Vaccines and drugs against *Neospora caninum*, an important apicomplexan causing abortion in cattle and other farm animals

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**Abstract:** The apicomplexan parasite *Neospora caninum* represents an important abortion-causing parasite in cattle. The economic impact of neosporosis has led to considerable investments to develop vaccines that would prevent infection and abortion. Live-attenuated vaccines have been shown to confer some protection against *N. caninum* infection, but may cause problems due to regulatory issues and other drawbacks. Therefore, efforts have been undertaken to develop recombinant subunit vaccines based on antigens involved in adhesion/invasion or other parasite–host cell interaction processes. Concerning chemotherapeutical agents, the currently known arsenal of active drugs that act against *N. caninum* is limited to a small number of compounds with suitable in vitro properties including low inhibition constants, parasitocidal effects, and low cytotoxicity. For in vivo studies, mostly small laboratory animal models that mimic cerebral infection, acute disease, and fetal loss upon infection during pregnancy have been applied for the assessment of vaccines or drug candidates. However, only a small number of recombinant vaccines and drug candidates have met the expectations, and small laboratory models for neosporosis need to be standardized in order to be able to compare the results of different laboratories. Few vaccines and compounds have made it into trials involving ruminant models such as cattle or sheep, including live-attenuated vaccines and the anticoccidial drug toltrazuril. We here summarize the current status of vaccine and drug development for neosporosis.

**Keywords:** neosporosis, anti-infective agents, chemotherapy, immunotherapy, vaccine, drug target

**Introduction**

Apicomplexan parasites are responsible for a variety of diseases in humans, pets, and/or farm animals, and are thus of high medical, veterinary, and economic importance. *Babesia*, *Besnoitia*, *Cryptosporidium*, *Eimeria*, *Neospora*, *Sarcocystis*, *Theileria*, and *Toxoplasma* are all relevant in the context of causing diseases in farm animals, with a great socioeconomic impact worldwide. *Neospora caninum* is phylogenetically most closely related to *Toxoplasma gondii*, but distinct from *Toxoplasma* with regard to several biological features including the life cycle, host range, and pathogenicity. Canids, namely dogs, wolves, dingoes, and coyotes, represent definitive hosts of *N. caninum*, and besides cattle also sheep, goats, and many more species have been reported as intermediate hosts. Humans do – to our present knowledge – not serve as intermediate hosts for *N. caninum*. Three infective stages of *N. caninum* have been identified to date. These are 1) tachyzoites, which represent the disease-causing and rapidly proliferating stage, which can be transmitted vertically from dam to offspring...
during pregnancy; ii) slowly replicating bradyzoites that form tissue cysts, and which are orally infective; and iii) sporozoites enclosed in oocysts, the end products of a sexual process taking place in the intestine of the definitive host followed by sporulation in the environment, which are also orally infective. Although a sylvatic cycle for N. caninum has been demonstrated, its importance as reservoir for the transmission to domestic animals has not been definitely elucidated.

Neosporosis in cattle causes annual losses of approximately 1.3 billion US dollars through abortion, stillbirth, or birth of weak offspring. In addition, N. caninum infection can result in birth of clinically healthy, but persistently infected calves, which in turn then vertically transmit the parasite to the next generation. Control options to limit the economic impact of neosporosis that have been proposed and modeled include i) testing and culling of seropositive animals, ii) discontinued breeding with offspring from seropositive cows, iii) vaccination of susceptible and infected animals, and iv) chemotherapeutical treatment of calves from seropositive cows. However, the most effective option is not always the most economic one and the suitability of any of these options, including considerations on potential treatment options for dogs, has to be carefully assessed for each case prior to implementation.

In this review, we will focus on the current state of the art of vaccine and drug development against neosporosis, and where appropriate, we will extrapolate to other apicomplexan parasites.

