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Clioquinol: To harm or heal

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ABSTRACT

Clioquinol, one of the first mass-produced drugs, was considered safe and efficacious for many years. It was used as an antifungal and an antiprotozoal drug until it was linked to an outbreak of subacute myelo-optic neuropathy (SMON), a debilitating disease almost exclusively confined to Japan. Today, new information regarding clioquinol targets and its mechanism of action, as well as genetic variation (SNPs) in efflux transporters in the Japanese population, provide a unique interpretation of the existing phenomena. Further understanding of clioquinol's role in the inhibition of cAMP efflux and promoting apoptosis might offer promise for the treatment of cancer and/or neurodegenerative diseases. Here, we highlight recent developments in the field and discuss possible connections, hypotheses and perspectives in clioquinol-related research.

Contents

1. Introduction

Clioquinol (5-Chloro-7-iodo-quinolin-8-ol) (Fig. 1) was first developed in 1899 as a topical antiseptic. It was subsequently repurposed to treat traveler's diarrhea, as well as certain fungal and protozoal infections of the gastrointestinal tract (Cahoon, 2009). For many years, it was considered safe, and in the 1930s clioquinol became available over-the-counter in Europe, the US and Asia.

2. Subacute myelo-optic neuropathy: clioquinol's fall from grace

While versatile and used worldwide, clioquinol became associated with a mysterious illness primarily confined to Japan. Between 1958

Abbreviations: ABC, ATP-binding cassette; AD, Alzheimer's disease; ATP, adenosine triphosphate; cAMP, 3′,5′-cyclic adenosine monophosphate; Cdk2, cyclin-dependent kinase 2; cGMP, cyclic guanosine monophosphate; CRE, cAMP response elements; CREB, cyclic adenosine monophosphate response element-binding protein; ECM, extracellular matrix; ER, endoplasmic reticulum; 5-FU, 5-fluorouracil; GPCR, G protein-coupled receptor; ICE, inhibitors of cAMP efflux; LC3-II, microtubule-associated protein 1 light chain 3; LTP, long-term potentiation; MDR1, multidrug resistance protein 1; MMSE, mini-mental state examination; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein 1; PBT2, (5,7-dichloro-2-((dimethylamino)methyl) 8-quinolinol; PKA, protein kinase A; cAMP, dependent protein kinase; ROS, reactive oxygen species; sAC, soluble adenylyl cyclase; SNP, single nucleotide polymorphism; SMON, subacute myelo-optic neuropathy; WT, wild type..

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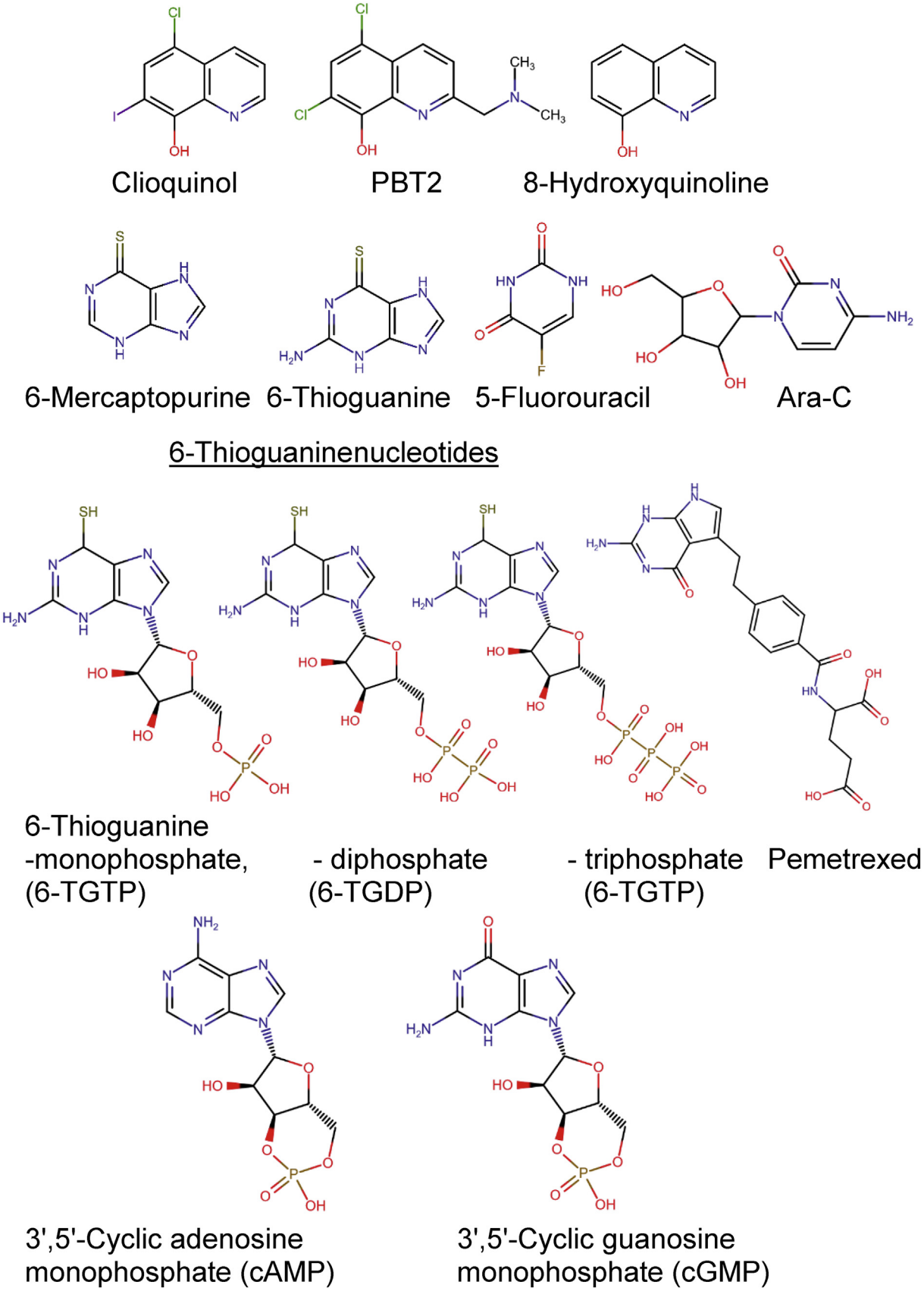

