing β-amyloid protein (Aβ), neurofibrillary tangles (NFT) of hyperphosphorylated tau protein, and progressive neuronal loss are considered the main hallmarks of the disease [1,2]. According to the "amyloid hypothesis", accumulation of the 42 a.a.-long Aβ protein, which might result from either an excessive production (derived from missense mutations in specific genes linked to familial AD) or a reduced cerebral clearance (as observed in late-onset AD), leads to the aggregation of monomeric Aβ$_{1-42}$ species into higher molecular weight oligomers [3].

Aß oligomers exert a neurotoxic role in AD animal models and patients and they can also contribute as a trigger of tau hyperphosphorylation leading to the formation of NFT, synaptic and neuronal loss, which are closely associated with memory deficits in AD patients [2].

Different molecular mechanisms have been proposed to explain the neurotoxic effects of Aβ oligomers, including the amplification of NMDA toxicity [4], the loss of the canonical Wnt pathway, a signalling pathway essential for maintaining neuronal homeo-

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stasis [5], and the activation of cell cycle in differentiated neurons (reviewed by Herrup et al. [6]).

Activation of cell cycle is considered an early event in the pathogenesis of AD [6]. In cultured neurons, synthetic Aß reproduces the neuronal cell cycle re-entry observed in transgenic AD animals, and in the human AD brain [7,8]. Neurons must permanently withdraw from the cell cycle to form synaptic connections [9], and use a large part of the cell cycle apparatus to control synaptic plasticity by decoupling the expression of cell cycle proteins from DNA replication (reviewed by Ueberham and Arendt [10]). Consequently, cell cycle activation in AD neurons might lead to synaptic failure and cognitive deficits that precede neuronal death [9].

Cytostatic drugs that act as cyclin-dependent kinase (CDK) inhibitors, including flavopiridol, are protective in cultured cortical neurons challenged with synthetic Aβ [7], and have been studied as neuroprotective agents in animal models of both spinal cord injury [11] and stroke [12,13]. In the present study, we demonstrate that flavopiridol, an inhibitor of CDKs [14], can rescue the memory deficits induced by the intracerebroventricular injection of $Aß_{1-42}$ oligomers in CD1 mice [15].

2. Materials & methods

2.1. Animals

Seven-week old male CD1 mice were obtained from Harlan laboratories (Italy). CD1 mice were individually housed, with free access to chow and water, in an air-conditioned room, with a 12-h light–dark cycle. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Preparation of Aß oligomers

Synthetic human $Aβ_{1-42}$ oligomers were prepared according to the original protocol of Klein's group [16]. Briefly, the $Aβ_{1-42}$ lyophilized peptide, purchased from Novus Biologicals (Littleton, USA), was dissolved in TFA (1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then, TFA was evaporated under a gentle stream of argon, and 1 ml HFIP was added to the peptide. After 1 h incubation at 37 °C, the peptide solution was dried under a stream of argon, and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying in a lyophilizer for 1 h, and Aβ(1–42) then resuspended in 5 mM anhydrous dimethyl sulfoxide (DMSO) before dilution to 100 μM in ice-cold cell culture medium DMEM-F12. Samples of Aβ(1–42) at the concentration of 100 μM were incubated for 72 h at 4 °C and then stored at −20° until use. $Aβ_{1-42}$ oligomers were used in neuronal cultures at a final concentration of 1 μM (see Supplementary data). For *in vivo* experiments, $Aβ_{1-42}$ oligomers were diluted from the stock in DMEM solution (100 μM) in sterile 0.1 M PBS (pH 7.4) at a final concentration of 10 μM and then injected i.c.v.

2.3. Drugs and treatment

Flavopiridol was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in DMSO at the initial concentration of 10 mM. The final concentration of DMSO applied to the cultures was 0.1%, whereas it was 1% for *in vivo* studies conducted in CD1 mice.

