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REVIEW

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Clioquinol: Review of its Mechanisms of Action and Clinical Uses in Neurodegenerative Disorders

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SUMMARY

Clioquinol was produced as a topical antiseptic and marketed as an oral intestinal amebicide in 1934, being used to treat a wide range of intestinal diseases. In the early 1970s, it was withdrawn from the market as an oral agent because of its association with subacute myelo-optic neuropathy (SMON), a syndrome that involves sensory and motor disturbances in the lower limbs and visual changes. The first methods for determining plasma and tissue clioquinol (5-chloro-7-iodo-8-quinolinol) levels were set up in the 1970s and involved HPLC separation with UV detection, these were followed by a more sensitive GC method with electron capture detection and a gaschromatographic-massspectrometric (GC-MS) method. Finally, an HPLC method using electrochemical detection has proved to be as highly sensitive and specific as the GC-MS. In rats, mice, rabbits, and hamsters, clioquinol is rapidily absorbed and undergoes first-pass metabolization to glucuronate and sulfate conjugates; the concentrations of the metabolites are higher than those of free clioquinol. Bioavailabilty versus intraperitoneal dosingis about 12%. Dogs and monkeys form fewer conjugates. In man, single-dose concentrations are dose related, and the drug's half-life is 11–14 h. There is no accumulation, and the drug is much less metabolized to conjugates. Clioquinol acts as a zinc and copper chelator. Metal chelation is a potential therapeutic strategy for Alzheimer's disease (AD) because zinc and copper are involved in the deposition and stabilization of amyloid plaques, and chelating agents can dissolve amyloid deposits *in vitro* and *in vivo*. In general, the ability of clioquinol to chelate and redistribute metals plays an important role in diseases characterised by Zn, Cu, Fe dyshomeostasis, such as AD and Parkinson's disease, as it reduces oxidation and the amyloid burden. Zinc chelators may also act as anticancer agents. Animal toxicity studies have revealed species-specific differences in neurotoxic responses that are related to the serum levels of clioquinol and metabolites. This is also true in humans, who form fewer conjugates. The results of studies of Alzheimer patients are conflicting and need further confirmation. The potential therapeutic role of the two main effects of MPACs (the regulation of the distribution of metals and antioxidants) has not yet been fully explored.

Introduction

Clioquinol was produced as a topical antiseptic and marketed as an oral intestinal amebicide in 1934, being used to treat a wide range of intestinal diseases including lambliasis, shigellosis, chronic nonspecific diarrhea, and traveller's diarrhea. In the early 1970s, it was withdrawn from the market as an oral agent because of its association with subacute myelo-optic neuropathy (SMON), a syndrome that involves sensory and motor disturbances of the lower limbs and visual changes. The common clinical picture was one of abdominal pain and/or diarrhea followed by painful dysesthesia within a few days or weeks. These symptoms are due to the symmetrical demyelinization of the lateral posterior funiculi of the spinal cord, optic nerve, and peripheral nerves [1,2]. SMON was very common in Japan, where it reportedly affected about 10,000 people.

The dose (between 300 mg/day and 3.5 g/day) and period of treatment before the onset of symptoms varied widely, as did the incidence of the disease. There is still controversy concerning the association between clioquinol and SMON, which was extremely rare outside Japan [3]. The reason for the neurological side effect among Japanese is unknown, but it has been related to concomitant vitamin B deficiency [4].

The evidence indicating that SMON was caused by clioquinol is circumstantial, and there are still doubts substantiated by extremely valid data [5]. These are further supported by the fact that

SMON was largely unknown outside Japan or before 1955 despite the large international sales of clioquinol, and disappeared before the drug was withdrawn from the market.

Clioquinol is currently available as a topical formulation for the treatment of skin infections, but its ability to act as a zinc and copper chelator [6] has induced many researchers to consider its possible use in the treatment of Alzheimer disease. It was therefore reintroduced by Prana Biotechnology for this indication on the basis of the metals hypothesis of Alzheimer's disease [7] and has since been followed by a new quinoline derivative (PBT2) as a candidate disease-modifying drug [8].

Bioanalytical Methods

The first methods for determining plasma and tissue clioquinol levels were set up in the 1970s by Japanese researchers in order to allow correlations to be made with the drug's toxic effects. Separation was performed by means of high performance liquid chromatography (HPLC) with ultraviolet (UV) detection; however, this method lacked sensitivity and specificity, and thus required complex extraction procedures [9–11].