Fig. 1. Chemical structures of substrates of ABCC4, ABCC5 and ABCC11, and other related compounds. Clioquinol (5-choloro-7-iodo-8-quinolinol), PBT2 (5,7-dichloro-2-((dimethylamino) methyl) 8-quinolinol) and 8-hydroxyquinoline are ionophore/chelator drugs. 6-Mercaptopurine, 6-thioguanine, 5-fluorouracil, Ara-C (cytosine arabinoside), pemetrexed and 6-thioguanine nucleotides are cancer and immunosuppressor drugs and substrates of ABCC4 and ABCC11 transporters. cAMP and cGMP are natural ABCC4, ABCC5 and ABCC11 substrates.

and 1970, Japanese physicians observed a number of cases that, at first, were characterized by mild abdominal pain and/or diarrhea (Kono, 1971). These symptoms were often followed by a myelitis-like illness that exhibited an array of neuropathological changes consisting of "pseudosystemic degeneration involving the peripheral nerves, post-lateral columns of the spinal cord and retrobulbar optic nerves" (Kono, 1971). The new illness was designated as subacute myelo-optic neuropathy or SMON, with unknown etiology. It affected thousands of people who were blinded, paralyzed or confined to wheelchairs for the rest of their lives (Cahoon, 2009). 10,000 diagnoses were made and nearly 5% of cases were fatal (Meade, 1975). Multiple hypotheses on the etiology of SMON were considered: intoxication by industrial waste or pesticides; a metabolic disorder or vitamin deficiency, and involvement of viral or bacterial infections (Kono, 1971), none of which were confirmed by subsequent studies. SMON patients presented with a green "fur" on their tongues, and green-pigmented urine and feces. Surprisingly, this green pigment was identified as the Fe (III) chelate of clioquinol (Kono, 1971). Moreover, the number of SMON monthly cases closely correlated with the number of clioquinol tablets consumed, and only patients that took clioquinol exhibited symptoms of the disease (Kono, 1971). This necessitated a ban of clioquinol sales in September 1970. As a result, the monthly SMON incidence went from ~ 150 in July to 1 in October (Kono, 1971). However, one mystery that remained was that SMON was primarily endemic to Japan. Internationally, the 1969 sales of clioquinol per capita in 15 studied countries were higher than in Japan, yet there were no reports of SMON (Meade, 1975). Some researchers speculated that familial (genetic) factors could account for the higher incidence of SMON in Japanese clioquinol users (Kono, 1971).

3. Clioquinol makes a comeback against neurodegenerative diseases

One of clioquinol's first-identified mechanisms of action relates to its ability to interact with metals. The idea that the accumulation of the "wrong" metals in the human brain can cause Alzheimer's disease (AD) dates back to the late 1970s (Eichhorn, 1979). In a more recent study, researchers found a significant decrease in serum manganese level in AD subjects vs. healthy individuals but concluded that neither lead nor manganese levels represented biomarkers in the studied cohort (Hare et al., 2016). Another study found that the copper content was increased in AD platelets. The patient stratification using MMSE score (see glossary) revealed a larger copper increase in the group with more significant impairment. They detected a 1700% copper increase in severe cognitive impaired patients and only a 112% increase in the mild-to-moderate cognitively impaired group. As a result, platelet copper was envisioned as a potential diagnostic biomarker for AD (Yevenes Ugarte et al., 2014). Specific roles for rubidium and potassium in AD were also proposed, since both metal ions are decreased across all intracellular compartments in the AD brain (Roberts et al., 2016). Nevertheless, these and other data motivated studies to assess metals as markers in AD pathogenesis.

In an effort to develop a novel therapeutic options for AD, Ashley Bush and collaborators reported results from a drug repurposing screen aimed at identifying efficient metal chelators among existing antibiotics and anti-inflammatory drugs (Helmuth, 2000). In 2001, clioquinol was reported to dissolve β-amyloid deposits in postmortem human tissues (Cherny et al., 2001). In a mouse AD model, clioquinol was also reported to inhibit plaque formation (Helmuth, 2000). Subsequently, Dr. Bush's group introduced the novel concept of metallostasis, a dysfunction of metal trafficking in the brain, where metals are redistributed into inappropriate compartments (Ayton, Lei, & Bush, 2013). This hypothesis, originating from early clioquinol-related findings, is based upon a triad of transition metals: Fe, Cu and Zn (Cuajungco & Faget, 2003; Grasso et al., 2011; Squitti, 2012). Today, several groups are attempting to improve clioquinol-based structures (Fig. 1) (Huntington Study Group Reach, 2015; Liang et al., 2015). Clioquinol and its next generation metal chelator derivative, PBT2 (5,7-dichloro-2-((dimethylamino) methyl) 8-quinolinol; Fig. 1), have shown "cognitive and plasma biomarker effect[s]" for 36 patients in a Phase II clinical trial in AD. However, a subsequent Phase II/III trial was terminated because of a toxic impurity, an unwanted by-product of the drug synthesis (see http://www.alzforum.org/therapeutics/clioquinol). The following trials showed satisfactory safety and tolerability of PBT2 in AD and Huntington's disease patients, with the conclusion that larger and longer future trials will be required to establish any potential therapeutic benefits (Huntington Study Group Reach, 2015; Lannfelt et al., 2008). Hence, future "large-scale phase 3 trials of [metal based therapeutic approaches] are warranted" (Ayton, Lei, & Bush, 2015). More recent reports indicate that clioquinol was also capable of preventing the loss of *substantia nigra* cells in the Parkinsonian human A53T transgenic mouse model, as the accumulation of nigral iron (Fe) in aggregates containing α-synuclein represents an important feature of Parkinson's disease pathogenesis (Billings et al., 2016). The effect of clioquinol upon cognitive and motor function in this mouse model was attributed to its ability to prevent a Fe-synuclein interaction as a moderate-affinity metal chelator (Finkelstein et al., 2016).

