In vitro maturation of *Toxoplasma gondii* bradyzoites in human myotubes and their metabolomic characterization

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The apicomplexan parasite *Toxoplasma gondii* forms bradyzoite-containing tissue cysts that cause chronic and drug-tolerant infections. However, current in vitro models do not allow long-term culture of these cysts to maturity. Here, we developed a human myotube-based in vitro culture model of functionally mature tissue cysts that are orally infectious to mice and tolerate exposure to a range of antibiotics and temperature stresses. Metabolomic characterization of purified cysts reveals global changes that comprise increased levels of amino acids and decreased abundance of nucleobase- and tricarboxylic acid cycle-associated metabolites. In contrast to fast replicating tachyzoite forms of *T. gondii* these tissue cysts tolerate exposure to the aconitase inhibitor sodium fluoroacetate. Direct access to persistent stages of *T. gondii* under defined cell culture conditions will be essential for the dissection of functionally important host-parasite interactions and drug evasion mechanisms. It will also facilitate the identification of new strategies for therapeutic intervention.
**Toxoplasma gondii** is an apicomplexan parasite that infects most warm-blooded animals, including an estimated third of all humans. Similar to other infectious protozoa, including the apicomplexan *Plasmodium vivax*, the kinetoplastid *Trypanosoma cruzi* and *Leishmania* spp., *T. gondii* forms specialized persistent stages that resist immune responses and many medical treatments. *T. gondii* bradyzoites reside, surrounded by a glycan-rich cyst wall, predominantly in brain and muscle tissue and can be transmitted through ingestion of undercooked meat products. These largely asymptomatic infections can cause recurring disease in immune-weakened individuals that are lethal if untreated.

The rapid proliferation of tachyzoites and the slow replication or dormancy of bradyzoites exert distinct demands on the metabolism of *T. gondii*. The rate of proliferation and the metabolic state are closely interrelated. They have been shown to independently affect lethality of antibiotics against bacterial microbes, with metabolism being the dominant factor. While multiple metabolomic investigations on the tachyzoite stage of *T. gondii* revealed unexpected features of key metabolic pathways such as the GABA-shunt of the tricarboxylic acid (TCA) cycle, a constitutively active gluconeogenic fructose 1,6-bisphosphatase, and the presence of a non-oxidative pentose phosphate pathway, the metabolism of bradyzoites has only been characterized indirectly, largely due to experimental constraints. In contrast to tachyzoites, bradyzoites depend on the turnover of the storage polysaccharide amylopectin by glycogen phosphorylase, and the hexose kinase and on a dedicated isoform of lactate dehydrogenase. This indicates that glycolytic degradation of glucose plays an important role in bradyzoites. Similarly, the relevance of mitochondrial and amino acid metabolism has been inferred. Bradyzoite formation is induced by a number of electron transport inhibitors, including atovaquone, rotenone, oligomycin and antimycin. Moreover, *T. gondii* cysts survive extended atovaquone exposure in vivo, indicating that their mitochondrial electron transport chain (mETC) is not strictly essential. Similarly, bradyzoites can also be induced by limiting supply of exogenous amino acids, and their viability depends on proteolysis in their plant-like vacuolar compartment. However, our ability to investigate such mechanisms remains very limited by the lack of adequate in vitro culture models.

Current methods to experimentally generate mature *T. gondii* tissue cysts are confined to murine infections with cystogenic *T. gondii* strains. The number of obtainable cysts from this in vivo model is low, precluding their metabolic analysis with current mass spectrometry-based approaches. In contrast, in vitro models are scalable and the stage conversion of *T. gondii* can be induced by a variety of treatments, such as alkaline, heat and chemical stress, nutrient starvation, excess adenosine, inhibitors of parasite protein kinases as well as host cell-dependent factors such as cell cycle arrest and low glycolytic flux. In addition, the infection of C2C12 murine skeletal muscle cells, neurons, and primary murine brain cells facilitates spontaneous stage conversion of cystogenic strains. Generally, maturation times of in vitro cysts remain brief, but cysts develop detectable tolerance against short exposure to low doses of pyrimethamine after three days and become orally infectious to mice at high doses after five days of culture.

Here, we developed a human myotube-based in vitro culture system that is scalable and enables long-term maturation of *T. gondii* cysts. The cysts resemble in vivo cysts in their ultrastructure, tolerance to antiparasitics and temperature stress and infectiousness to mice. Mass spectrometry-based metabolic profiling revealed a distinct metabolome that renders tissue cysts insensitive toward the aconitase inhibitor sodium fluoroacetate (NaFAc).

**Results**

**KD3 human myoblasts differentiate into multinucleated myotubes.** *T. gondii* persists in skeletal muscle tissue, but robust in vitro culture systems supporting long-term maturation of tissue cysts in these natural host cells are lacking. We tested an immortalized human skeletal muscle cell isolate (KD3) for its ability to support the culture of *T. gondii* tissue cysts. Subconfluent myoblast cultures were differentiated into myotubes through serum starvation for five days until cells fused to multinucleated tubes that were expressing the myosin heavy chain protein and developing spontaneous contraction activity (Supplementary Movie 1, 2). Accordingly, the myogenic index, which reflects the fraction of nuclei residing in cells containing three or more nuclei, rose to 0.3 (Fig. 1B).

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**Fig. 1 KD3 human myoblasts form multinucleated myotubes.** A Immunofluorescence images of KD3 myoblasts and myotubes after 5 days of differentiation, stained for myosin heavy chain (MF20) and DNA (DAPI). Representative of at least two independent experiments. Scale bar indicates 100 µm. B As a morphological parameter of myotube formation, the myogenic index (number of nuclei residing in cells containing three or more nuclei, divided by the total number of nuclei) was measured in at least ten different randomly selected locations by CCEAN software. Values are expressed as mean of two individual experiments performed in triplicates. Source data are provided as a Source Data file.
KD3 human myotubes support development of matured tissue cysts of several *T. gondii* strains. Next, KD3 myotubes were tested for their ability to support long-term maturation of *T. gondii* cysts. We infected myotubes with Pru-tdTomato parasites, a type II Prugniaud strain constitutively expressing the tandem (td) Tomato variant of the red fluorescent protein, for up to 21 days. Evaluation of the ultrastructure of the encysted parasites by electron microscopy (Figs. 2A, S1) revealed hallmark characteristics of bradyzoites, including abundant amylpectin granules posteriorly positioned nuclei after seven days of infection. The formation of the cyst wall is indicated by the apparent deposition of electron-dense vesicular-tubular material along the parasitophorous vacuolar membrane throughout the course of infection (Fig. S1D)37.