Vaccination against neosporosis

Considerable efforts have been made to develop vaccines against N. caninum, as well as the closely related T. gondii to reduce oocyst shedding in the final hosts (cats for Toxoplasma and canids for Neospora, respectively), and tissue cyst formation in mammals, but to date only a live-attenuated vaccine for prevention of ovine toxoplasmosis (Toxovax) has been licensed, and it is not approved for use in other species. As the mathematical modeling of the costs of the management practices used for the control of neosporosis indicate that vaccination could be an efficient intervention strategy, several research groups have recognized the need for the development of effective vaccines against neosporosis to be used in cattle.

N. caninum has developed distinct adaptations to its intracellular lifestyle and efficient mechanisms to achieve host cell invasion and subsequent intracellular survival have evolved. Knowledge of the molecular basis of these processes is essential for understanding the pathogenic mechanisms underlying infection and for designing strategies to combat neosporosis. The postgenomic era of apicomplexan cell biology offers powerful experimental tools that can be exploited to improve our understanding of parasite survival strategies and pathogenicity.

Selected vaccination studies in mice are summarized in Table 1, and respective studies in cattle and other farm animals are compiled in Table 2. These comprise trials using live or attenuated N. caninum tachyzoites, tachyzoite extracts, or specific polypeptides expressed in various systems. The experimental design of a typical vaccine trial in the mouse model is as follows: i) animals receive a first immunization and one or two vaccine boosts of the antigen of interested emulsified in a suitable adjuvants. Control animals receive a placebo inoculum; ii) in a nonpregnant model, the animals are then challenged with a given dose of freshly isolated parasites. To assess efficacy in a pregnant model, animals are mated prior to challenge infection. In most cases, cell culture-derived tachyzoites have been used, since access to oocysts is restricted. However, this does not represent the natural infection mode; and iii) parameters linked to neosporosis have been measured, such as survival of dams and offspring, clinical symptoms (most notably due to multiple organ failure at the early time points and neurological symptoms at later stages of infection), parasite burden in different organs, especially the brain, and humoral and cellular immune responses. In the mouse model, transplacental transmission of N. caninum to the offspring causes in most cases acute, generalized neosporosis followed by early death of newborns within 30 days. Thus, the mere survival of the newborns during a 4-week period after birth is already a good parameter to measure protection.

Most recombinant subunit vaccine candidates assessed to date have been antigens that represent surface proteins, heat shock proteins, or were derived from proteins that are released from the apicomplexan-specific secretory organelles named micronemes, rhoptries, and dense granules. Most of these antigens were expressed in Escherichia coli, and have been applied either as monovalent vaccines or in different combinations as polyvalent antigen cocktails (Table 1). The selection of suitable vaccine candidates has been largely guided by the idea to target the host cell–parasite interactions that lead to host cell entry. The initial host cell recognition is mediated by parasite surface antigens, while the actual invasion process is dependent on specific molecular interactions between host receptors and parasite ligands secreted from micronemes, rhoptries, and...
Table 1 Summary of selected vaccine studies involving neosporosis in mice