Nonetheless, the mechanism of clioquinol's action, at first thought to be related to Cu/Zn or other metal chelating properties, is still debated and considered important (Cahoon, 2009). Alternative theories have suggested that the accumulation of aggregated proteins in neurodegenerative diseases like AD could be attributed to defective clearance of misfolded or aggregated proteins in astrocytes and neurons. Autophagy, a cellular degradation process that targets unnecessary cytosolic proteins to the lysosomal compartment, seemed a plausible candidate for the pathogenesis of AD. In fact, it was found that clioquinol induces autophagy (Park et al., 2011), or that it can reverse arrested autophagy (Seo, Lee, Cho, Yoon, & Koh, 2015). At micromolar concentrations, clioquinol up-regulated phosphatidylethanolamine conjugated to the protein LC3-I, a marker of autophagy. The microtubule-associated protein 1A/1B-light chain 3 (LC3) cytosolic form is referenced as LC3-I, and the phosphatidylethanolamine conjugate as LC3-II (Tanida, Ueno, & Kominami, 2008). This effect appears to be related to the ionophore properties of clioquinol rather than its zinc chelation properties, as an alternative zinc chelator was shown to reduce LC3-II accumulation. Moreover, the addition of zinc increased the accumulation of LC3-II, confirming the role of zinc in the induction of autophagy (Park et al., 2011). While "arrested autophagy" could contribute to AD (Seo et al., 2015), data relating clioquinol and autophagy are limited. Because targeting the autophagic pathway is envisioned as a promising approach in cancer therapy (Levy, Towers, & Thorburn, 2017), it is not surprising that clioquinol is under study in cancer-related fields.

Nonetheless, several reports indicate that autophagy induction by clioquinol is model-dependent. For example, in human hepatoma cells, clioquinol induced neither autophagy nor apoptosis, although it triggered cell cycle arrest in the S-phase. This effect was accompanied by down-modulation of cell cycle proteins, including Cdk2 and cyclins D1 and A2, as well as up-regulation of p21 and p27 (Huang, Wang, Chen, Zhang, & Shi, 2015). As transition metals are unlikely to be unique targets of clioquinol, other potential clioquinol targets were later identified. ABC transporters represent one class of potential targets.

4. Clioquinol may be a rising star against cancer too

Due to multiple reported cellular effects, clioquinol has also been studied for use against cancer, where modulating membrane transport is of interest. Originally, ATP binding cassette (ABC) transporters (see glossary) attracted attention because of their abilities to efflux the nucleoside-like drugs that are used for chemotherapy in cancer or immunosuppression in autoimmune diseases (Fig. 1). The idea that ABC transporters have normal physiological functions, that include active transport of metabolites and signaling molecules, was later accepted (Fletcher, Haber, Henderson, & Norris, 2010). Given their altered metabolism, cancer cells rely upon membrane transport more than normal

cells. Moreover, up-regulated expression of membrane transporters was associated with poor overall survival and a high degree of resistant disease in acute myeloid leukemia (Chigaev, 2015). Another concept links aberrant membrane transport in cancer with evasion of programmed cell death. The efflux of the pro-apoptotic second messenger, 3′,5′-cyclic adenosine monophosphate (cAMP, Fig. 1), is proposed as one such cell survival mechanism (Copsel et al., 2011; Perez, Smagley et al., 2016). Three transporters from the ABCC family, ABCC4, ABCC5 and ABCC11, are reported to efflux cyclic nucleotides. Furthermore, the increased expression of these transport proteins is known to be associated with worse prognosis in leukemia (Copsel et al., 2011; Guo et al., 2009).

To test the hypothesis that blocking cAMP efflux triggers the accumulation of cAMP and stimulates cAMP-dependent downstream signaling, we developed a screen using a fluorescent cAMP analog to identify compounds that block active cAMP efflux (Perez, Simons, Smagley, Sklar, & Chigaev, 2016). Surprisingly, one of the identified drugs was clioquinol. In support of our hypothesis, treatment with clioquinol or other Inhibitors of cAMP Efflux (ICE) resulted in increased phosphorylation of the cAMP-responsive element-binding protein (CREB; Ser133), a classical cAMP downstream effector that activates target genes. Furthermore, it triggered dose-dependent hallmarks of the cAMP-dependent intrinsic apoptotic pathway. While no apparent correlation between ABCC4 (MRP4) expression and cAMP efflux was found, we concluded that alternate ABC transporters capable of cAMP efflux, such as ABCC5 (MRP5) and ABCC11 (MRP8), could contribute to the removal of cAMP from different cells (D. R. Perez et al., 2016). These studies suggest that clioquinol and other ICE can target cAMP efflux in cancer via ABC transporters. A recent report showed the effect of clioquinol on another ABC transporter, ABCB1 (MDR1 or P-gp) (McInerney et al., 2018). A combination of clioquinol with zinc and copper significantly increased the expression of the ABCB1 protein. This was accompanied by a change in the ability of cells to efflux the ABCB1 substrate (McInerney et al., 2018). Thus, there could be an underappreciated connection between ABC transporters, efflux substrates and clioquinol.