*T. gondii* strains differ greatly in their capacity to form tissue cysts in vitro and in vivo38 and their maturation is accompanied by sequential regulation of marker protein expression39. To test the suitability of KD3 myotubes to culture tissue cysts of multiple *T. gondii* strains, we monitored the formation of tissue cysts of eight parasite strains of three major isotypes under CO2-deplete conditions at neutral and basic pH. Stage conversion was detected by staining the cyst wall with *Dolichos biflorus* agglutinin (DBA) and antibodies against the CC2 protein40 and by the absence of cysts in vitro and in vivo38 and their maturation is accompanied by staining the cyst wall with DBA-positive and remained stable without being overgrown by tachyzoites for 21 days. This was also true for the hard to differentiate RHΔku80 (Fig. S2). We observed almost complete differentiation of type III VEG parasites into DBA- and CC2-positive and SAG1-negative cysts. All type II strains and the type III NED strain showed intermediate maturation (between 35 and 63%) that was considerably increased in basic pH to 73% and 83%, respectively. Also, wild-type type I strains GT1 and RH exhibited differentiation up to 30% that increased to 77% and 55% at basic pH, respectively. As expected, the high-passage laboratory strain RHΔku80 did not lose SAG1 signal at neutral pH but required basic conditions (Fig. 2C).

To directly relate the capability of KD3 myotubes to generate mature bradyzoites to existing culture methods, we differentiated Pru-tdTomato parasites for up to 21 days in HFF, KD3 myoblasts and KD3 myotubes by bicarbonate starvation at pH 7.4 (Fig. S3A) and pH 8.3 (Fig. S3B). In both conditions, parasites in myoblasts and HFF cells lysed host cells after 14 and 21 days respectively. Only in myotubes, Pru-tdTomato parasites formed an increasing fraction of DBA-positive and SAG1-negative cysts, while SAG1-positive and DBA-negative tachyzoites remained undetectable.

We also tested spontaneous stage conversion under standard bicarbonate-replete conditions as observed in the murine skeletal muscle cell line C2C1241. To this end, we infected myoblasts, myotubes and HFF cells with Pru-GFP parasites which express GFP through the bradyzoite-specific *LDH2* promoter42 for 96 h (Fig. S4). GFP-negative tachyzoites continued to proliferate in HFF cells and myoblasts leading to host cell lysis. In contrast, proliferation of *T. gondii* appeared

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**Fig. 2 Time- and pH-dependent cyst maturation of type I, II, and III *T. gondii* strains in KD3 myotubes.** A Electron microscopy of 7-, 14- and 21-day-old Pru-tdTomato tissue cysts in KD3 myotubes (days post infection; d.p.i.). Cysts show a distinct wall (CW) and are densely packed with parasites containing amylpectin granules (A) and nuclei (N). Representative of at least two independent experiments. B Immunofluorescence imaging of KD3 myotobes infected with type I (RH, RHΔku80, GT1), type II (ME49, NTE, Pru-tdTomato) and type III (NED, VEG) parasites. Cyst formation was induced for 7, 14, and 21 days under neutral or basic pH and stained with anti-CC2, anti-SAG1 antibodies, DBA and DAPI. Shown are representative images of data shown in (C) of tachyzoite controls infected with RHΔku80 for 24 h (upper panel), 14-day-old intermediate cysts (middle panel) and mature cysts (lower panel) of the NED strain. Scale bar indicates 5 μm. C Relative numbers of SAG1-negative but DBA- and CC2-positive cysts counted from three independent blinded experiments performed in triplicates. At least 25 DBA-positive cysts per strain, time point and replicate, respectively, were counted. Values represent means ± SEM. Source data are provided as a Source Data file.

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attenuated and spontaneous formation was indicated by DBA staining and GFP expression (Fig. S4).

Together, these data show that KD3 myotubes enable the culture of tissue cysts of multiple parasite strains at physiological pH over the course of 21 days and augment spontaneous stage conversion.

**KD3 myotubes-derived tissue cysts harbor pepsin- and temperature stress-resistant bradyzoites.** To test whether in vitro bradyzoites also develop functional hallmarks of in vivo bradyzoites, such as resistance to temperature stress and pepsin digestion, we infected myotubes with Pru-tdTomato parasites and then differentiated parasites for up to 35 days (Fig. 3A). Intracellular cysts were digested with pepsin for 20, 40, and 60 min, the reaction mix was then neutralized and cysts were seeded onto HFF cells to allow re-differentiation in tachyzoite medium under CO2-replete conditions. Untreated and pepsin-digested tachyzoite cultures served as positive and negative controls, respectively. Growth of both, untreated tachyzoite and bradyzoite cultures, was observed by an increase in red fluorescence (indicative of tachyzoite replication) after two and seven days, respectively (Fig. 3B). Interestingly, all pepsin-treated bradyzoite cultures recovered after 7, 9, and 14 days, depending on the length of the pepsin digest, while pepsin-digested tachyzoite cultures did not recover even after a period of 21 days. We quantified pepsin resistance across the time course of cyst maturation by calculating a resistance score (RS) (see “Methods”) based on the pepsin-inflicted growth delay (Fig. 3C). Already after 14 days all digested cultures recovered with an RS between 0.4 and 0.5. Pepsin resistance continued to increase over the course of 28 days up to an RS of 0.8 (Fig. 3C).

In human transmission scenarios, encysted bradyzoites survive extended cold-storage and heat stress. To test whether in vitro bradyzoites would survive under these conditions, cysts were matured for 35 days and treated with a combination of (1) storage at 4 °C for up to seven days, (2) a 5 min heat shock at 55 °C or 65 °C and (3) pepsin digestion for 60 min (Fig. 3D). Storage at room temperature (RT) for two days served as a positive control and did not decrease viability in comparison with untreated cysts, as indicated by an RS of 1. Different combinations of storage at 4 °C for four or seven days and heat shock at 55 °C did lower the RS to a range between 0.75 and 0.65, while heating to 65 °C effectively killed tissue cysts as expected. These results demonstrate that myotube-grown encysted bradyzoites develop traits that are important for oral transmission of tissue cysts as it occurs with cysts in muscle tissues of farm animals.