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Ref</th>
<th>Year</th>
<th>Setup</th>
<th>Results</th>
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<tbody>
<tr>
<td>Nc-1 tachyzoites</td>
<td>50</td>
<td>1998</td>
<td>A/j, Balbc, C57BL/6; vaccinated with live Nc-1 tachyzoites; challenge after various days with <em>Toxoplasma gondii</em> tachyzoites.</td>
<td>Complete protection in mice vaccinated with <em>Neospora caninum</em> against acute infection by <em>T. gondii</em>. Early stimulation of CD8+ T-cells.</td>
</tr>
<tr>
<td>SAG1, SRS2 (rE, DNA) alone or combined; crude somatic antigen</td>
<td>51</td>
<td>2003</td>
<td>C57BL/6, vaccine + Ribi (2×), challenge 28 days after the first injection (proteins). pcDNA vector with genes im, challenge 69 days after first injection. Euthanasia after 21 days.</td>
<td>Protection with crude antigen. No protection with recombinant antigens as compared to adjuvant control. Protection with pcDNA in combination with recombinant antigens.</td>
</tr>
<tr>
<td>MIC3 (rE)</td>
<td>52</td>
<td>2003</td>
<td>C57BL/6, vaccinated with MIC3 + Ribi (3×), challenge 7 days after last boost, euthanasia after 21 days.</td>
<td>Reduced cerebral infection in MIC3 vaccinated mice as compared to adjuvant control. Th2-type humoral response associated with protection.</td>
</tr>
<tr>
<td>MIC1 (rE and DNA)</td>
<td>53</td>
<td>2005</td>
<td>C57BL/6, recMIC1 (3× ip), pcDNA-MIC1 (3× im) alone or in combination. Challenge, euthanasia after 21 days.</td>
<td>No clinical symptoms in vaccinated mice. Cerebral infection reduced in mice vaccinated with recombinant protein, enhanced in group with combined vaccination.</td>
</tr>
<tr>
<td>Nc-1 tachyzoites (γ-irradiated)</td>
<td>55</td>
<td>2006</td>
<td>C57BL/6, vaccinated with irradiated tachyzoites (2×). Challenge 6 weeks after last boost (lethal, 107; sublethal, 106). euthanasia 25 days after challenge.</td>
<td>All lethally challenged control mice died within 1 week, all vaccinated mice survived. Protection associated with mixed Th1/Th2 response.</td>
</tr>
<tr>
<td>MIC1, MIC3, GRA2, GRA6, SRS2 (in <em>Brucella abortus</em>)</td>
<td>56</td>
<td>2007</td>
<td>C57BL/6, vaccinated with live <em>B. abortus</em> expressing the antigens (2×), lethal challenge × days after last boost. Euthanasia after 28 days.</td>
<td>All control mice died within 8 days. Complete protection by MIC1 and GRA6.</td>
</tr>
<tr>
<td>MIC1, MIC3, GRA2, GRA6, SRS2 (in <em>B. abortus</em>)</td>
<td>58</td>
<td>2007</td>
<td>C57BL/6, vaccinated with live <em>B. abortus</em> expressing the antigens (2×), mating, sublethal challenge. Euthanasia of pups after 21 days.</td>
<td>Protection against vertical transmission by <em>B. abortus</em> expressing antigens.</td>
</tr>
<tr>
<td>MIC4 (native, rE, DNA)</td>
<td>20</td>
<td>2007</td>
<td>C57BL/6, vaccinated 3× in 4-week interval, sublethal challenge, euthanasia after 21 days.</td>
<td>Mice of all vaccinated groups showed neosporosis symptoms and had an increased mortality as compared to the control group.</td>
</tr>
<tr>
<td>ROP2 (rE)</td>
<td>19</td>
<td>2008</td>
<td>C57BL/6, vaccinated 3× in 2-week interval either with Freund’s incomplete or saponin, challenge, euthanasia after 35 days.</td>
<td>No symptoms in vaccinated mice, reduced parasite burden in brains of vaccinated mice. Th1- or Th2-type humoral response depending on the adjuvant.</td>
</tr>
<tr>
<td>ROP2 + MIC1 + MIC3</td>
<td>59</td>
<td>2009</td>
<td>BALB/c, single vaccines or in combination (3×), mating, challenge at day 7 postmating, euthanasia of dams and nonpregnant mice after 30 days.</td>
<td>Reduced vertical transmission by ROP2 alone or in combination. Humoral and cytokine responses associated with a Th2 immune response.</td>
</tr>
<tr>
<td>Nc expressing TgSAG1</td>
<td>61</td>
<td>2010</td>
<td>BALB/c, vaccinated 2× with 107 Nc-1 expressing TgSAG1 or GFP. Challenge 4 weeks after last boost with <em>T. gondii</em> (500 tachyzoites).</td>
<td>Moderate protection by Nc/GFP, good protection by Nc/TgSAG1. Immune response Th1-dominant.</td>
</tr>
<tr>
<td>PDI, ROP2, MAG1 (rE)</td>
<td>62</td>
<td>2010</td>
<td>C57/BL6, vaccinated with saponin as adjuvant (ip) or intranasally (in) with cholera toxin as adjuvant (3×, 15 days intervals). Challenge 2 weeks after last boost, euthanasia after 28 days.</td>
<td>Reduced cerebral loads with ROP1 (ip, in) and PDI (in only). Protection against clinical symptoms only by PDI (in).</td>
</tr>
<tr>
<td>Nc expressing NcSAG4</td>
<td>63</td>
<td>2011</td>
<td>Female BALB/c, vaccinated twice with Nc-1 expressing SAG4, some were mated, challenge at day 7 of gestation.</td>
<td>Protection against vertical transmission by Nc-1 wt and Nc-1 expressing SAG4, not associated with constant Th1- or Th2-type immune response.</td>
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</table>