5. ATP-binding cassette transporters, potential culprits in clioquinol-associated subacute myelo-optic neuropathy?

Clioquinol's potential involvement with ABC transporters brings us back to SMON. Could transporter inhibition contribute to its neuropathological symptoms? We propose that a natural (genetic) impairment of normal transporter function or capacity, combined with increased transport inhibition by clioquinol, could trigger the etiology of SMON. Toward this end, we focus on the fact that SMON was almost completely confined to the Japanese population and was seldom reported in other countries (Kono, 1971; Meade, 1975). While high doses of clioquinol were sufficient to partially recapitulate human pathology in several animal models, only a few cases of the disease were reported outside of Japan.

The familial aggregation of the SMON cases, previously interpreted in terms of the infectious nature of SMON etiology (Kono, 1971), could reflect a genetic component related to single-nucleotide polymorphisms (SNPs; *see glossary*) in cAMP-transporting ABC pumps. Multiple non-synonymous SNPs that can potentially affect a transporter's function have been identified, and surprisingly, ABCC4 and ABCC11 SNPs are elevated in the Japanese population.

6. Single nucleotide polymorphisms in ABCC4 and ABCC11 in Japanese population dramatically increase sensitivity to nucleotide-like drugs

6.1. ABCC4

The ABCC4 SNP rs3765534 (G2269A, E857K) dramatically reduces transporter function by interfering with protein membrane localization (Krishnamurthy et al., 2008). This SNP is widespread in the Japanese population (> 18% allelic frequency) but is significantly less in all other studied groups: Ashkenazi Jews, Africans north of the Sahara, Pacific Islanders, European Americans, African Americans, Middle Easterners and Chinese Americans (Krishnamurthy et al., 2008). In Japanese patients with inflammatory bowel disease, the allelic frequency of ABCC4 G2269A was 14.7%, with levels of the substrate 6-thioguanine significantly higher in patients with the ABCC4 variant alone than in patients with the WT allelotype. This result, together with experiments in murine models, suggests that the decreased ability to efflux 6-thioguanine nucleotides (Fig. 1) accounts for thiopurine-induced hematopoietic toxicity in patients with the ABCC4 G2269A SNP (Krishnamurthy et al., 2008). In another study, the relationship between ABCC4 G2269A, increased thiopurine sensitivity and leukopenia was also reported (Ban et al., 2010).

Additional SNPs in ABCC4 with a high frequency in the Japanese population are C912A and C559T (compare 0.3 and 0.14 respectively in the Japanese population vs. 0.02 and 0 in the Caucasian population), and these have been shown to affect 6-mercaptopurine (Fig. 1) sensitivity in childhood ALL (Tanaka et al., 2015). The variant G559T correlated with diminished ABCC4 protein expression in human liver samples (Janke et al., 2008) and reduced drug efflux function (Abla et al., 2008). ABCC4 expression in the livers of patients carrying the non-synonymous C912A variant was only ~54% of control expression. Hence, ABCC4 SNPs that negatively affect expression or efflux function of the transporter and thus, increase patient sensitivity to nucleotide-like drugs are enriched in the Japanese population.

6.2. ABCC11

The SNP rs17822931 (G538A; G180R) in ABCC11 was first discovered as a genetic variant that determines earwax type in humans (Yoshiura et al., 2006). WT ABCC11 is associated with brownish, sticky, wet-type earwax and axillary osmidrosis (see glossary) (Nakano, Miwa, Hirano, Yoshiura, & Niikawa, 2009; Toyoda et al., 2009). The G180R is related to the formation of dry-type earwax (Yoshiura et al., 2006). The G180R variant lacks N-linked glycosylation necessary for stable protein expression, and therefore, undergoes rapid proteasomal degradation (Toyoda et al., 2009). As a result, the secretion of earwax is dramatically changed (Fig. 2).

In the Japanese population, the frequency of the G538A allele is very high. Accordingly, it varies from 0.71–0.99 in different prefectures of Japan (Super Science High School, 2009). In populations of European or African origin, it is extremely rare (0.00–0.03). The geographical distribution of this allele is believed to be related to the migration of the Yayoi people, who came to Japan 3000–1800 years ago (Super Science High School, 2009). It worth noting that this distribution is somewhat similar to the distribution of the annual incidence or prevalence rates of SMON, with the highest rates in the southwestern part of Japan, especially in the Kinki or Kansai and Shikoku prefectures (compare maps in references (Super Science High School, 2009) and (Kono, 1971)).

Similar to ABCC4, several studies also indicated the importance of this SNP in nucleotide-like drug sensitivity. Uemura et al., 2010 analyzed the effect of the ABCC11 SNP genotype (G538A, G/G, G/A, and A/A) in a set of 13 adenocarcinoma cell lines. They found that A/A homozygotes were significantly more sensitive to pemetrexed (Fig. 1), as compared to the combined G/G and G/A groups. The authors concluded that SNP (G538A) in the ABCC11 gene represents an important determinant of pemetrexed sensitivity (Uemura et al., 2010).