To obtain a non-Tg model of AD, $Aβ_{1-42}$ oligomers were administered directly into the brain via i.c.v. injections. Synthetic human $Aβ_{1-42}$ oligomers were diluted in sterile 0.1 M PBS (pH 7.4) at a final concentration of 10 μM. Sterile 0.1 M PBS was injected i.c.v. into control animals (vehicle). $Aβ_{1-42}$ oligomers were administered i.c.v. (2 μl) using a microsyringe with a 28-gauge stainless-steel needle 3.0-mm-long (Hamilton). I.c.v. injection was used because of its simplicity with respect to stereotaxis in mice and to ensure diffusion of $Aβ_{1-42}$ in the whole brain [17]. Two microliters of a 10 μM solution were injected, that is 20 pmol of Aβ monomer equivalent. We therefore injected 0.09 μg of Aβ in the adult mouse brain weighing around 500 mg. Assuming that soluble Aß oligomers are freely diffusing in CSF and then in the brain, their final concentration would be 0.18 μg/g. Before performing Aß injection, mice were randomly allocated to the five experimental groups ($n = 10$ per group): vehicle, Aβ, Aβ + Flavo 0.5 mg/kg, Aβ + Flavo 1 mg/kg, Aβ + Flavo 3 (mg/kg). Three days after Aß injection, flavopiridol was administered intraperitoneally (i.p.) for 11 days.

2.4. Passive-avoidance test

Passive-avoidance test was performed as previously described [18]. The apparatus for the step-through passive-avoidance test was an automated shuttle-box divided into an illuminated compartment and a dark compartment of the same size by a wall with a guillotine door. In the experimental session, each mouse was trained to adapt to the step-through passive-avoidance apparatus. In the adaptation trial, the animal was placed into the illuminated compartment. After 10 s, the door between these two boxes was opened and the mouse was allowed to move into the dark compartment freely. The learning trial was similar to the adaptation trial except that the door was closed automatically as soon as the mouse stepped into the dark compartment and an inescapable foot-shock (0.2 mA, 2 s) was delivered through the grid floor. The retention of the passive-avoidance response was measured 1 and 7 days after the learning trial. The latency to re-enter the dark compartment was recorded. No foot-shock was delivered during the retention test. The first and second memory retention test were carried out 15 and 21 days after i.c.v. administration of $Aβ_{1-42}$. Flavopiridol was administered i.p. for 11 days, starting on day 3 after $Aβ_{1-42}$ injection. On day 14, one hour after the drug treatment, mice were submitted to the learning trial of the passive avoidance task.

2.5. Object recognition test

The object recognition test was performed as previously described [19]. Three days (from day 11 to day 13) before training (day 14), mice were habituated to the new context (a plastic Box 50 cm long, 35 cm wide, and 15 cm high) for 10 min/day. Two different objects (plastic boxes) were placed in the arena to let mice familiarize with exploration. On day 14, mice, previously treated for 11 days with i.p. injections of flavopiridol (0.5 mg/Kg or 1 mg/Kg) or vehicle, forty-five minutes after the last injection of flavopiridol, underwent the first trial (T1) of ORT consisting in exploring two identical objects (glass bakers upside-down) placed in the central part of the box, equally distant from the perimeter. T1 lasted 10 min, a time sufficient to learn the task. The second trial (T2) was performed 24 h later (day 15) to test memory retention. Mice were presented with two objects, respectively a "familiar" (i.e. the one used for T1) and a "novel" object (a ceramic cup). The latter object was placed on the left or the right side of the box in a randomly but balanced manner, to minimize potential biases due to a preference for particular locations. To avoid olfactory cues, the objects and the apparatus were cleaned with 70% ethanol after each trial. Exploration, defined as the mouse pointing its nose toward the object from a distance not >2 cm (as marked by a reference circle), was manually evaluated by an investigator blind with respect to treatment. In particular, the following parameters were studied: (i) discrimination (D) index calculated as "exploration of novel object minus exploration of familiar object/total exploration time";

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