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# Clozapine-induced agranulocytosis: Evidence for an immune-mediated mechanism from a patient-specific *in-vitro* approach

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ABSTRACT

Use of the atypical antipsychotic clozapine (CZP) is compromised by the risk of potentially fatal agranulocytosis/granulocytopenia (CIAG). To address this, we have established a simple, personalized cell culture-based strategy to identify CIAG-susceptible patients, hypothesizing that an immunogenic and possibly haptene-based mechanism underlies CIAG pathophysiology.

To detect a putative haptene-induced response to CZP *in vitro* exposure, a traditional lymphocyte stimulation assay was adapted and applied to patient-specific peripheral blood-derived mononuclear cells (PBMC). 6 patients with a history of CIAG, 6 patients under CZP treatment (without CIAG) and 12 matched healthy controls were studied.

*In vitro* CZP exposure, even at strikingly low levels, resulted in significantly increased proliferation rates only in CIAG patients' PBMC. Other parameters including cell viability and mitogen-induced proliferation were also affected by *in vitro* CZP exposure, yet there was no significant difference between the groups.

This personalized approach is a starting point for further investigations into a putative haptene-based mechanism underlying CIAG development, and may facilitate the future development of predictive testing.



## 1. Introduction

The atypical antipsychotic clozapine (CZP), introduced in 1960, remains a first-choice in treatment-resistant schizophrenia (Baldessarini and Frankenburg, 1991; Hasan et al., 2012; Nielsen et al., 2013; Dold and Leucht, 2014). Despite its excellent antipsychotic properties and a very low probability of causing debilitating and stigmatizing extrapyramidal side effects, the clinical utility of CZP is compromised by the occurrence of rare, but potentially life threatening side effects. One of the most important side effects is clozapine-induced neutropenia, which may eventually develop into life-threatening clozapine-induced agranulocytosis/granulocytopenia (CIAG) (Drew, 2013). This led to the partial withdrawal of CZP from the markets and strong restrictions to its use in the mid-1970s. Depending on the severity, the incidence of CIAG ranges from 3% for milder cases of granulocytopenia (Nooijen et al., 2011) (between 500 and 1500 granulocytes/nl) and 0.8% for full-blown CIAG (Nooijen et al., 2011), which is defined by <-500 granulocytes/nl. Interestingly, in cases with marked CIAG, the occurrence appears to be independent of the clozapine dosage (Alvir et al., 1993).

Although numerous studies have identified risk factors including higher age (Alvir et al., 1993; Barak et al., 1999) and female gender (Alvir et al., 1993), there are no functional tests or biomarkers available to reliably assess a patient-specific risk for the emergence of CIAG under CZP treatment. One of the main reasons for the lack of predictive testing is the fact that, to date, the molecular underpinnings of CIAG remain largely unknown. Numerous studies have addressed putative interactions between CZP and the immune system at different levels (*in vivo, ex vivo, in vitro*), suggesting immune-mediated mechanisms to be involved in both its side effects, and potentially even its clinical efficacy (Leykin et al., 1997; Hinze-Selch et al., 1998; Basta-Kaim et al., 2006; Hu et al., 2012; Ribeiro et al., 2013; Park et al., 2015). Unfortunately, most studies have either not specifically studied CIAG patients, or have pursued experimental approaches that have failed to detect distinct anomalies in patients with CIAG or other severe side effects. Therefore, current experimental evidence for a molecular mechanism underlying the emergence of CIAG is limited and it remains subject of debate whether an autoimmune-like process is involved in its pathophysiology or if CIAG development is based on primary cytotoxic mechanisms.

Hallmark studies by Uetrecht and colleagues have revealed that oxidative CZP degradation is likely to involve the formation of several

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metabolites, including those with reactive nitrenium ions, which can covalently bind to leukocytes (Liu and Uetrecht, 1995; Uetrecht et al., 1997; Williams et al., 2000). It is believed that such covalent modification of cell surface structures either results in direct toxicity, or leads to the haptenic formation of an antigenic structure that will elicit toxicity indirectly *via* a targeted immune response (Pirmohamed and Park, 1997; Williams et al., 2000; Pereira and Dean, 2006; Goldstein et al., 2014). Although an earlier attempt by Guest and colleagues to experimentally generate and detect antigen-specific T-cells in CIAG was unsuccessful (Guest et al., 1998), numerous well-designed studies have demonstrated a potential association of CIAG with certain HLA-haplotypes (HLA-B38, DR4, DQw3, DQB1), a finding that again supports the hypothesis of an immune-mediated pathophysiology (Lieberman et al., 1990; Athanasiou et al., 2011; Goldstein et al., 2014). In further support of an autoimmune etiology, CZP-treatment is well-known to be associated with an increased incidence of coagulopathies, vasculitis, myocarditis and parotitis. An autoimmune pathophysiology has been suggested to underlie all of these conditions (Hinze-Selch et al., 1998; Kilian et al., 1999; La Grenade et al., 2001; Penaskovic et al., 2005; Voulgari et al., 2015).