Thus, an increased sensitivity to nucleotide-like drugs directly related to ABCC protein polymorphisms has been reported in Japanese patients (Ban et al., 2010; Janke et al., 2008). The cyclic nucleotides, cAMP and cGMP, are well known natural substrates of ABCC transporters that exhibit significant structural similarity with anticancer drugs, including pemetrexed, methotrexate, cytosine arabinoside, and 9′-(2′-phosphonyl-methoxyethyl)adenine, as well as 5-fluoro-2′-

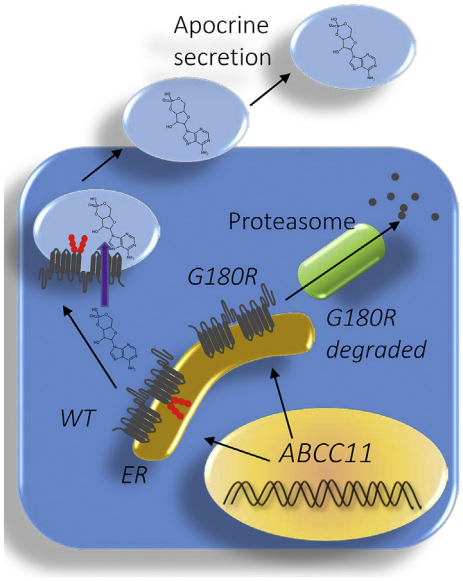

Fig. 2. Schematic illustration of the role of SNP rs17822931 (G538A; G180R) in ABCC11 functional activity (modified from (Toyoda, Gomi, Nakagawa, Nagakura, & Ishikawa, 2016)). The G180R variant lacks N-linked glycosylation (red circles) necessary for stable protein expression, and is subsequently targeted for rapid proteasomal degradation (Toyoda et al., 2009). The precursors of the odorous compounds are not loaded into apocrine vesicles that are released by the cell, and therefore, the loss of transporters results in dry earwax and odorless sweat. Loading apocrine vesicles by the WT protein is shown by the purple arrow.

deoxyuridine 5′-monophosphate, an active metabolite of 5-FU (5-fluorouracil) (Fig. 1) (Toyoda & Ishikawa, 2010). Is it plausible that the decreased function of the ABC transporter in patients carrying SNPs, in a manner similar to cancer drugs, also diminishes cAMP efflux? Would patients with SNPs in ABCC4 and ABCC11 transporters be vulnerable to clioquinol, while patients carrying wild type alleles would be resistant? While testing these hypotheses would require additional studies, if true, clioquinol could be safe for a large fraction of the world's population and could allow broad repurposing. Tests for detecting mutant ABCC4 and ABCC11 alleles have already been developed and validated in large human populations (Super Science High School, 2009), thus allowing for easy identification of good candidates for clioquinol treatment.

7. Possible beneficial effects of single nucleotide polymorphisms in ABCC transporters

It noteworthy that SNPs in cAMP transporters are not entirely harmful. They may provide benefits for other conditions, like cancer. While the physiological function of ABCC11 beyond earwax and axillary glands is unclear, a role in cancer is possible. Caucasians and African–Americans showed approximately four-fold higher rates of breast cancer mortality as compared to women of Japanese and Taiwanese origin. The international mortality and frequency rates for breast cancer were reported to be associated with the frequency of the allele for wet-type earwax (Petrakis, 1971). A direct study of ABCC11 G538A association with breast cancer conducted in Japanese women revealed that the WT (538G) allele frequency in cancer patients was higher than in the control group and moderately associated with the risk of breast cancer (Ota et al., 2010). Since WT ABCC11 has the ability to efflux cyclic nucleotides (cAMP and cGMP), the finding that a functionally "defective" transporter allele has a protective effect against cancer supports the idea that cAMP efflux transporters can contribute to the evasion of apoptosis and that impaired cAMP removal helps to eliminate cancer cells (Perez, Smagley et al., 2016). In Caucasian women, no significant relationship between breast cancer risk and G538A allele has been found (Beesley et al., 2011) (Lang et al., 2011). One explanation for this phenomenon could be that the presence of fully functional ABCC11 paralogs (such as ABCC4, for example) in the Caucasians population compensates for the function of the mutated transporter. Since the Japanese population is enriched in SNPs that can also down modulate ABCC4 function, it is plausible that a specific set of SNPs in ABCC transporters (rather than a single SNP) is required to provide a cancer-protective function.

If down-modulation of transporter function is beneficial for cancer patients, then transporter up-regulation should have the opposite effect. Indeed, a high level of ABCC11 expression in breast tumor tissue was associated with worse disease-free survival (Yamada et al., 2013). Typically, such data are interpreted as though an increased expression of the transporter causes efflux of the chemotherapy drug, providing a survival benefit for malignant cells. However, the authors argued that only a fraction of the patient cohort used in this study (~30.9%) had received 5-fluorouracil (Fig. 1), a known substrate of ABCC11 used for breast cancer therapy. Therefore, they posited that drug "efflux alone cannot explain the association" between transporter overexpression and poor disease-free survival. The authors concluded that "some other function of ABCC11 may contribute to the phenomenon" (Yamada, Ishikawa, Takabe, & Endo, 2013). Thus, the efflux of another substrate, possibly of a nucleotide nature, may provide a plausible explanation. The recent discovery that type 10 soluble adenylyl cyclase (sAC; *see glossary*) produces cAMP as a result of oncogenic stress and functions as a tumor suppressor protein may also provide indirect support for the notion of a specific role of cAMP in cancerogenesis (Ramos-Espiritu et al., 2016).

Furthermore, it is well established that in Japan, the rates of AD are lower than in other developed countries. This difference is usually attributed to differences in diet, low in cholesterol and saturated fat (Grant, 2014). However, since the SNPs in cAMP transporters seem to lower, or at least negatively correlate, with the risk of breast cancer, is it possible that a similar relationship exists in AD?

8. Could clioquinol's effect on cAMP be relevant to clioquinol's action in Alzheimer's disease?

The ability to modify the efficacy of synaptic transmission in response to neural activity is termed synaptic plasticity. It underlies the capacity of neuronal networks to adjust to external stimuli, and to process information. Long-term plasticity, occurring over tens of minutes to years, appears to impact memory and learning. Experimentally studied as long-term potentiation (LTP; see glossary), it may represent the measurable cellular correlate of learning and memory. Consequently, the etiology of AD pathology is shifting from β-amyloid deposits and neuronal death toward synaptic dysfunction as an early cause of the disease. LTP impairment was one of the first quantifiable outcomes supporting this idea (Pozueta, Lefort, & Shelanski, 2013). However, the molecular mechanisms of this process are unclear.