(Continued)
dense granules. The timely release of these ligands from apical organelles to the parasite surface is crucial for receptor engagement and invasion.17 Therefore, the majority of antigens investigated to date have originated from selected surface-associated or secreted proteins. At this point, it is noteworthy to mention that a mere database-dependent research for vaccine candidates may lead on the wrong track. An example is the rhoptry protein ROP18. It is a pseudogene in *N. caninum*, but expressed in the closely related *T. gondii*. *N. caninum* expressing TgROP18 as a

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**Table 1 (Continued)**

<table>
<thead>
<tr>
<th>Vaccine</th>
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<tbody>
<tr>
<td>Cyclophilin, SRS2 (rE)</td>
<td>64</td>
<td>2011</td>
<td>Female BALB/c; antigens alone or in combination with adjuvants (sc, 2×; 2-week interval). Control with irrelevant bacterial antigen. Challenge 3 weeks after last boost. Euthanasia 3 weeks after challenge.</td>
<td>Humoral response against antigens. Higher protection against cerebral infection when cyclophilin was present. Lower protection with SRS2 alone.</td>
</tr>
<tr>
<td>MIC1-MIC3-ROP2 (chimeric, rE)</td>
<td>65</td>
<td>2011</td>
<td>Female BALB/c; immunized with combinations of antigenic domains from MIC1, MIC3, and ROP2 with saponin as adjuvant (ip, 3×; 2-week interval). Challenge 2 weeks after last boost. Euthanasia 36 days post-challenge.</td>
<td>Complete protection by one combination only (MIC3-1-R), correlated with lower parasite load in brains in nonpregnant mice.</td>
</tr>
<tr>
<td>Nc tachyzoites (live)</td>
<td>66</td>
<td>2012</td>
<td>Female BALB/c; immunized with live Nc Spain H-1 tachyzoites (sc, 2× at 3-week interval). For pregnant model, mating, challenge ad mid gestation with Nc Liv.</td>
<td>Reduction of neonatal mortality, reduction of vertical transmission, and lower cerebral parasite load in nonpregnant mice.</td>
</tr>
<tr>
<td>Nc tachyzoite extract</td>
<td>68</td>
<td>2012</td>
<td>BALB/c; vaccinated with different amounts of extract formulated with various adjuvants (sc, 2×; 2-week interval). Challenge at 38 days postvaccination with Nc-1. Euthanasia 21 days post-challenge.</td>
<td>Immune responses depend on formulation, no protection.</td>
</tr>
<tr>
<td>MIC1-MIC3-ROP2 chimeric (rE)</td>
<td>69</td>
<td>2013</td>
<td>BALB/c; vaccine + saponin or Freund’s incomplete (3×), pregnant.</td>
<td>Protection in combination with saponin in the nonpregnant model, associated with Th1/Th2 response. No protection in pregnant model. With Freund’s, limited or no effects, Th1 response.</td>
</tr>
<tr>
<td>SRS2-GRA7-fusion in adenovirus</td>
<td>70</td>
<td>2013</td>
<td>BALB/c, vaccinated (im) with recombinant adenovirus containing the fusion or GFP as a control (3×).</td>
<td>IFN-γ and IL-4 levels elevated in adenovirus infected mice as compared to control mice. Higher levels with SRS2-GRA7-fusion than with GFP.</td>
</tr>
<tr>
<td>BAG1, BSR4, MAG1, SAG4 (rE)</td>
<td>71</td>
<td>2013</td>
<td>BALB/c, vaccinated with four bradyzoite antigens with PBS or bitter gourd extract as adjuvants (im, 2×). Challenge with Nc Liv 3 weeks after last boost.</td>
<td>Antigen specific IgG1 and IgG2 and IFN-γ responses to all antigens. Protection from acute infection and lower parasite load in mice vaccinated with BAG1, MAG1, and SAG4.</td>
</tr>
<tr>
<td>PDI (rE)</td>
<td>72</td>
<td>2013</td>
<td>BALB/c, female, vaccinated with PDI and cholera toxin (subunits A and B or subunit B alone) as adjuvants, intranasal (3×, 2-week interval), mating, challenge at day 7 postmating.</td>
<td>Good protection against cerebral infection by PDI with subunits A and B as compared to cholera toxin alone. No effect with subunit B alone. No protection against vertical transmission.</td>
</tr>
<tr>
<td>SAG1, SRS2, MIC3 (BmNPB)</td>
<td>73</td>
<td>2014</td>
<td>Vaccination with BmNPB displaying antigens or wt BmNPB (im, 3× at 2-week interval). Challenge with Nc Liv after last challenge.</td>
<td>Reduced parasite load in brains in all groups vaccinated with BmNPB as compared to placebo. No effect due to displayed antigens.</td>
</tr>
<tr>
<td>SAG1 + Hsp20 + Gra7 (rE)</td>
<td>74</td>
<td>2014</td>
<td>Cattle, vaccine + stimulating complexes or complexes alone (2×), mating, challenge after 70 days with Nc-1, slaughter after 104 days.</td>
<td>Immune responses against all three proteins. Nc detection in lungs in all three groups in CNS and lungs in all three groups, no significant differences.</td>
</tr>
</tbody>
</table>