Summarized, these data indicate that KD3 myotubes support maturation of *T. gondii* tissue cysts for 35 days. These in vitro bradyzoites develop resistance to pepsin and temperature stresses,
myotube-derived tachyzoites, 50 or 500 35-day-old Pru-tdTomato in vitro cysts or 10 Pru-tdTomato in vivo cysts. Brains and blood from moribund animals were collected starting from 12 days post infection. Values are expressed as means and SEM from two independent experiments consisting of measuring their diameters (Fig. 4A). Maturation over the course in vivo cysts, we estimated relative volumes of in vitro cysts by facilitate the comparison of infectivity between in vitro and tissue cysts prepared 35 days post infection from brain tissue. To myotube-derived tachyzoites and as positive control 10 in vivo mean diameter of 56 µm22. Assuming spherical cysts, this implies of 35 days increased the average diameter from 8.8 to 11 µm. This

\[ p < 0.0001 \]  

\[ p = 0.0015 \]

Bradyzoite age [days]

which are functional hallmarks of mature in vivo bradyzoites and required properties for both experimental oral infection of mice and environmental transmission to humans.

KD3 myotube-derived tissue cysts are orally infectious to mice. To assure oral infectivity to animals, groups of KD3 myotube-derived tissue cysts are orally infectious to mice and environmental transmission to humans. Cysts develop resistance against antiparasitics. *T. gondii* tissue cysts largely resist current toxoplasmosis treatments. To test whether cysts cultured in myotubes mirror drug resistance in vivo, we exposed 14-, 21-, and 28-day-old Pru-tdTomato-expressing cysts to antiparasitics for seven days and monitored their re-differentiation into tachyzoites under CO2-replete conditions as a measure of bradyzoite viability (Fig. 5A). Figure 5B shows the fluorescence signal observed over 49 days, including 14 days of cyst maturation, seven days of pyrimethamine treatment, and 28 days of re-differentiation. Growth of DMSO- and pyrimethamine-treated parasites was indicated by an increase in fluorescence above background at 14 and 24 days post treatment, respectively (Fig. 5B). Next, 14-, 21-, and 28-day-old tissue cysts were treated with 5 µM and 20 µM pyrimethamine, 20 µM sulfadiazine, and 0.1 µM of 1-hydroxy-2-dodecyl-4(1)-quinolon (HDQ), an inhibitor of dihydroorotate dehydrogenase (DHOD)43 and alternative NADH dehydrogenase44 (Fig. 5C). Bradyzoite cultures survived all tested treatments. Interestingly, while resistance against sulfadiazine, HDQ or low doses of pyrimethamine was already fully developed after 21 days, indicated by an RS of 1, resistance against

| Stage | Origin | Tz | Tz | Cyst | Cyst | Cyst |
|-------|--------|----|----|------|------|------|
| Inoculum | HFF | 10^4 | 10^4 | 50 | 500 | 10 |
| Seroconversion | 1/10 | 2/10 | 8/10† | 9/10† | 4/5† |
| Brain cysts§§ | 1/1 | 2/2 | 1/3 | 4/4 | 4/4 |

Fig. 4 In vitro cysts are orally infectious to mice and lead to manifestation of chronic infection. A Quantification of cyst diameter of 7-, 14-, 21, 28, and 35-day-old Pru-tdTomato in vitro tissue cysts. Encysted bradyzoites were stained with DBA and DAPI. Images of 50 cysts were randomly recorded and the diameter was measured by defining the DBA stain as the outline of the cysts. Values are expressed as means and SEM from one experiment. B Reaction index and cyst number per brain of mice that were orally infected with 10^4 HFF- or myotube-derived tachyzoites, 50 or 500 35-day-old Pru-tdTomato in vitro cysts or 10 Pru-tdTomato in vivo cysts. Values are expressed as means and SEM from two independent experiments consisting of five mice per group. C Quantification of cyst burden in brains of infected mice. Only seropositive mice from one experiment were analyzed for brain cysts. D Summary of in vivo infection experiments. § Seroconversion of moribund, but alive animals were checked throughout the experiment. §§ Brain cyst burden was only determined in seropositive animals of one experiment. Source data are provided as a Source Data file.

Inoculum 10 4 104 50 500 10 Origin HFF MT MT MT Mouse Stage Tz Tz Cyst Cyst Cyst Bradyzoite age [days]

\[ \text{Brain cyst burden was only determined in seropositive animals of one experiment. Source data are provided as a Source Data file.} \]
Resistance scores of tachyzoites (0) and 14-, 21-, and 28-day-old bradyzoites treated with pyrimethamine (PYR), sulfadiazine (SULF) or 1-hydroxy-2-dodecyl-4(1H) quinolone (HDQ) and bumped kinase inhibitor (BKI) 1553 and BKI1294. Shown are means of three independent experiments with five replicates each and SEM (*p = 0.0002 for HDQ treatment, p = 0.0002 for BKI1553 and p = 0.0017 for BKI1294 treatments, two-tailed Mann-Whitney U tests). Source data are provided as a Source Data file.

**Fig. 5.** *T. gondii* in vitro bradyzoites develop broad drug tolerance. **A** Experimental design for in vitro drug tolerance assay. Pru-tdTomato cysts and tachyzoite controls were drug treated at the indicated concentrations for one week. Following the treatment, medium was changed to bicarbonate-replete medium to induce re-differentiation to proliferating tachyzoites and tdTomato fluorescence was monitored. **B** Raw fluorescence intensities of a representative experiment during cyst maturation for 14 days, 7 days of treatment with 20 μM pyrimethamine or 0.2% DMSO as solvent control and re-differentiation for 28 days. Black dotted line indicates the limit of detection. Data represents the means of one experiment with five replicates. **C, D** Resistance scores of tachyzoites (0) and 14-, 21-, and 28-day-old bradyzoites treated with pyrimethamine (PYR), sulfadiazine (SULF) or 1-hydroxy-2-dodecyl-4(1H) quinolone (HDQ) and bumped kinase inhibitor (BKI) 1553 and BKI1294. Shown are means of three independent experiments with five replicates each and SEM (*p = 0.0023 for 20 μM pyrimethamine treatments, p = 0.025 for sulfadiazine treatment and p = 0.0002 for HDQ treatment, p = 0.035 for BKI1553 and p = 0.0017 for BKI1294 treatments, two-tailed Mann-Whitney U tests). Source data are provided as a Source Data file.