Because the consolidation of LTP requires a series of transitions between engaged and disengaged cell adhesion molecules, like integrins (Fig. 3; *see glossary*), it is possible that the stabilization of one state due to a signaling dysfunction would significantly perturb the outcome of the entire process. As integrin function depends upon the cAMP/PKA/CREB pathway (Chigaev et al., 2015; Chigaev & Sklar, 2012; Chigaev, Smagley, & Sklar, 2014; Chigaev, Waller, Amit, & Sklar, 2008), clioquinol and other ICE compounds shown to up-regulate the cAMP-signaling pathway were also able to deactivate integrins (Perez, Smagley et al., 2016). The observation that anti-integrin antibodies or antagonists that inhibit adhesive interactions were capable of preventing the β-amyloid peptide-induced inhibition of LTP (Wang et al., 2008), suggests that the de-adhesion or integrin deactivation step of the LTP consolidation sequence (step #1, Fig. 3) is likely affected in AD. The fact that pharmacologically-increased intracellular cAMP was also capable of preventing the inhibition of LTP induced by β-amyloid peptides

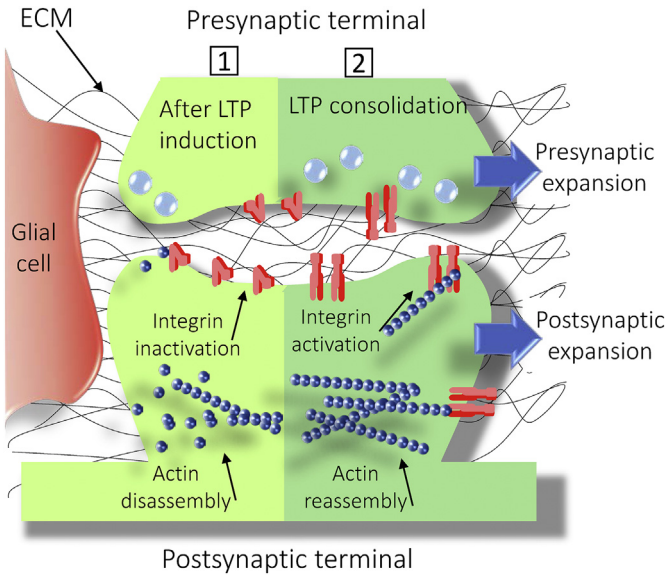

Fig. 3. A model for the role of integrin in consolidation of LTP. Cellular integrins modulate their molecular conformation, rapidly and reversibly changing from a low ligand-binding affinity non-adhesive (bent) state to a high ligand-binding adhesive state (extended) under the control of "inside-out" signaling originating from GPCRs. The addition of integrin antagonists or anti-integrin function-blocking antibodies, as well as their genetic disruption in murine models, results in the reduction of LTP stabilization and a deficiency in hippocampal-dependent working memory (McGeachie, Cingolani, & Goda, 2011). The model includes two major steps: 1) Immediately after induction of LTP, integrins that normally stabilize synaptic structure are inactivated. This results in integrin disengagement as well as disassembly of the postsynaptic actin network and extracellular matrix (ECM). 2) LTP consolidation. Large morphological changes and synaptic reassembly after LTP induction allow for the insertion of new molecules and the expansion of the synapse. After a period of instability, new integrins are inserted and engaged stabilizing the new morphology and function of the synapse via transition from low to high integrin affinity (McGeachie et al., 2011).

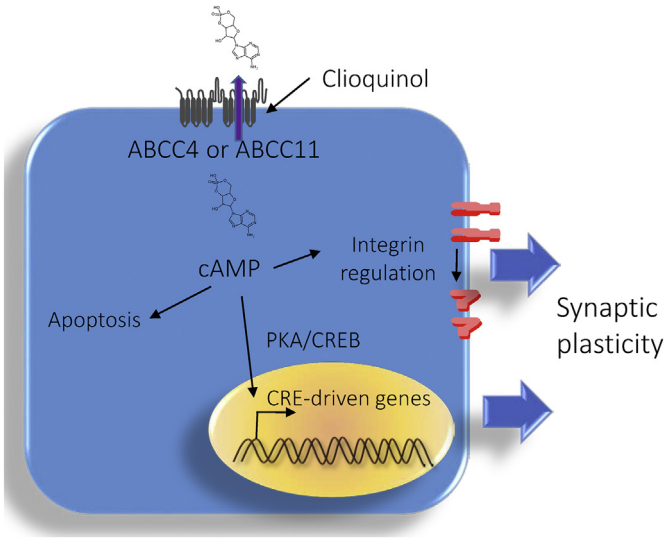

Fig. 4. A model that summarizes hypotheses relating cAMP accumulation with the pathogenesis of SMON, AD and other diseases. Excessive accumulation of cAMP that may result from SNPs in ABCC4 and ABCC11 transporters targeted by clioquinol, can trigger apoptosis that can be devastating. In certain cancers, where cAMP efflux supports malignant cell survival, blocking the efflux represents a novel cancer targeting mechanism. In Japanese and Taiwanese women, SNPs in ABCC transporters that lower transporter activity can have a natural protective role against cancer. Additional accumulation of cAMP resulting from the effects of clioquinol can enhance the expression of CRE-driven genes that may indirectly affect synaptic plasticity. Cytosolic cAMP can directly modulate the integrin activation state and thus regulate synaptic plasticity.