**Abbreviations:** BmNPB, *Bombyx mori* nucleopolyhedrovirus; CNS, central nervous system; im, intramuscular; ip, intraperitoneal; sc, subcutaneous; rE, recombinantly expressed in *Escherichia coli*; DNA, DNA vaccine; Ref, reference; wt, wild type; PDI, protein disulfide isomerase; PBS, phosphate buffered saline; Nc, Neospora caninum; Nc Liv, *N. caninum* Liverpool isolate.
transgenic become more virulent for mice than control transgenics.18

Vaccine studies, as presented in Tables 1 and 2, are difficult to compare since the models employed exhibit a large degree of variation, not only with respect to the vaccine to be tested but also with respect to the mouse lines, the Neospora strains, the timespans between vaccination and challenge, challenge and vaccination procedures, vaccine formulations (adjuvants), etc. Nevertheless, some conclusions can be drawn from these studies: i) vaccination with live or attenuated N. caninum tachyzoites has emerged to be the most efficient for the protection of mice as well as cattle against acute infection and prevention of vertical transmission; ii) concerning subunit vaccines, some have exhibited good protection (eg, ROP220), others were ineffective or even exhibited antiprotective effects (eg, MIC420); and iii) it is difficult to establish correlations between effects of a given vaccine and respective immune responses.

As mentioned earlier, the recombinant vaccines used in the studies are based on proteins involved in adhesion and penetration of the host cell. Therefore, it cannot be excluded that a protective effect of antibodies raised against these proteins is due to a direct impairment of adhesion and invasion by the parasite21 rather than due to the stimulation of a specific cellular immune response. Since these effects could be mimicked (in theory) by directly applying the respective antibodies, one would speak about immunotherapy rather than vaccination.22 Another difficulty in interpreting the results with recombinant vaccines comes from the fact that many of them were expressed in E. coli and affinity purified from crude extracts. Therefore, contaminations with immune-modulating agents from bacterial origin such as lipopolysaccharides cannot be ruled out, and controls with irrelevant proteins expressed in the same system or with lipopolysaccharide-depleted protein fractions23 should always