The metabolome of *T. gondii* depends on the host cell type and parasite stage. The metabolome is directly linked to the mode of action of many antiparasitics and may also be associated with their efficacy. However, the metabolome of bradyzoites and its differences to tachyzoites remains largely unknown. To test whether bradyzoites possess a distinct metabolome, we compared the abundance of metabolites between ME49 parasites in their bradyzoite form with intracellular tachyzoites in both HFF cells and myotubes (Fig. 6). Parasites were differentiated into cysts for 28 days, quenched with ice-cold phosphate-buffered saline (PBS), released from cells by needle passage, and purified from host cell debris via binding to DBA-coated magnetic beads. Intracellular tachyzoites were prepared by needle passage and filtered to remove host material. We also estimated artefacts that are introduced by differences in quenching and preparation procedures of tachyzoites and bradyzoites. We supplemented isolated tachyzoites with beads and processed them similarly to bradyzoites. To correct for background metabolites from contaminating host material and magnetic bead preparations, we included uninfected myotubes and bead-only controls into our experimental antiparasitics and are suited for compound screens against chronically infectious forms of *T. gondii*. 

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**Image Details:**

- **A** Experimental design for in vitro drug tolerance assay. Pru-tdTomato cysts and tachyzoite controls were drug treated at the indicated concentrations for one week. Following the treatment, medium was changed to bicarbonate-replete medium to induce re-differentiation to proliferating tachyzoites and tdTomato fluorescence was monitored.

- **B** Raw fluorescence intensities of a representative experiment during cyst maturation for 14 days, 7 days of treatment with 20 μM pyrimethamine or 0.2% DMSO as solvent control and re-differentiation for 28 days. Black dotted line indicates the limit of detection. Data represents the means of one experiment with five replicates.

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- **Fig. 5.** *T. gondii* in vitro bradyzoites develop broad drug tolerance. **A** Experimental design for in vitro drug tolerance assay. Pru-tdTomato cysts and tachyzoite controls were drug treated at the indicated concentrations for one week. Following the treatment, medium was changed to bicarbonate-replete medium to induce re-differentiation to proliferating tachyzoites and tdTomato fluorescence was monitored. **B** Raw fluorescence intensities of a representative experiment during cyst maturation for 14 days, 7 days of treatment with 20 μM pyrimethamine or 0.2% DMSO as solvent control and re-differentiation for 28 days. Black dotted line indicates the limit of detection. Data represents the means of one experiment with five replicates. **C, D** Resistance scores of tachyzoites (0) and 14-, 21-, and 28-day-old bradyzoites treated with pyrimethamine (PYR), sulfadiazine (SULF) or 1-hydroxy-2-dodecyl-4(1H) quinolone (HDQ) and bumped kinase inhibitor (BKI) 1553 and BKI1294. Shown are means of three independent experiments with five replicates each and SEM (*p = 0.0023 for 20 μM pyrimethamine treatments, p = 0.025 for sulfadiazine treatment and p = 0.0002 for HDQ treatment, p = 0.035 for BKI1553 and p = 0.0017 for BKI1294 treatments, two-tailed Mann-Whitney U tests). Source data are provided as a Source Data file.
These samples were treated identically to bradyzoite samples. All preparations were extracted in 80% acetonitrile and analyzed on a HILIC-UHPLC-LC/MS. Blank and host cell background-subtracted ion intensities were normalized to the total ion count per sample and log2-fold changes of metabolites between experimental conditions calculated (Fig. S8A–F, Supplementary Data 1). Principal component (PC) analysis indicated that bradyzoites in the purified tissue cysts differed from tachyzoites along PC1, while myotube- and HFF-derived tachyzoites were separated in PC2 (Fig. 6A). Bead-supplemented tachyzoite controls that underwent incubation with similar purification procedure as tissue cysts and were incubated with...
beads for one hour were indistinguishable from pure tachyzoites. Uninfected host cell controls were similar to bead-only samples, indicating minimal metabolite alterations and contamination due to the purification procedures (Fig. S8E). The two clusters of HFF-derived tachyzoites represent two independent experiments whose variation manifests itself after host-background subtraction (Fig. S8D). We quantified the levels of 71 metabolites, excluding those that we detected in uninfected controls. The levels of 26 metabolites varied between parasite stages, 10 varied between types of host cells, while 24 depended on both factors, and 11 remained invariant (Fig. 6B–D). Comparing bradyzoites to tachyzoites from the same host cell type (Fig. 6E), we noted that the relative abundances of amino acids and sugar derivatives were generally increased, while vitamins, derivatives of amino acids, nucleic acids and metabolites associated with the TCA cycle were significantly decreased. Fatty acids (FAs) and related molecules are variably affected by stage conversion. When comparing tachyzoites from HFF cells with those from myotubes (Fig. 6E), we found that the levels of amino acids and sugars were higher in HFF-grown tachyzoites, while FAs increased in abundance. The host cell type had mixed effects on other metabolite classes.

These data reveal distinct metabolic features in T. gondii bradyzoites involving various metabolite classes and the mitochondrial TCA cycle.