(Wang, Rowan, & Anwyl, 2009) additionally supports this idea. Thus, cAMP regulation of cell adhesion through integrin receptors provides an unexpected link between clioquinol and synaptic plasticity (Fig. 4).

9. The cAMP/PKA/CREB signaling pathway as a possible target in Alzheimer's disease

Discovered in a screen designed to identify compounds that block the efflux of a cAMP fluorescent derivative, clioquinol was reported to up-regulate CREB Ser133 phosphorylation (see Fig. 3 in reference (Perez, Smagley et al., 2016)). It was also shown that mice expressing a constitutively active form of CREB (VP16-CREB) in hippocampal neurons facilitated LTP (Li et al., 2013). The enhanced expression of CRE-driven genes facilitated the priming of synapses leading to the stimulation of synaptic capture (Barco, Alarcon, & Kandel, 2002). Thus, increased cAMP signaling, via administration of caffeine (Zeitlin, Patel, Burgess, Arendash, & Echeverria, 2011), or environmental enrichment (*see glossary*) by means of β2 adrenergic receptor/adenylyl cyclase stimulation, can be beneficial in animal models of AD (Li et al., 2013). Moreover, because impaired CREB phosphorylation represents an essential pathological component of AD, the induction of CREB phosphorylation (Ser133) in response to different classes of drugs/compounds has been proposed as a mechanistic marker for identifying compounds potentially beneficial for treating AD (Scott Bitner, 2012). However, the cause of the cAMP-dependent pathway dysfunction in AD remains unknown.

Earlier models for AD pathology proposed PKA dysfunction, where oxidative stress mediated by the β-amyloid peptide, ROS and mitochondrial dysfunction were envisioned to contribute to AD pathogenesis (Du et al., 2014). Alternative β-amyloid peptide-independent models have also been considered (Chen et al., 2012; Liang, Liu, Grundke-Iqbal, Iqbal, & Gong, 2007; Shi et al., 2011). Nevertheless, the role of cAMP efflux and cAMP-efflux transporters in AD has never been considered despite:

1. cAMP-efflux transporter expression on neurons (Hartz & Bauer, 2011),
2. elevated ABCC4 expression in AD brains (Wijesuriya, Bullock, Faull, Hladky, & Barrand, 2010),
3. increased cerebrospinal fluid cAMP levels in AD patients (Martinez et al., 1999),
4. increased cAMP immunostaining in cerebral cortical and meningeal vessels correlated with β-amyloid immunostaining localized in the "selectively vulnerable targets of neurodegeneration" (Martinez, Hernandez, & Hernanz, 2001). Furthermore, ABC transporters are generally considered good drug targets in neurological diseases (Qosa et al., 2016).

Extracellular deposits of β-amyloid peptides and neurofibrillary tangles (aggregates of hyper-phosphorylated tau protein) are two pathological hallmarks of AD. However, it is difficult to correlate the severity of clinical manifestations of the disease, such as dementia, and these pathological features (Murphy & LeVine III, 2010). We propose that dysregulation of neuronal cAMP signaling, and specifically cAMP efflux, contributes to the pathology of AD. The idea that these protein assemblies can cause synaptic dysfunction and impair LTP has gotten more attention from the scientific community. One of the first breakthrough findings here was the observation that β-amyloid peptides were capable of inhibiting the cAMP/PKA/CREB pathway and LTP. Elevation of the cAMP content in neurons was sufficient to reverse this inhibition (Vitolo et al., 2002). Today, a large body of literature supports the notion that additional stimulation of the cAMP/PKA/CREB pathway leads to subsequent CREB phosphorylation in AD (Fig. 4).

The cAMP-dependent signaling pathway and multiple cAMP-downstream effectors also play significant roles in triggering neuronal apoptosis (Atkins et al., 2007; Kraft et al., 2013; Zhao et al., 2008). Numerous stress factors, including ischemia and reperfusion, traumatic

brain injury, etc., are reported to stimulate cAMP-dependent signaling. Surprisingly, the signaling pathway that activates apoptosis in neurons is very similar to the pathway that induces the death of cancer cells. In neurons, apoptosis induced by reperfusion after simulated ischemia results in increased cAMP accumulation, mitochondrial depolarization, and effector caspase activation (Chagtoo et al., 2018). These steps parallel the apoptosis induced in leukemic cells by clioquinol and other ICE (Perez, Smagley et al., 2016). In both cases, apoptosis was prevented with KH7, a specific inhibitor of the cAMP-producing enzyme sAC. Moreover, the three transporters reported to actively efflux cAMP from cells were detected on the protein level in neurons (ABCC5 and ABCC11), astrocytes and microglia (ABCC4 and ABCC5), and endothelia (ABCC4 and ABCC5) (Hartz & Bauer, 2011), providing a target for clioquinol and other ICE in the CNS. Given the potential relationship of CNS transporters, neuronal function, and clioquinol, is it possible that clioquinol's capacity to inhibit cAMP efflux, rather than its chelator/ionophore activity, provides any of the speculated benefits that were reported in AD? If true, could other ICE compounds provide similar potential benefits? Indeed, two ICE compounds, cryptotanshinone and parthenolide, were claimed to prevent, alleviate or treat AD or AD symptoms ((Mei et al., 2009; Yoo & Park, 2012; Yu et al., 2007; Zhang, Qian, Zhang, & Wang, 2016) and patent applications US 2004/0039050 Al, US 2015/0164858 Al). Thus, it is plausible that ICE may restore activation of downstream cAMP signaling. Hence, a story that started with clioquinol and a mysterious disease confined to Japan, leads to novel questions and hypotheses about AD pathogenesis.