Table 2 Summary of selected vaccine studies against neosporosis in farm animals

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Ref</th>
<th>Year</th>
<th>Setup</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neospora caninum tachyzoites</td>
<td>76</td>
<td>2003</td>
<td>Seronegative ewes, vaccinated with tachyzoites + adjuvant (2×). Challenge during pregnancy.</td>
<td>Humoral response against N. caninum in vaccinated ewes higher than in control ewes. Lower N. caninum DNA levels in lambs from vaccinated ewes.</td>
</tr>
<tr>
<td>Natural infection by N. caninum</td>
<td>77</td>
<td>2003</td>
<td>Naturally infected and naive cows, challenged at week 10 of gestation.</td>
<td>Natural infection protects against abortion induced by challenge, but not against vertical transmission.</td>
</tr>
<tr>
<td>N. caninum tachyzoites (killed)</td>
<td>78</td>
<td>2004</td>
<td>Ewes, vaccinated with tachyzoites + adjuvant (2×). Challenge 30 days after last boost.</td>
<td>Protection against abortion, but not against vertical transmission.</td>
</tr>
<tr>
<td>N. caninum tachyzoites (killed)</td>
<td>79</td>
<td>2004</td>
<td>Field trial with dairy cattle. No challenge.</td>
<td>Reduction of abortion from 20% in the placebo group to 11% in the vaccinated group. Vaccination increases the risk of vertical transmission. In one of five herds, vaccination reduced abortion.</td>
</tr>
<tr>
<td>N. caninum tachyzoites (killed)</td>
<td>80</td>
<td>2012</td>
<td>Clinical trial with a killed tachyzoite vaccine (Bovilis Neoguard) on five dairy farms (sc, 2×; 4-week interval).</td>
<td>Increased IgG1 and IFN-γ levels in vaccinated animals as compared to controls. Stimulation of CD4+ and CD8+ cells.</td>
</tr>
<tr>
<td>N. caninum tachyzoite extract</td>
<td>81</td>
<td>2013</td>
<td>Cattle, aqueous tachyzoite extract at various concentrations with soybean based adjuvant (2×). No challenge.</td>
<td>IgG and IFN-γ levels increased as compared to controls. Lower parasite load in brains in cattle immunized with 50 μg.</td>
</tr>
<tr>
<td>GRA7 (rE)</td>
<td>82</td>
<td>2013</td>
<td>Culture, GRA7 (50–200 μg) entrapped in oligomannose microsomes (sc, 2×). Challenge with Nc-1 27 days after last boost. Euthanasia at 85–87 dpi.</td>
<td>Strong IgG and IFN-γ responses postimmunization. No fetal loss in immunized but not challenged heifers. In challenged heifers, 50% protection against fetal loss.</td>
</tr>
<tr>
<td>N. caninum tachyzoites (live)</td>
<td>83</td>
<td>2013</td>
<td>Seronegative heifers, immunized with Nc-Spain H1 (2×), challenge with Nc-1 postmating (2×).</td>
<td>Protection against abortion by vaccination, best with live tachyzoites iv.</td>
</tr>
<tr>
<td>N. caninum tachyzoites (live or frozen)</td>
<td>84</td>
<td>2013</td>
<td>Cattle, 96 seronegative animals, immunized with Nc-Nowra (sc or iv, 1×), mating. Pregnant heifers were challenged.</td>
<td>Immune responses against antigens. No IFN-γ response. No protection against vertical transmission.</td>
</tr>
<tr>
<td>SAG1 + HSP20 + GRA7</td>
<td>74</td>
<td>2014</td>
<td>Pregnant heifers, immunized with recombinant proteins formulated with ISCOM (sc, 2×). Challenge with Nc-1 at day 70 of gestation.</td>
<td>Immune responses against antigens. No IFN-γ response. No protection against vertical transmission.</td>
</tr>
</tbody>
</table>