**Nature T. gondii tissue cysts develop resistance against the aconitase inhibitor sodium fluoroacetate.** We hypothesized that diminished pools of TCA metabolites in bradyzoites would be consistent with lower importance in this stage. To estimate the reliance of bradyzoites on the TCA cycle, we used the aconitase inhibitor NaFAc to inhibit the TCA cycle flux in T. gondii6. Continuous exposure to NaFAc arrests tachyzoite replication with an IC50 of 168 µM, and parasite replication was minimal at 500 µM (Fig. 7A). Next, we tested the ability of tachyzoites and bradyzoites to survive exposure to 0.5–5.0 mM NaFAc for seven days. 28-day-old cysts recovered after treatment with a RS of approximately 0.8, while tachyzoites were only able to survive 0.5 mM NaFAc, but not higher concentrations (Fig. 7B). To exclude that tolerance of bradyzoites toward NaFAc relies on exclusion of this inhibitor from the cyst47, we sought to measure marked direct metabolic effects of this inhibitor on tachyzoite and bradyzoite stages6. We treated 28-day-old ME49 cysts and intracellular myotube-cultured tachyzoites with NaFAc for seven and three days, respectively, and analyzed the metabolomes by HILIC-UHPLC-LC/MS (Fig. 7C, D, Supplementary Data 2). The PC analysis revealed that solvent- and NaFAc-treated tachyzoites form distinct clusters that are separated along the PC2 axis. In contrast, treatment of tissue cysts did not lead to a separation from untreated controls (Fig. 7C). NaFAc treatment affected the relative abundance of 33 metabolites in tachyzoites, but changed only 8 metabolites in bradyzoites (Fig. 7D). Aconitate levels in both parasite stages were increased significantly upon NaFAc treatment (Fig. 7D). These data are consistent with a NaFAc-mediated inhibition of aconitase in bradyzoites and show that extended exposure to high doses of this inhibitor, in contrast to tachyzoites, is well tolerated.

**Discussion**

Persistence mechanisms of important protozoan pathogens, including T. gondii, T. cruzi, Leishmania spp. and P. vivax, remain largely understudied, and there are inadequate or no treatments available against chronic infections with these organisms2. The limited availability of methods to culture persistier cells restricts the approaches to study the link between persistence and metabolism7,48. Here, we report a cell culture model based on immortalized human skeletal muscle cells36 that supports maturation of tissue cysts of type I, II and III T. gondii strains. These myotube-grown bradyzoites exhibit key characteristics including typical ultrastructural features, stress resistance, and tolerance toward antimicrobials.

Our in vitro system is scalable, enabling measurements of bradyzoite metabolites via mass spectrometry. To this end, we established a protocol that allows isolation and purification of cysts in large quantities and without the use of Percoll gradients22, making them available for mass spectrometry-based metabolomics. We obtained an estimated yield of 2 × 10^6 cysts per two T150 culture dishes that were sufficient for one technical replicate and represented a volume-adjusted tenfold improvement to the bradyzoite yield from the infection of one mouse22. PC analysis of the impact of host cell type and the parasite stage indicates mostly independent effects on the metabolome (Fig. 5A). Stage conversion leads to changes that are largely homogeneous across classes of metabolites such as amino acids, vitamins, TCA cycle intermediates, and nucleic acids. This indicates the operation of distinct metabolic homeostasis mechanisms in bradyzoites.

Bradyzoites harbor lower levels of several TCA cycle-associated metabolites that are consistent with a lower reliance on this pathway (Fig. 6E). This interpretation is supported by an NaFAc-induced slight accumulation ofaconitate (Fig. 7B). Extensive accumulation of citric acid andaconitate has previously been shown to occur in extracellular tachyzoites already after four hours of treatment6. We attribute the lower accumulation ofaconitate we observe in bradyzoites to the extended treatment over seven days and the longer quenching and purification procedure required for intracellular parasites. Importantly, this accumulation ofaconitate suggests that NaFAc is available to bradyzoites, that theaconitate is active and inhibited by NaFAc. This interpretation also agrees with previous findings of an active TCA cycle enzyme, isocitrate dehydrogenase, in lysates of four-week-old bradyzoites from mice19 and with continued transcription of TCA-cycle-related genes30. Interestingly, NaFAc exerts comparatively limited effects on other non-TCA cycle-related metabolites in bradyzoites. It is noteworthy that our data do not exclude the possibility that parts of the TCA cycle remain
operational and may process anaplerotic substrates, such as glutamine to generate NADH for subsequent ATP production through the mETC. However, full resistance to the inhibitor of the alternative type 2 NADH-dehydrogenase TgNDH2 (Figs. 5C and S7C, D) suggests that this source of ATP would equally be dispensable. HDQ appears to only partially decrease the mitochondrial membrane potential in bradyzoites (Fig. S7A, B), indicating the presence of HDQ-independent sources of reduction equivalents or a very slow rate of mitochondrial ATP synthesis concomitant with negligible depolarization of the membrane potential. Bradyzoites may rely increasingly on glycolytic ATP production, as indicated by increased activity of
glycolytic enzymes in this stage. The number of *T. gondii* cysts in brain tissue of latently infected mice depends on the turnover of large amylopectin pools and on the expression of several glycolytic enzymes including hexokinase and lactate dehydrogenase. Also, the pyruvate dehydrogenase E1 subunit transcript is less abundant in four-week-old bradyzoites.

Interestingly, transcriptomic analyses of *P. vivax* hypnozoites, a persistent stage of this malaria parasite, indicates a similar metabolic shift toward glycolysis.

We also found that a number of amino acids were accumulated in bradyzoites (Fig. 6E). We attribute these changes directly to stage conversion and not to differences in media composition. The affected amino acids, except for proline, which can also be synthesized from glutamate, are less concentrated in the Roswell Park Memorial Institute (RPMI)-based bradyzoite medium compared to the DMEM-based medium used for tachyzoites. All of these amino acids have been shown to be imported by extracellular tachyzoites. *T. gondii* tachyzoites express several Atp1-dependent Amino Acid Transporter proteins, all of which remain transcriptionally expressed in bradyzoites (Fig. 6F). This suggests that bradyzoites retain the ability to take up amino acids from their environment. Interestingly, restriction of some exogenous amino acids has been used to facilitate stage conversion of *T. gondii* cysts. While the differentiation efficiency of all tested *T. gondii* strains can be further increased by additional non-physiological stressors such as basic pH, it is not needed for extended cultures. Strikingly, even highly virulent RH-derived parasite sub-strains did not overgrow the culture (Fig. 2C). Resistance of bradyzoites against peptidase digestion and temperature stress develops after 14 days post infection and increases until 28 days post infection (Fig. 3C). The onset of tolerance toward inhibitors of well-established drug targets including the calcium-dependent protein kinase (TgCDPK1), dihydrofolate reductase-thymidylate synthase (TgDHFR-TS) and the DHOD (Fig. 5C, D) follows similar timing and is shared with *T. gondii* cysts in the brain of chronically infected mice.