Even more complex gaschromatographic (GC) methods with electron capture detection after acetylation were developed by two independent groups: Jack and Riess [12], who used it to study the plasma and urine levels of clioquinol in humans, and Chen et al. [13], who determined conjugate metabolites in the biological fluids of various animals. Both methods used solvent extraction with a sensitivity of 50 ng/mL.

A subsequent highly sensitive gaschromatographic-massspectrometric (GC-MS) method was developed using benzene extraction and the conversion of clioquinol into pentafluorobenzyl ether [14].

More recently, a method using a Pd(II) complex was set up to determine clioquinol levels in pharmaceutical preparations but it has never been used in biological samples [15].

Finally, a sensitive and highly specific HPLC method using electrochemical detection that does not require complex extraction procedures has been used to determine plasma and tissue concentrations in hamsters [16]; as it is as sensitive and specific as the GC-MS method, it can also be easily used to monitor plasma levels in humans.

Pharmacokinetics and Metabolism

Animal Studies

Pharmacokinetics (PK) and metabolism have been studied in rodents (mice, rats, rabbits, and hamsters) and dogs [17–20]. In rats, clioquinol is rapidily absorbed and undergoes first-pass metabolism to glucuronate and sulfate conjugates: the metabolites therefore reach higher concentrations than those of free clioquinol. Similar results have also been obtained in mice and rabbits, whereas monkeys, dogs, and man form much lower concentrations of metabolites and free clioquinol concentrations are higher than those of the metabolites [17].

In hamsters, clioquinol is rapidly absorbed after a single intraperitoneal (i.p.) injection and after oral (p.o.) dosing: peak plasma concentrations are reached after 30 min, and the drug's apparent elimination half-life is about 2 h [20]. There is no accumulation in peripheral tissue or brain as its concentrations after repeated doses are similar to those observed after a single dose. Clioquinol crosses the blood–brain barrier, although to a lesser extent than that suggested by its relatively high liposolubility: it has been found in brain tissue and cerebrospinal fluid (CSF), but the brain:plasma ratio was only 20%, and 7% of this was found in the CSF [20].

The amount of clioquinol metabolized to conjugated compounds is much lower in hamsters (30% glucuronate, and there hardly any sulfate metabolites) than in rats which, in comparison with the free compound, have fourfold higher concentrations of glucuronate metabolites and 10-fold higher concentrations of sulfate metabolites [18–20].

Plasma clioquinol concentrations are much lower after p.o. than after i.p. administration, and the relative bioavailabilty is about 12% [20]: this low level of absorption was obtained using an oral suspension in carboxymethylcellulose and justifies the use of a surfactant [17–18] as in the case of the pharmaceutical formulation.

Percutaneous absorption has been studied in dogs and leads to a bioavailability of more than 50%, and the application of 3% cream (in a total of 5 g) for 28 days reduces body weight, and causes liver necrosis and severe neurological symptoms [21]. As a consequence, the Committee on Drugs of the American Academy of Pediatrics has recommended that topical products containing clioquinol (and diiodohydroxyquinoline) should not be used [22].

Human Studies

There are few PK studies in humans. Jack and Riess [12] used a sensitive GC-electron capture method to study the PK of single doses (250–1500 mg of clioquinol powder) and found that plasma concentrations were dose related and the drug's half-life ranged from 11 to 14 h.

Clioquinol is much less metabolized to form conjugates in humans than in rodents [13,14]. Dose/concentration ratios in hamster are similar to those found in other rodents, but much lower than those found in humans: at doses of 250 or 500 mg the dose/concentration ratio in humans is 0.64–1.4 [12,20], which means that humans have a mean of 33 times the concentrations of free clioquinol found in hamsters.

Clioquinol in man has an half-life between 10 and 14 h [12]. Repeated administrations (500 mg three times a day) generate an accumulation that reaches steady-state in about 5 days with an inter-subject variability in the range of one order of magnitude. Less than 1% of clioquinol is detectable in urine as about 95% is present as glucuronate and 3% as sulfate conjugates. It has been found experimentally that bile salts improve bioavailabilty by acting as suspending agents and increasing intestinal reabsorption [19].

Pharmacological Effects

Clioquinol is used in what is often claimed to be the life-saving treatment of acrodermatitis enteropathica because of its ability to

improve the plasma levels and intestinal absorption of zinc (Zn) by chelation. However, the chelation is not confined to Zn but may also include iron (Fe) and copper (Cu) [23]. There have been reports of greenish urine [24], feces, and tongue [25] after clioquinol administration due to iron chelate excretion (urine, feces) and deposits (tongue) [5].