Abbreviations: iv, intravenous; ISCOM, immune stimulating complex; Ref, references; sc, subcutaneous; dpi, days post-infection.
be included. A fusion of the recombinant antigen with a lipid may facilitate recognition by a toll-like receptor of type 2 and modify the subsequent immune responses.24

In mice, studies with vaccines on acute infection models are often terminated after 3–4 weeks post-challenge. It is thus very difficult to extrapolate the immune responses observed in these studies to chronic infections as observed in cattle. Moreover, the immune responses of rodents and ruminants show highly dissimilar characteristics. In mice, elevated levels of IFN-γ have been correlated to increased IgG2a and IgG3 production, high IL-4 to increased IgG1 and IgE synthesis, and TGF-β to IgA. In cattle, the situation is less polarized, and the classical roles of many cytokines in the laboratory mouse do not extrapolate entirely or at all to cattle.25 Therefore, a suitable approach for the selection of suitable vaccine candidates would be to perform in vitro assays with bovine CD4+ cytotoxic lymphocytes, as done by Staska et al.26 The candidate polypeptides inducing the highest level of IFN-γ secretion should then be retained for further assessment.

**Drugs as an option to intervene in neosporosis?**

Until recently, chemotherapeutic treatment of seropositive farm animals infected with *N. caninum* has not been regarded as an economically viable option, due to the potentially rather long withdrawal period during which milk or meat from drug-treated animals remains unacceptable.27 Thus, there are no effective and safe anti-Neospora drugs on the market. However, the inherent difficulty in identifying an efficacious vaccine that has the ability to protect against *N. caninum* infection in pregnant animals renders a chemotherapeutic approach more interesting. Experimental studies have revealed potentially interesting effects of several compounds in vitro and in laboratory animal models in vivo.28 These compounds were mainly derived from screenings against *Plasmodium*. Their application against *Neospora* would thus constitute a good example of drug repurposing.29

**Identification of suitable chemotherapeutics**

The strategies to identify antiparasitic agents against apicomplexan parasites are discussed elsewhere.1,30 Briefly, drug candidates can be initially identified by in vitro tests, where suitable host cells (eg, fibroblasts) are infected with *N. caninum* tachyzoites in the presence of the test compounds or a solvent control. After a given time period, ie, when the controls show a high level of infection, the experiment is stopped and the tachyzoites are quantified by a suitable method. On the one hand, quantitative real-time polymerase chain reaction will provide information on the respective parasite loads. However, a suitable alternative for drug screening is the transgenic *N. caninum* Ne-1 isolate-derived strain expressing *E. coli* beta-galactosidase under the control of a GRA1 promoter.31 At first, these in vitro studies will provide inhibition constants (eg, IC50 values), and data concerning host cell toxicity can be obtained using standard viability assays such as AlamarBlue. Furthermore, in vitro studies allow to assess whether a compound is parasitocidal or parasitostatic, whether it affects intracellular parasites, extracellularly located parasites or both, and they are suitable to assess the risk of resistance formation. Combined with morphological and structural investigations (eg, scanning electron microscopy and transmission electron microscopy), such in vitro studies can provide an initial characterization of the effects of a given compound. An example of a detailed study dealing with such aspects has been performed with *T. gondii* strains and pentamidine derivatives.32

In vivo drug assessments in mice can be performed using the same models as described earlier for the vaccine studies. Ideally, a standardized mouse model should be used in order to render different experiments with different compounds performed in different laboratories comparable. At best, only compounds that have been characterized in terms of their toxicity, stability, pharmacokinetic properties, and bioavailability are used for in vivo experiments. After inoculation of *N. caninum* tachyzoites, one should ideally allow the parasite 2–3 days to establish the infection prior to initiation of treatment, and protective effects against acute infection and against placental transmission are analyzed. In the latter case, pregnant mice are infected and treated during pregnancy. Since it cannot be ruled out that a given compound could affect pregnancy and offspring, in some cases, controls with uninfected dams have to be included.