Hence, our in vitro model will facilitate dissecting the molecular basis of the impressive drug tolerance and stress resistance of *T. gondii* cysts. Expectedly, in vitro cysts are also orally infectious to mice (Fig. 4A–D). Comparing estimated volumes of in vitro cysts and in vivo brain-derived cysts, our data suggest that 10 in vivo cysts are equivalent to 500 in vitro cysts in this regard. Surprisingly, 3 out of 20 mice that received tachyzoites via oral gavage seroconverted. It has been observed earlier that numbers greater than 1000 peptidase-sensitive tachyzoites were orally infectious to mice.

Electron microscopic analysis of the ultrastructure of myctube-derived tissue cysts revealed typical features such as a developing cyst wall, amylopectin granules, and posteriorly positioned nuclei in the bradyzoites (Figs. 2A, S1) Interestingly, we also found evidence of disintegrating bradyzoites at all time points up to 21 days post infection, (Fig. S1) which has also been described previously in mice after four and 8 weeks of infection. This parasite turnover likely contributes to the heterogeneous appearance with respect to cyst size and bradyzoite packaging density. A similar phenotypic heterogeneity has been ascribed to the ability of other intracellular pathogens to persist in the presence of stressors such as drugs and immune effectors.

In conclusion, we present a novel system to culture functionally mature *T. gondii* tissue cysts that surpasses typical in vivo systems through its scalability. Importantly, it allows cyst purification with methods compatible with subsequent mass spectrometry-based...
applications. We show that bradyzoites maintain a distinct metabolome that confers resistance to an aconitase inhibitor. This culture model will be valuable for uncovering new bradyzoite biology and for addressing drug tolerance.

Methods

Host cell lines and cultivation. All cultures were maintained in a 37 °C humidified CO₂ (10%) incubator as described previously7. Briefly, RH5ta human foreskin fibroblast (ATCC CRL-4001 HFF) monolayers were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 25 mM glucose (Sigma-Aldrich), 4 mM l-glutamine (Thermo Fisher Scientific), 1 mM sodium pyruvate (Capricorn Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin, 2% heat-inactivated bovine serum (FBS) (Capricorn Scientific) for 24 min and then with goat anti-rat-Alexa546 1:130 (Invitrogen), goat anti-rat-Alexa350 1:130 (Thermo Fisher Scientific), rabbit anti-mouse-Cy3 1:400 (Dianova), donkey anti-mouse-Cy5 1:3000 (Dianova) or streptavidin-Alexa488 1:5000 (Dianova), and streptavidin-Cy5 1:5000 (Jackson ImmunoResearch). Mitochondrial staining was done prior to fixation for 30 min with MitoTracker Deep Red FM (Thermo Fisher Scientific) at 200 nM in tachyzoite medium at 37 °C in a humidified CO₂ incubator. Cultures were chased for 15 min in tachyzoite medium and washed three times with PBS, fixed with 4% paraformaldehyde followed by immunofluorescence staining as indicated. Monochromatic images were recorded on a Zeiss ApoTome Imaging equipped with an Axio Cam MRc5 HighSpeed 5.5 Mega objective or on a Zeiss Axio Observer imager equipped with Plan-Neofluar 10x/0.3 objective or with a Plan-Apochromat 63x/1.4 oil objective and imported into ImageJ version 1.52a88 for coloring, the generation of overlays and quantification.

Pepsin digestion and in vitro oral transmission assay. KD3 myocyte cultures in 6-well plates were infected with 6 × 10⁶ extracellular Pru-tdTomato tachyzoites corresponding to an MOI of 10. Bradyzoite and bradyzoite formation were assessed on the indicated times in bradyzoite medium. Tachyzoite controls were infected for two days with 3.2 × 10⁶ parasites corresponding to an MOI of 5 in tachyzoite medium. On the day of experiment, pepsin digestion was performed for 20, 40, and 60 min at 37 °C as described previously87 using a modified pepsin solution. The buffered solution consisted of 1.01 g pepsin (Thermo Fisher Scientific), 0.166 g glycine (Thermo Fisher Scientific), 0.129 g NaCl (Sigma-Aldrich) and 17.8 ml of a 1 M HCl solution (Sigma-Aldrich), filled up to 200 ml with Milli-Q-water, resulting in a pH of 1.2 and supplemented with 5 mM glucose. Digestion was terminated by neutralization using a 1.2% sodium bicarbonate solution (pH = 8.3) containing 0.0195 g/µl phenol red as pH indicator dye. Digested infected and uninfected controls without pepsin were processed as cell scraper and harvested via centrifugation (1200 × g, 10 min). The supernatant was then removed, the pellets were resuspended in 450 µl DMEM tachyzoite medium and used to infect HFF cells in a 96-well plate in triplicates, if not indicated differently. Parasite recovery was monitored by measuring tDTomato fluorescence intensity (excitation 554 nm/emission 589 nm) in a plate reader (Tecan Infinite M200 PRO) using Tecan i-control software v2.0.10.0 every second day for up to 21 days. Medium was carefully exchanged without perturbing infected monolayers after each measurement.

For the oral transmission assay, scraped monolayers and controls were challenged by the indicated combination of stresses: cold stress (4 °C for 4 and 7 days), heat stress (55 or 65 °C for 5 min), incubation at RT for two days, and pepsin digestion (60 min).

The RS was calculated as the difference of days passed between the detection of viable tachyzoites and treated tachyzoites. This data was normalized to the number of days of total observation to account for differences in initial cyst loads and the resulting re-growth periods between experiments:

\[
RS = 1 - (\#\text{days}_{\text{treatment}} - \#\text{days}_{\text{vehicle}}) / \#\text{days}_{\text{observed}}
\]

An RS of 0 indicates no recovery from the treatment and a RS of 1 means equal growth to the vehicle-treated control, indicating full resistance to the treatment.

In vivo oral transmission assay. KD3 myocyte cultures in 6-well plates were infected with 1.9 × 10⁶ Pru-ttdTomato tachyzoites corresponding to a MOI of 0.3. Bradyzoite formation was induced for indicated times in bradyzoite medium. Tachyzoite cultures were prepared in T25 cell culture flasks by infecting KD3 myotubes and human fibroblasts with 3.6 × 10⁶ tachyzoites corresponding to an MOI of 2 for 2 days in tachyzoite medium. The cultures were then prepared for shipping and sent to Freiburg at RT within 24 h. The cultures were resuspended in PBS, fixed, and stored in the fridge until further processing.