Further evidence regarding the underlying molecular mechanisms of CIAG would significantly advance the development of diagnostic and predictive strategies such as biomarkers to better assess the potential risk of CIAG. Given the life-threatening nature of CIAG, a reliable predictor would be of significant benefit. If its etiology is autoimmune-mediated, with clozapine acting as a haptene-inducing agent, we hypothesized that a quantifiable and functional reaction could be assessed using metabolically active, patient-specific immunocompetent cells in an *in vitro* approach. While earlier studies have focused on clozapine's immunomodulatory effects in schizophrenic patients without any signs of CIAG (Rapaport et al., 1991; Hinze-Selch et al., 1998), here we aimed to establish a simple, functional *in vitro* immune assay that screens for putative differences between CZP-treated patients with a normal treatment course and those who recently experienced clinically significant CIAG (defined as being severe enough to discontinue CZP treatment). The primary readout of the assay is a nonradioactive alternative to the well-established lymphocyte proliferation assay (LPA) (Froebel et al., 1999). This is a nonspecific, yet highly sensitive strategy to detect and quantify a potential antigen-specific response in patient-specific peripheral blood-derived mononuclear cells (PBMC). Based upon long-term cultivation of unsorted, naïve PBMCs, adhering and *in vitro* differentiating monocytes may provide the cellular basis for the presentation of haptenic macromolecules possibly formed upon drug exposure. *In vitro* stimulation and priming of antigen-specific lymphocytes represents a long-known approach to detect, quantify and even therapeutically exploit an immune reaction in a patient-specific manner (Delespesse et al., 1975; Jenkins et al., 2013; Wolfraim et al., 2013; Ramadan, 2014). Therefore a simple, functional, personalized cell culture-based approach was designed to discriminate former CIAG- from non-CIAG prone patients. Our experimental approach was hypothesized to detect a proliferative response in unsorted PBMCs from CIAG patients. In analogy to a recently shown *in vitro* proliferative response to beta-lactam antibiotics in PBMC cultures from sensitive patients (Jenkins et al., 2013), we chose to compare cell cultures from 6 patients with schizophrenia currently on CZP medication with 6 patients formerly exposed to CZP with a history of severe CIAG, and 12 healthy controls matched for age and gender. Differential effects of CZP exposure were systematically investigated in these patient-specific PBMC cultures.

## 2. Material and methods

### 2.1. Patients

A total of 24 individuals were recruited. The CIAG group was formed of 6 outpatients with a known history of CIAG occurring for the first time during treatment in our department and meeting DSM-IV and ICD-10 diagnostic criteria for schizophrenia. The disease- and treatment control group was formed of 6 schizophrenic patients currently on CZP treatment for at least six months without any history of CZP-associated side-effects (daily dosage 100–425 mg, mean 275 mg). Additionally, 12 drug-free individuals with no history of schizophrenia made up the healthy control group. All groups were matched for age and gender. Exclusion criteria for all patients were prior diagnosis of a severe internal, chronic inflammatory or other comorbid psychiatric disorder as assessed by endocrinological (thyroid dysfunction), inflammatory and infectious parameters (serum levels of C-reactive protein). All individuals gave written informed consent before participation in the study. All procedures had been approved by the university's local ethics committee and were in accordance with the Helsinki Declaration of 1975 as revised in 1983.

### 2.2. PBMC isolation

20 ml of peripheral venous blood was drawn using heparinized collection tubes (BD Vacutainer). Within the next 4 h, 2.5 parts of whole blood were diluted with 1.5 parts of Dulbecco's phosphate buffered saline (D-PBS) without $Ca^{++}/Mg^{++}$ (Biochrom AG Berlin). The diluted blood was carefully layered on top of 1 part of Histopaque 1077 (Sigma-Aldrich). After centrifugation for 20 min. at 800 $\times g$, cells were harvested from the Histopaque layer, diluted with 3 parts of D-PBS, again centrifuged for 15 min. at 600 $\times g$. The resulting pellet was resuspended in 3 parts of D-PBS and again centrifuged for 15 min. at 300 $\times g$. The clean pellet was finally resuspended in RPMI 1640 Medium (Biochrom AG Berlin), containing 10% FCS (Biochrom), 1% Penicillin/Streptomycin (10,000 U/10,000 μg per ml; Biochrom), and 2-Mercaptoethanol (1.43 mM; Sigma-Aldrich). Cells were counted and their concentration was adjusted as required to yield the required final cellular density of 10,000 cells per well of a flat-bottom 96-well-plate.

### 2.3. Lymphocyte proliferation assay and drug exposure

All substances and cell culture materials were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise stated. The assay followed the well-established ACTG consensus method with minor modifications (Froebel et al., 1999). In brief, the assay measures the ability of antigen-specific lymphocytes to undergo clonal proliferation following recognition of antigenic structures that are added to, or possibly generated within, a PBMC culture. After an incubation period of several days in the presence of antigenic structures, proliferation of antigen-specific T-cells may result in increased cellular density. Importantly, the assay only measures proliferation, and cannot discriminate between different T- or B-cell subsets. To avoid potential hazards and logistic problems with $^{3}H$-labelled thymidine, we chose to use a commercially available single-step colorimetric assay (CellTiter 96® AQueous One Reagent, Promega), performed according to the manufacturer's instructions using a standard plate photometer (CLARIOstar Multimode Plate Reader, BMG Labtech, Germany).

Cells were seeded at an initial density of 10,000 cells per well into flat-bottom 96-well tissue culture plates, including cell-free control conditions. Immediately after seeding, cells were exposed to CZP ranging from 0 (VEH only) to 80 μM. Each assay condition was additionally stimulated with the unspecific mitogen Phytohemagglutinine-L (PHA-L; 1 μg/ml) as a positive control. Following an incubation period of 96 h at 37 °C in a humidified atmosphere containing 5% $CO_2$, the MTS-containing working solution was prepared according to the manufacturer's instructions and added to each well. After an additional 4 h of incubation at 37 °C, absorbance of the synthesized water-soluble formazan dye was detected at 490 nm. For each individual, at least two independent assays were run and each treatment condition within each assay was run at least in triplicate. Results were calculated as proliferative responses relative to the unstimulated/untreated control condition