**Effective compounds against neosporosis – an overview**

Selected studies on drugs against neosporosis are compiled in Table 3. Most experimental treatments have been performed with toltrazuril, a triazinone derivative effective against various coccidians including *Eimeria*,33 and commercialized under the trivial name Baycox™. The mode of action of toltrazuril and of its main metabolite toltrazuril sulfone (ponazuril) is not only related to the inhibition of dihydroorotate dehydrogenase and therefore pyrimidine biosynthesis, but also to the inhibition of the respiratory chain of the parasite.34 Whereas the effects against coccidian
infections are well documented in poultry, as well as in cattle, it remains unclear whether toltrazuril is a suitable drug against neosporosis in cattle (Table 3). Thiazolides, including nitazoxanide, the mother compound of this class, exhibited interesting effects against *N. caninum* in vitro, but failed in vivo when orally applied, and even showed acute toxicity when applied intraperitoneally (Table 3). This is most likely due to induction of host cell apoptosis. The most promising drug candidates for neosporosis treatment come from compounds initially developed against *Plasmodium* such as artemisinin and pentamidine derivatives and spiroindolones, a novel class of antimalarials, inhibiting a Na⁺-efflux pump in *Plasmodium*. Among other drug targets, calcium-dependent kinase I (CDPK1) in *N. caninum* deserves particular interest. CDPK1 is essential for microneme secretion, host cell invasion, and egress of *T. gondii*. A particular class of inhibitors, bumped kinase inhibitors, has bulky C3 aryl substituents, which can enter and block a hydrophobic pocket in the adenosine triphosphate binding site due to a small (glycine) gatekeeper residue. BKIs selectively inhibit CDPK1 from apicomplexans, exhibiting a good structure–activity relationship but do not inhibit mammalian kinases because these have larger gatekeeper residues adjacent to the hydrophobic pocket thereby blocking the entry of the bulky C3 aryl group. Some BKIs, especially BKI-1294, exhibited interesting effects against *N. caninum* in vitro, but do not inhibit mammalian kinases: they are, however, not directly parasitocidal.
foreskin fibroblast monolayers for more than 20 days at 2.5 μM BKI-1294, parasitocidal effects have been observed. Similar findings have been obtained for different strains of T. gondii, where death of intracellular parasites is preceded by the formation of large, multinucleated complexes with a deregulated gene expression as evidenced by the expression of bradyzoite as well as tachyzoite antigens. A similar induction of bradyzoite antigen expression was observed when treating N. caninum-infected fibroblast monolayers with artemisone and respective derivatives.

Toward a strategy against neosporosis

During the last decade, a number of in vitro and in vivo studies revealed some promising vaccine and drug candidates against neosporosis. None of them could, however, achieve full protection against transplacental transmission of N. caninum, the goal that should ultimately be achieved in cattle. Nevertheless, the promising results of both approaches could potentially be translated into a combined immuno-chemotherapeutical approach. The simplest model of an immune-chemotherapy would consist in applying a live- or attenuated vaccine together with a compound with high efficacy as shown in previous in vitro and in vivo studies. Such an approach has long been developed to vaccinate against theileriosis, with cattle being inoculated with live sporozoites and immediately treated with buparvaquone, the only drug currently available against *Theileria parva* and *Theileria annulata*, so far.

Another model could consist in applying suitable chemotherapeutics together with polypeptides acting as classical vaccines or immune-stimulators. Recombinant proteins produced in *E. coli* or via another suitable expression system may contain impurities and are very expensive, especially when produced in high purity at large scales. On the other hand, the chemosynthesis of peptides has become increasingly cost-effective. Highly antigenic peptides could thus be produced by chemosynthesis, coupled to a high molecular weight carrier to render them immunogenic and/or to a suitable TLR-ligand, and could then be coapplied with a suitable chemotherapeutic agent.

Taken together, these encouraging results indicate that the ultimate goal of a one-shot therapy against neosporosis in cattle could become feasible. More in vitro as well as in vivo research using appropriate and, most importantly, standardized animal model is, however, required to reach this goal.

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