For electron microscopy, 3.2 × 10⁶ Pru-tdTomato tachyzoites corresponding to an MOI of 10 were incubated for 24 h, unless indicated otherwise. For bradyzoite experiments, independent of strain and host cell background, myocyte monolayers were infected with 1.3 × 10⁸ extracellular tachyzoites corresponding to an MOI of 0.1 and incubated for the indicated times. Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4) to release intracellular parasites. The cyst solution was placed on a glass slide and cysts were counted based on the fluorescence of tDTomato in a fluorescence microscope. For in vivo cyst quantification, one C57BL/6JRj mouse was infected with 1 × 10⁶ T. gondii tachyzoites via intraperitoneal injection. Thirty days post injection, the mouse was euthanized by cervical dislocation. The brain was harvested in 2 ml PBS and sent to Freiburg at RT within 24 h. The brain was thoroughly washed and the resulting re-growth periods between experiments:

\[
RS = 1 - (\#\text{days}_{\text{treatment}} - \#\text{days}_{\text{vehicle}}) / \#\text{days}_{\text{observed}}
\]

An RS of 0 indicates no recovery from the treatment and a RS of 1 means equal growth to the vehicle-treated control, indicating full resistance to the treatment.

Immunofluorescence assay. Uninfected host cells were grown on monolayers on 12 mm round glass coverslips (TPP) and infected for 24 h, unless indicated otherwise. For bradyzoite experiments, independent of strain and host cell background, myocyte monolayers were infected with 1.3 × 10⁸ extracellular tachyzoites corresponding to an MOI of 0.1 and incubated for the indicated times. Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4) to release intracellular parasites. The cyst solution was placed on a glass slide and cysts were counted based on the fluorescence of tDTomato in a fluorescence microscope. For in vivo cyst quantification, one C57BL/6JRj mouse was infected with 1 × 10⁶ T. gondii tachyzoites via intraperitoneal injection. Thirty days post injection, the mouse was euthanized by cervical dislocation. The brain was harvested in 2 ml PBS and sent to Freiburg at RT within 24 h. The brain was thoroughly washed and the resulting re-growth periods between experiments:

\[
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\]

An RS of 0 indicates no recovery from the treatment and a RS of 1 means equal growth to the vehicle-treated control, indicating full resistance to the treatment.
clear-bottomed 96-well plate and quantified at ×20 magnification using DBA positivity as a first criterion and tdTomato positivity to confirm brain cyst.

Mouse infections. Groups of five 10-week-old male and female mice (C57BL/6Rj) were obtained from certified breeders (Janvier Labs, Route du Genest, 53940 Le Genest-Saint-Isle, France) and kept under specific-pathogen-free conditions in the local animal facility (Department for Microbiology and Hygiene, Freiburg). Mice were kept on a day/night cycle of 14 and 10 h at 20–22 °C and a relative humidity between 30% and 55%. Mice were fed feeding diet (Altromin) and non-automated drinking water ad libitum and housed in green line type 2 cages (Tecniplast). All animal experiments were performed in accordance with the guidelines of the German animal protection law and the Federation for Laboratory Animal Science Associations. Experiments were approved by the state of Baden-Württemberg (Regierungspressurisdiktion Freiburg; reference number 35–9185.81/A-19/89).

Mice were infected by oral gavage with 50 or 500 freshly prepared in vitro-generated Pru-tdTomato cysts, 10 freshly prepared in vivo-derived Pru-tdTomato cysts or 10⁴ myotube- or HFF-derived extracellular Pru-tdTomato tachyzoites in a total volume of 200 µl sterile PBS.

Infected mice were monitored and weighed daily for the duration of the experiment. Relative weight loss was calculated based on the weight at the day of infection. Brains and blood from moribund animals were collected starting from 12 days post infection. After 30 days post infection, blood samples of all surviving mice were collected from the facial vein. Blood was allowed to clot at RT for 30 min before centrifugation (10,000 × g, 10 min). Serum was collected and either immediately analyzed or stored at −80 °C until further analysis. Animals were euthanized by cervical dislocation. Brains were harvested and cysts per brain sample were blindly counted as described previously:

#Cysts/brain = cysts in 50 µl × 40

Seroconversion was evaluated via ELISA as described previously89. Serum from non-infected (n.i.) mice were included to calculate the cut-off:

cut-off = mean Absorbance⁴⁵⁰nm−57⁰nm(n.i.) + 2 × standard deviation of Absorbance⁴⁵⁰nm−57⁰nm(n.i.)

An individual cut-off was calculated per each individual ELISA. In order to compare the T. gondii specific-antibody titers from different independent experiments a reactivity index was calculated per sample:

Reactivity index = mean Absorbance⁴⁵⁰nm−57⁰nm/cut-off

Reactivity index values higher than one were considered as positive.

Cyst isolation. Uninfected and infected myotubes were prepared. In both cases, two T150 dishes were pooled into one sample. For bradyzoite samples, myotubes were infected with 3.2 × 10⁴ Pru-tdTomato tachyzoites corresponding to a MOI of 0.3 and cyst formation was induced for indicated time. On the day of harvest, infected samples and uninfected host cell controls were placed on ice, medium was removed and monolayers were washed three times with ice-cold PBS. Cells were then harvested by scraping into 10 ml ice-cold 0.05% BSA in PBS per T150 dish. Cysts were released from the monolayer via performing a 23 G needle (Sterican®) 25 times with a syringe and collected via centrifugation (1200 × g, 10 min, 0 °C). The supernatant was removed, the pellet was resuspended carefully in ice-cold 2% BSA in PBS containing 200 µl DBA-coupled beads (preparation described below) and samples were incubated for 1 h at 4 °C with gentle shaking. Subsequently, the samples were placed in a magnetic stand on ice, washed five times with 0.1% BSA in PBS to remove cell debris, followed by two washing steps with PBS to remove residual BSA. Cysts and beads were then collected via centrifugation (1200 × g, 10 min, 0 °C), shock frozen in liquid nitrogen and stored at −80 °C until extraction. Tachyzoite samples were generated in T150 dishes by infecting myotubes and HFF cells with 3.2 × 10⁷ tachyzoites corresponding to an MOI of 3 for 48 h before the seven-day drug pulse was applied in tachyzoite medium. Parasite recovery was monitored by measuring tdTomato fluorescence for 28 days as described above. Medium was exchanged after 24 h. Myotubes were treated with either 10% DMSO (Thermo Fisher Scientific) or 0.2% DMSO (Thermo Fisher Scientific) as solvent control. Cysts were washed three times with ice-cold PBS and monolayers were scraped and harvested using a 23 G needle. Tachyzoites were filtered-purified through a 3 µm filter (Whatman) and PBS-washed by centrifugation (1200 × g, 10 min, 0 °C) three times. All samples were extracted simultaneously in 80% acetonitrile for LC/MS analysis as described below. Bead-only controls were processed equally. Bead-supplemented tachyzoite controls were processed equally to cyst samples, replacing washing steps via magnetic stand by centrifugation (1200 × g, 10 min, 0 °C).

