REVIEW

Department of Pharmacology & Toxicology[1], University of Veterinary and Animal Sciences; Department of Theriogenology[2], University of Veterinary and Animal Sciences, Lahore, Pakistan

Metronidazole and the immune system

L. Shakir[1], A. Javeed[1], M. Ashraf[1], A. Riaz[2]

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Dr. Aqeel Javeed, Department of Pharmacology & Toxicology, University of Veterinary and Animal Sciences, Abdul Qadir Jilani Road, Lahore, Pakistan, 54600
aqeelvet@hotmail.com

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Metronidazole (MTZ) is a nitroimidazole antibiotic used mainly for the treatment of infections caused by susceptible organisms, particularly anaerobic bacteria and protozoa. Distinct from its antibiotic, amoebicidal, and antiprotozoal effects, MTZ displays immunopharmacological behaviour. This review outlines multiple effects of MTZ on different aspects of immunity, including innate and acquired immunity, and also highlights the immunopharmacological behaviour of MTZ in terms of its relevance to inflammation, delayed type hypersensitivity (DTH) and graft versus host disease (GVHD).

1. Introduction

Nitroheterocyclic chemicals have been used in a wide variety of applications, ranging from food preservatives to antibiotics. The 5-nitroimidazoles are a well-established group of antiprotozoan and antibacterial agents. They contain a heterocyclic structure consisting of an imidazole-based nucleus with a nitro group, NO2, in position 5. Metronidazole [1-(hydroxyethyl)-2-methyl-5-nitroimidazole] is prescribed for infections of protozoal origin (tricomoniasis, giardiasis and amebiasis) and also for infections by anaerobic micro-organisms (Galmier et al. 1998; Menendez et al. 2001). It is also used to cure gastric infections caused by Helicobacter pylori and in Crohn's disease (Tsai and Chen 2003; Dilger et al. 2007). In addition, MTZ has also been used as a radiosensitizer for hypoxic or tumor cells (Menendez et al. 2002). MTZ is highly and repeatedly prescribed because of reinfections, inappropriate prescribing and self-medication.

All investigational new drugs should be evaluated for their potential to produce immunomodulating effects. Most antiprotozoal drugs greatly alter the recipient's immune response. Some drugs potentiate the immune response while others suppress the immune response (Hanson 1981). MTZ has multiple effects on different aspects of immunity. In this review, we will highlight the effects of MTZ on different components of the innate and adaptive immune system, such as: changes in the levels of different cytokines of the immune system, anti-inflammatory effects, neutropenia and inhibiting the generation of reactive oxygen species (ROS), reduction in the number and function of macrophages, lymphocyte proliferation. and damage to lymphocyte DNA. We will also discuss the effect of MTZ on delayed type hypersensitivity (DTH) and graft versus host disease (GVHD) and lastly its effect on the humoral component of immunity.

2. Metronidazole and innate immunity

The early replication and spread of infectious microorganisms is suppressed by the innate component of immunity (Le Bon and Tough 2002). MTZ and ciprofloxacin have a great effect on innate immunity in monoblasts by shutting down certain bacterial pattern recognition programs (TLR-1 and TLR- 5) and up-regulating others (TLR-4 and TLR-7).

Cytokines can be produced in all tissues but mostly in cells which are subject defense, development and repair (Hopkins 2003). Cells of the innate immune system produce cytokines such as IFN (α and β), GM-CSF, TNF-α, IL-12, IL-15, IL-18, and IL-1 (Belardelli and Ferrantini 2002). As the level of cytokines controls the response of the immune system, so a fall in their level corresponds to immunosuppression. MTZ treatment has been involved in decreased cytokine levels. Treatment with oral and vaginal MTZ induced a decrease in cytokine levels in pregnant women with bacterial vaginosis. Cervical levels of IL -1beta, IL-6, and IL -8 were considerably lowered after 4 weeks as they recovered from bacterial vaginosis (Yudin et al. 2003). Similarly, another study reported that MTZ treatment in children with giardiasis complicated and uncomplicated by allergy played a role in converting high levels of TNF-α,sIL-2R, IL-1β, IL-6, IL-8, CRP, and nitric oxide to normal levels (Bayraktar et al. 2005). The combination of MTZ therapy and Escherichia coli strain M-17 resulted in massive reductions in the colonic levels of the cytokines IL-12, IL-6, IL-1beta and IFN-γ in mice (Fitzpatrick et al. 2008). In addition, the continuous use of MTZ and aztreonam decreased lung neutrophil content (MPO concentration), TNF and IL-1 mRNA expression (Mercer-Jones et al. 1998).

2.1. Metronidazole and inflammation

Inflammation protects the body from harmful substances, directing components of the immune system to the site of injury or infection. But inflammation may play a role in the development of a variety of serious conditions that affect many people. MTZ prevented the progression of inflammatory changes in the lungs of guinea pigs suffering from anaerobic pneumonia by reducing anaerobic flora, and activating a local immune reaction (Shroit et al. 1986). Similarly, intestinal inflammation was considerably decreased after 2–12 weeks treatment with MTZ (800 mg/day) while patients were maintained on an

unchanged non-steroidal anti-inflammatory drug (Bjarnason et al. 1992). A central early event in the inflammatory process is the migration of leukocytes from the blood stream to tissue (Savilahti et al. 2004). The anti-inflammatory activity of MTZ is related to its effect on leukocyte endothelial cell adhesion. It reversed the enhanced leukocyte attachment and emigration responses induced by indomethacin and was also effective in blocking the decrease in leukocyte rolling velocity and increased leukocyte adherence/emigration induced by exposure of venules to leukotriene B4 in rats (Arndt et al. 1994).