As it is a mitochondrial toxin, clioquinol/Zn chelate has been considered to be the ultimate cause of SMON [26]. Metal chelation makes clioquinol more liposoluble and consequently more bioavailable. Furthermore, the chelate comes into contact with the intestinal tract before other tissues such as the CNS, and it has been reported that the abdominal symptoms of SMON precede its neurological symptoms. The intestine is where Zn, Fe, Cu, and many other chelatable components are available for chelation, and the relationships between food/intestinal flora/food contaminants/viruses/bile salts (which make clioquinol more biovailable) create a complex determining the activity (benefit > risk) or toxicity (risk > benefit) of clioquinol. The content of Zn in cereals, pulses, vegetables, meat, and fish varies 3–50 times and, as it is 3–50 times greater than that of Cu, Zn chelation predominates over Cu chelation. Furthermore, foods such as the octopus contain five times more Zn than cauliflower, and the average Zn content in the most common meat eaten by humans is as high as 3.5 mg per 100 g); the presence of clioquinol in the intestine could increase the amount of Zn to toxic levels.

Some authors have recently shown that clioquinol potently inhibits the 20S proteasome by acting through Cu-dependent and Cu-independent mechanisms [27], and causes cell death because of the consequent intracellular accumulation of misfolded protein. Cu binding is also associated with the reverse aggregation of beta-amyloid, which explains clioquinol's potential role in the treatment for malignancies and AD. Clioquinol's Cu (and Zn) binding in the synapses is considered beneficial because it may reduce beta-amyloid aggregation, although the real point is that clioquinol has to cross the BBB in order to bind these transition metals and modify amyloid deposition.

Clioquinol is considered to be a metal protein attenuating compound (MPAC) that acts as a zinc (Zn), copper (Cu), and iron (Fe) chelator [6]. Metal chelation is a potential therapeutic strategy for Alzheimer's disease (AD) because all three metals are involved in the deposition and stabilization of amyloid plaques, and chelating agents can dissolve amyloid deposits by preventing metal-Aß interactions [28,29]. Aß protein has copper and zinc binding sites and these ions are enriched in amyloid plaques in transgenic mice model of AD and in patients [30–33]. It is thought that the recently described clioquinol-induced dissolution of Alzheimer-like plaques in experimental models is related to its chelating properties [34]. In transgenic mice with Alzheimer symptoms and high Aß peptide levels, 9 weeks' oral clioquinol treatment has led to a 49% decrease in brain Aß deposition and an improvement of symptoms [34].

The binding of clioquinol with Cu, Zn, and Fe could lead to different effects. It is well known that Cu homeostasis is altered in the brains of AD patients, who have high extracellular and low intracellular Cu levels. Clioquinol seems to upregulate matrix metalloproteases (MMP2 and MMP3), bringing the Cu that activates epidermal growth factor receptor (EGFR) to the cell membrane, opening the cascade of mitogen-activated protein kinase (MAPK) pathways, and leading to the enhanced degradation of secreted Aß [35].

Aß can bind Cu^{2+} with high affinity and becomes an Aß radical ($A\beta^{\bullet}$) that can oxidize lipids and proteins; this reaction also generates Cu^{+} which, together with molecular oxygen (O_2), becomes superoxide ($O_2^{\bullet}$) and can be immediately transformed into H_2O_2 [8,36] by oxidizing the closest substrate (e.g. dopamine, cholesterol, fatty acids).

Clioquinol increases the cell influx of Cu and seems to regulates its transfer back from cerebral plaques into surrounding cells. Consequently, its effect is related to the redistribution of Cu [37], and may be positive or negative depending on the availability of intracellular Cu. The amount of Cu in the brain decreases during aging, and related enzymatic activities such as cytochrome clioquinol oxidase and superoxidedismutase (SOD) may be restored by an increase in Cu availability [8].

Zn chelating activity seems to be more directly involved in heme production. Zn is necessary for the heme synthesis, which is known to be increased in the brain of AD patients [38] and is considered to be one of the more important causes of oxidative stress, whereas clioquinol binds Zn, thus reducing heme synthesis and the related oxidative stress. Furthermore, heme precursors such as succinyl-CoA are more available for Krebs' cycle and are likely to increase the production of adenosine triphosphate (ATP) [38], which is very helpful because of the shortage of energy in AD brain.