Preparation of beads. Coupling of Dynabeads™ MyOne™ Streptavidin T1 (Thermo Fisher Scientific) to DBA was done as described in the manufacturer’s protocol. Briefly, 200 µl beads/sample were resuspended in 1 ml PBS by vortexing, washed three times with PBS in a magnetic stand, and resuspended in 1 ml PBS containing 50 µg DBA/sample. The tube containing the DBA-magnetic bead mixture was incubated on a rotary mixer for 45 min at RT. Uncoupled DBA was washed by removing the washed beads three times with PBS. After washing, the DBA-coated beads were resuspended in 2 ml PBS containing 2% BSA.

Untargeted metabolic analysis in vitro cysts. For metabolome measurements of tissue cysts and tachyzoites, tachyzoite isolation, cyst maturation and isolation were performed as described above. Metabolites were extracted in 80% acetonitrile (Carl Roth) and 20% water (Carl Roth) containing internal standards (p-hydroxyphenylacetic acid (PAP), 4′-iodoacetophenone (CAPS), 4′,5′-diaminopyrimidine (DAP), and 6′-amino-5′-iodoacetophenone (6AIA) (Sigma-Aldrich)). The supernatants were transferred to MS vials for immediate LC/MS analysis. 5 µl of each sample were collected to generate a pooled biological quality control (PBOC). Twenty microliters of the in vitro cysts, bead control and host cell background samples, and 5 µl of the tachyzoite samples were injected. The injection order of the samples was randomized, and samples and PBOQs were injected periodically. The samples were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) via 70k/MS1 scans, with intermittent 35k data-dependent 55k MS2 scans in positive and negative mode separately.

Chromatographic separation was achieved on a Vanquish Flex fitted with 2 Q-Exactive UPLC BEH Amide column (Waters). Running a 25 min linear gradient starting with 90% eluent A (10 mM ammonium carbonate in acetonitrile)/10% eluent B (10 mM ammonium carbonate in water) and ending with 40% eluent A/60% eluent B, followed by washing and equilibration steps.

XCalibur 4.2.47 software and Compound Discoverer 3.1 software (Thermo Fisher Scientific) was used for recording and for peak detection, combination of adducts, and compound annotation, respectively. Metabolite identifications were based on either retention time and accurate mass match to an in-house library of 160 authentic standards, or by matching accurate mass and MS2 fragments to mzCloud database (mirrored offline in m/z vault v2.12.15 in May 2020) (Thermo Fisher Scientific). Data were exported to Excel for grouping, combination of datasets from positive and negative ionization runs and blank subtraction. Compound ions with a coverage less than 50% in at least one sample group were excluded. All missing values were gap-filled with either the respective mean of each sample group or the global minimum intensity when undetected in a sample group. After normalization to the internal standard, the fractional abundances for each sample were calculated and centered between 1 and −1.

Raw data, data curation steps, and performed statistics are accessible in Supplementary Data 1 and Supplementary Data 2.

Drug tolerance assays. Myotubes in 96-well plates were infected with 2.4 × 10⁴ T. gondii Pru-tdTomato tachyzoites corresponding to a MOI of 0.1 and cyst formation was induced for indicated times in bradyzoite medium at ambient CO₂ levels, followed by a drug pulse of 7 days with respective vehicle as solvent control. Tachyzoite controls were infected with Pru-tdTomato tachyzoites corresponding to a MOI of 1 in tachyzoite medium for 2 h before the seven-day drug pulse was applied in tachyzoite medium. Parasite recovery was monitored by measuring tdTomato fluorescence for 28 days as described above. Medium was exchanged after 24 h. Drug tolerance assays were done as described above for pepin digestion and the oral transmission assay.

(i) Developing drug tolerance. Cysts were generated for indicated times and treated with established antiparasitics (pyrimethamine, sulfadoxine (Sigma)) and HDQ (a kind gift of Wolfgang Böhme), two bupiramide inhibitors (BK1294, BK1553)90,91 or 0.2% DMSO (Thermo Fisher Scientific) as solvent control at the indicated concentrations for seven days. Relative tachyzoite controls were generated by infecting KD3 myotubes with 2.4 × 10⁴ Pru-tdTomato tachyzoites corresponding to a MOI of 1 in tachyzoite medium before treatment.

(ii) Drug resistance of matured cysts vs. tachyzoites. Cyst formation was induced for indicated time and pulsed for seven days with indicated concentrations of sodium fluoride (abcd GmbH) or 0.5% H₂O as solvent control. Relative tachyzoite controls were generated as described above.

(iii) ICCÖ assay tachyzoites. Growth of tachyzoite controls treated with the indicated concentrations of sodium fluoride was monitored throughout the seven-day drug pulse. The half inhibitory concentration (IC₅₀) values were determined at day seven by fitting the dose-response curve by non-linear regression in GraphPad Prism v8 and v9.

Statistical analysis. All tests, except statistics of metabolite abundances, were performed in GraphPad Prism v8 and v9 as detailed in the results section. Statistical significance of metabolite changes was calculated in Microsoft Excel. PC analyses were computed using ClustVis 2.092.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Authors can confirm that all relevant data are included in the paper and/or its supplementary information files. The mzCloud database is available here: https://www.mzcloud.org./. ToxodB is available at https://toxodb.org/. The LCMS data generated in this study has been deposited in the metabolomics workbench database (https://www.metabolomicsworkbench.org) under accession code Study ID ST002051 and
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