10. Concluding remarks and future perspectives

The public health impact of AD is expected to increase in the next 30–50 years, yet there is no effective preventative strategy or treatment that will dramatically reverse its debilitating effects. The main difficulty is our lack of understanding of the disease pathogenesis. A deeper understanding of the AD pathological process will help us to identify better therapeutic targets and will facilitate the development of new therapeutic strategies and drugs aimed at overcoming the disease progression and burden.

Here, we have briefly reviewed the history of the drug clioquinol and highlighted multiple mechanisms of action that have been attributed to the beneficial effects of the drug, as well as its severe side effects during different historical periods. Further interest in the use of clioquinol was aroused by the finding that it inhibited plaque formation in AD mouse models, possibly by its ionophore properties. A key concept here is the ability of clioquinol to block cAMP efflux from cells and thus, to trigger the phosphorylation of CREB Ser133, a classical cAMP effector that activates target genes. This finding provided a connection to possible targets of clioquinol: ABCC4 and ABCC11, transporters that normally efflux numerous endogenous substrates, including cAMP. A further analysis revealed the presence of SNPs in both ABCC4 and ABCC11, capable of reducing transporter function and at the same time present with a high frequency in the Japanese population. Modern studies showed that these SNPs are critical for patient sensitivity to cancer and immunosuppressor nucleotide-like drugs, substrates of ABCC4 and ABCC11 transporters. Thus, this line of research provides a plausible explanation for the SMON phenomenon: patients that carry SNPs in ABC transporters that dramatically affect nucleotide efflux are expected to be more sensitive to clioquinol. Since these SNPs are geographically restricted to Japan, this also accounts for the specific distribution of the disease. Today, these hypotheses can be experimentally verified, and as a result, might offer a path to the safer use of clioquinol in clinical practice.

Several alternative ideas about clioquinol's mechanism of action were explored by others, however, they did not result in significant breakthroughs. One exciting new concept presented here describes clioquinol's role in blocking cAMP efflux and increasing CREB phosphorylation. Because the dysregulation of the cAMP/PKA/CREB pathway is a hallmark of several diseases, it is plausible that the beneficial effects of clioquinol are due to its influence on this pathway. Moreover, the specific role of cAMP-related signaling in integrin inside-out deactivation provides an unexpected molecular mechanism that could link clioquinol effects with synaptic plasticity. This leads to a number of questions that can all be addressed using modern technologies. For example, it is important to know how blocking cAMP efflux affects LTP consolidation. Very limited data about the efflux of signaling mediators, including cyclic nucleotides, from the tetrapartite synapse elements are available. In addition, it is necessary to understand conformational and ligand-binding affinity changes in synaptic and glial integrins that occur under different conditions, and how changes in the extracellular environment affect the synapse. On a macro scale, studies of the signaling partners of the cAMP/PKA/CREB pathway and their alterations in aging, health and disease are also required. And finally, will it be possible to develop novel therapeutic strategies for AD by targeting active cyclic nucleotide efflux?

Today we still have much to learn about the pathological processes underlying neurodegenerative diseases. As the human population ages, the overall impact of AD is expected to grow. Recent research, surprisingly originating from studies of an old drug, clioquinol, and implicating cAMP-related signaling in the pathogenesis of AD, represent a promising approach that may lead to a better future for generations to come.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Glossary

ATP-binding cassette (ABC) transporter: A superfamily of active transporters that use energy from ATP hydrolysis to pump substrates across cell membranes. Certain ABC pumps participate in drug efflux from cancer cells, thus contributing to drug resistance. Defects in transport function may lead to drug sensitivity and adverse drug reactions. ABCC (subfamily C) members are also known as multidrug resistance proteins (MRPs). ABCC4 (MRP4), ABCC5 (MRP5) and ABCC11 (MRP8) are reported to efflux cyclic nucleotides (cAMP and cGMP).

Axillary osmidrosis: A condition characterized by increased foul odor in the underarm region. Specific SNPs in ABCC11 result in a loss of transporter activity, and therefore dramatically reduce body odor.

Environmental enrichment: Manipulations administered to laboratory animals, including expanded living space, physical exercise and engagement with novel objects that stimulate sensory perception and cognition.

Integrin: Integrins, cell adhesion molecules expressed on the majority of cells, are known to be critical for cell adhesion to endothelium, extravasation, homing and mobilization. They are also vital for the tetrapartite structure of neuronal synapses, where impairing integrin adhesion has been shown to damage synaptic transmission and the induction of LTP. Rapid changes in cell adhesion are controlled by integrin conformational changes rather than by changes in expression or trafficking. Cyclic AMP accumulation has been shown to down-modulate integrin-dependent adhesion.

Long-term potentiation (LTP): A sustained strengthening of neurological synapses resulting from recent synaptic activity. This particular type of synaptic plasticity is believed to underlie short-term memory and learning in mammals. LTP and other types of synaptic plasticity are controlled at least in part by integrins.

Mini-mental state examination (MMSE): A test administered by a health-care professional to evaluate a patient's mental skills (cognitive state). It includes a series of questions, and the score interprets the degree of mental state impairment. MMSE is one of the tests used to assess dementia.

Single nucleotide polymorphism (SNP): A common type of genetic variability wherein a single DNA nucleotide is changed. Two major types of SNPs exist: synonymous and non-synonymous substitutions. Non-synonymous SNPs alter the amino acid encoded, which may or may not result in a change of a protein function.

Soluble adenylyl cyclase (sAC): The protein encoded by the *ADCY10* gene catalyzes the formation of the second messenger cAMP from ATP. Its activity is stimulated by low pH, bicarbonate ion and CO_2. Also, sAC has been implicated as a tumor suppressor protein whose expression is down-modulated in several cancers. Decreased sAC expression promotes cellular transformation in vitro and stimulates cancer progression in vivo.