Together with glutamate, large amounts of Zn (200–300 μM) are released in the glutamatergic synaptic cleft [39] and may compete for binding with Cu, about 15 μM of which is released postsynaptically after NMDA-induced activation [8]. These physiological concentrations of both Zn and Cu are sufficient for nonfibrillar and amorphous Aß aggregation, but not β-sheets.

At a pH of 7.4, the binding of the two ions is equivalent whereas, at a pH of 6.6 (nearer to the medium acidic condition of AD brain: 6.8–70), Cu displaces Zn from Aß [8]. However, one of the most important buffers in the body, inorganic phosphate, is capable of modulating pH in the synapses and can also bind ions as it is a natural chelating agent of Zn [40] and also Fe and Cu (data on file). In this way, phosphates may be the arbiter of the preferential binding of Aß to Zn, Cu, and Fe.

The prevalence of Zn binding would increase the precipitation of Aß into nonfibrillar aggregates [41] without causing an oxidative cascade, whereas the prevalence of Cu would generate nonfibrillar aggregates and cause oxidative damage. For these reasons, despite the fact that both Cu and Zn precipitate Aß, it is thought that Zn has an overall protective effect on the brain [41]. However, nonfibrillar Aß can be neurotoxic regardless of whether it is precipitated by Cu or Zn, and the reduction in Cu and Zn production induced by MPACs is one of the goals of the treatment of AD [8].

Clioquinol can also bind Fe, and the characteristic "green hairy tongue" of subjects with clioquinol intoxication has been found to be a chelate of clioquinol with Fe^{3+} [42]. Its Fe chelating properties may initially be directed toward oxidation as Fe is essential for the Fenton reaction that transforms hydroperoxides into the hydroxyl radical $OH^{\bullet}$. Furthermore, the very high concentrations of

Fe in the brain of AD patients can bind Aß to generate an oxidative reaction that finishes with H_2O_2 generation [8], and finally aggregate Aß into nonamorphous fibrillar amyloid [43]. Consequently, iron chelation may be one of the key activities of clioquinol. Some authors consider Fe in concomitance with aluminium (Al) to be the only cause of the precipitation of Aß-sheets in senile plaque cores [44] and that the presence of Cu and Zn in the same plaques is merely adventitious as their binding may only contribute to the cohesiveness of the core material.

In general, ability of clioquinol to chelate and redistribute metals may play an important role in diseases such as AD and Parkinson's disease that are characterised by Zn, Cu, and Fe dyshomeostasis.

More mechanistically, the chelating properties of clioquinol are related to an indirect antioxidant effect that depends on pH and the relative concentration of the metals *in situ*.

In this perspective, it is not surprising that clioquinol may act as a pro-oxidant under some experimental conditions, such as in murine cortical cultures [45].

However, metal chelation is not the only effect of clioquinol because no excess of Cu or Zn has been detected in urine after treatment [7], and its activity seems to be definitely more directed toward redistributing the ions in the body.

Similiraties between prion protein (PrP) and Aß indicate that similar therapeutic strategies might applicable for the treatment of Alzheimer's disease and prion diseases [46].

It has been found in two *in vitro* experimental models of transmissible spongiform encephalopathy (TSE) that 12 days' treatment with clioquinol reduces PrP^{res} formation in cultured cells, and that the contact of clioquinol with partially purified PrP reduces PrP^{res} levels in a dose-dependent manner [47]. These findings suggest that the drug affects metal–protein interactions and PrP conversion processes; furthermore, by reducing Cu and Zn ions, it also acts as an antioxidant.

The antioxidant effect may explain the improvement in memory observed in clioquinol-treated hamsters intracerebrally infected with prions as the levels of these oxidative markers were reduced at the time the memory improved [48]. Although prion amyloid, and copper and zinc ions are also involved in the prion protein misfolding and aggregation that characterises the neuropathogenesis of TSEs [49], prion amyloid was not reduced at difference from intraperitoneally infected animals in which clioquinol was able to delay the onset of prion disease [50].

As amyotrophic lateral sclerosis (ALS) is considered a protein misfolding disease, and insoluble forms of superoxide dismutase accumulate in neural tissues [51,52], clioquinol has been tested in animal models at the ALS Therapeutic Development Institute (TDI) with some conflicting results (Rob Goldstein, personal communication).

More promising results have been obtained in an *in vitro* study of huntingtin-expressing cells, which expressed less mutant protein and showed a decreased death rate, and a transgenic mouse model of Huntington's disease, in which clioquinol improved the behavioral and pathological phenotypes [53]. These effects may be due to the downregulation of mutant huntingtin expression. Furthermore, since Huntington's disease, is characterized by oxidative stress, the binding of clioquinol to transition metals reduces the production of reactive oxygen species.

Clioquinol has been found to have anticancer activity *in vitro* and *in vivo*, however positive conclusions cannot be made because different zinc chelator/ionophore have no identical activity [54,55].

Toxicity

A number of toxicological studies have shown that clioquinol causes neurotoxicity, but only high doses give rise to neurological symptoms and spinal cord abnormalities in animals [27]. This is thought to be related to the conjugation's capacity to form easily excreted glucuronate or sulfate metabolites [17]. The human dose required to reach the levels that considered to be neurotoxic for animals (dogs, monkeys) is at least 10 times lower.

Toxicity studies have been carried out in various animals since it was discovered that clioquinol was associated with SMON syndrome. There are species-specific differences in neurotoxic responses to repeated doses of clioquinol: 60–150 mg/kg/day in mongrel dogs, 350–450 mg/kg/day in beagles, and 200–700 mg/kg/day in monkeys. These differences are related to serum clioquinol levels and therefore to its metabolic fate: toxic effects appear at a mean level of 14 μg/mL in dogs and 5 μg/mL in monkeys, both of which are lower than those at which toxic effects appear in rats [17–19]. In hamsters, toxic plasma and tissue clioquinol concentrations are also lower than those observed in rats, which show some signs of toxicity after the chronic administration of 200 mg/kg/day when the concentrations of free clioquinol are higher than 17 μg/mL [20], and 100 mg/kg/day in dogs at free clioquinol concentrations of about 14 μg/mL [17].

The differences in metabolism can also explain the relatively lower toxic doses (20–30 mg/kg/day) in the patients who developed the SMON syndrome. As the symptoms of SMON are similar to those of vitamin B_{12} deficiency, studies have been carried out in order to determine whether clioquinol alters the metabolism of the vitamin by chelating (57CO)-cyanocobalamin [56]: these led to the conclusion that clioquinol reduces the absorption and accumulation of the vitamin, and so a supplement should be given to patients receiving chronic doses of clioquinol.

Alternatively, the neurological side effects may have been related to the different formulation used in Japan or genetic susceptibilty to this effect among Japanese. Furthermore, neuropathy is a known side effect of the proteasome inhibitor bortezomib [28].

In addition to the SMON syndrome, other reported side effects of clioquinol include abdominal pain and green discoloration of the tongue, which may be related to its iron chelating effect and altered iron distribution in the tongue [25].

Clinical Studies

An open study of various doses of clioquinol found an improvement in the cognitive test performance of AD patients [57], who also showed a decrease in CSF Tau protein levels, a marker that is usually increased in AD patients.

A double-blind, placebo-controlled study of clioquinol in a population of patients with mild-to-moderate and severe AD who received up to 375 mg twice a day for 36 weeks found that plasma

$A\beta_{42}$ levels decreased after 24 weeks, particularly in the more severely affected patients. These patients also showed an attenuation in disease progression on the basis of cognitive tests [58].

On the other hand, a recent and detailed review of the clinical studies by Sampson et al. [59] found that clioquinol did not have any significant effect on cognition (as measured by the ADAS-Cog scale) may be because Phase II trial were underpowered to detect an effect on cognition.

Conclusions and Future Perspectives

The potential therapeutic role of the two main effects of MPACs (the regulation of the distribution of metals and antioxidants) has not yet been fully explored.

Analysis of the data suggests that the activity of clioquinol is related to its metal binding capacity as a chelating agent or ionophore. Its risk/benefit ratio depends on the amount of Cu, Zn, or Fe made more available or inactivated by clioquinol: more specifically, an imbalance of Cu and Zn in the synapses determines beta-amyloid precipitation or toxic Zn reuptake. This means that much depends on the relative presence of the individual metal, and so it is very difficult to predict its final clinical activity.

New compounds with a better toxicological profile than that of clioquinol (such as the new derivative PBT-2) will certainly stimulate their use. In fact there is the need to urgently find therapies for Alzheimer's disease for which no disease modifying agents are available.

Conflict of Interest

The authors have no conflict of interest.

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