MTZ and sulfasalazine minimized substantial bacterial translocation into the mesenteric lymph nodes, liver, and spleen in a chronic model (two daily injections of indomethacin by subcutaneous injection) in rats, indicating possible suppression of immunity (Yamada et al. 1993). Similarly, MTZ plays a vital role in the suppression of cap polyposis by decreasing the inflammatory infiltrate (Shimizu et al. 2002). MTZ has shown effective anti-inflammatory and anti-bacterial effects on septic microcirculation. In untreated CASP or LPS animal models, MTZ administration greatly decreased the enhanced leukocyte adhesion to the endothelium within the V1 venule (Lehmann et al. 2006). MTZ (0.5%) subgingival irrigation once daily combined with a simple oral hygiene regimen was comparatively more effective in decreasing chronic periodontitis as evaluated in a randomized, double-blind, placebo-controlled trial.

The anti-inflammatory functions of MTZ, as compared to its antibacterial ones, contribute to its superior role in acne vulgaris (Bannatyne 1999). MTZ, having both anti-inflammatory and antimicrobial activity, is effective in the treatment of rosacea. The antioxidant activity of MTZ helps to stop oxidative tissue damage *in vitro* (Miyachi 2001). The anti-inflammatory effect of MTZ partly contributes to its effective role in curing papulopustular rosacea. Neutrophil cell functions are affected by the antioxidant action of MTZ (Miyachi et al. 1986). Topical MTZ should be prescribed as first-line therapy because it inhibits chain reactions of the inflammatory process involved in rosacea (Millikan 2003).

Combined therapy with topical MTZ gel (1%) and doxycycline for mild to moderate rosacea was proved to be more effective and better tolerated in reducing inflammation than topical MTZ (1%) gel alone in a 16-week, randomized, double-blind, placebo-controlled study (Fowler 2007). Inflammatory cytokines (Interferon-γ) speed up inflammatory processes by activating immunocompetent cells (Kunikata et al. 2000). MTZ for porphyromonas gingivalis-treatment in ApoE+/– mice lowered the levels of proinflammatory cytokines (Amar et al. 2009).

2.2. Metronidazole and neutrophils

In the ascendant phase of infection, polymorphonuclear phagocytes (neutrophils, basophils, and eosinophils) are of much importance (Beutler 2004). MTZ concentrations within human polymorphonuclear leukocytes were similar to the extracellular level as determined by a velocity gradient centrifugation technique (Hand et al. 1987). Brief neutopenia has been observed after MTZ therapy in patients (Lefebvre and Hesseltine 1965). Similarly, MTZ decreased the percentage of neutrophils in Balb/c mice (Fararjeh et al. 2008). These findings show immunosuppression induced by MTZ.

Reactive oxygen intermediates are produced within the phagosome of neutrophils and macrophages (Belardelli and Ferrantini 2002). Reactive oxygen species (ROS) can cause oxidative damage to nucleic acids, sugars, proteins and lipids. MTZ together with palmitoleic acid greatly decreased ROS production by neutrophils which ultimately resulted in significant amelioration of rosacea and acne in humans (Akamatsu et al. 1990). MTZ significantly inhibited the generation of ROS from neutrophils which causes disruption of the integrity of the follicular epithelium, that in turn contributes to inflammation (Akamatsu and Horio 1998).

MTZ and tinidazole (derivatives of nitroimidazole) 1–4% ointments have anti-inflammatory, immunosuppressive and anti-itching effects on inflammatory dermatitis models (evoked by antigen, hapten and monoclonal anti-dinitrophenol IgE antibody) in mice. MTZ decreased neutrophil-generated free radicals at sites of inflammation with the aid of palmitoleic acid in the skin and inhibited oxidative tissue injury under *in vivo* conditions in mice (Nishimuta and Ito 2003). MTZ exerts antioxidant effects by decreasing ROS production through alteration of the neutrophil function and reducing ROS concentration through its ROS scavenging properties as shown in a simple skin lipid model system and a complex skin adapted it lipid system (Narayanan et al. 2007). MTZ together with 2% diphenhydramine hydrochloride, 0.1% zinc acetate cream and a moisturizer cause decrease of erythema and papule size.

MTZ at different concentrations has no effect on neutrophil chemotaxis to endotoxin-activated autologous serum, random motility and postphagocytic metabolic activity in humans (Anderson et al. 1979). Variations in individual values of neutrophil chemiluminescence indices (as determined by luminol dependent chemiluminescence) were observed after MTZ treatment (250 mg BID) because of the effect of MTZ and/or its metabolites on the blood cells of patients with urogenital chlamydial infection (Shchepetkin and Iur'ev 1998).

2.3. Metronidazole and macrophages

The heterogenous component of innate immunity is macrophages, which have diverse functions and phenotypes depending on their location and activation state (Bowdish et al. 2007). Important functions of macrophages include engulfing, killing microbes and supervision (Beutler 2004). MTZ has a limited capacity to enter monocytes, generating cellular concentrations equal to or less than the extracellular levels (Hand and King-Thompson 1989). The percentage of circulating peripheral blood monocytes was significantly decreased by MTZ. A range of concentrations (5, 10, 50, and 200 μg/mL) of MTZ caused a significant decrease in the phagocytic activity of peritoneal macrophages. The concentration of TNF-α was also significantly reduced by MTZ in a dose-dependent manner (Fararjeh et al. 2008). MTZ inhibited the ability of sonicated pouchitis flora to stimulate proliferation of mononuclear cells in an *ex vivo* cell culture assay in the presence of antigen presenting cells. In separate assays, with other stimuli, neither direct addition of MTZ nor of its metabolite influenced mononuclear cell proliferation (Bell et al. 2004). In an *in vitro* study with human peripheral blood mononuclear cells, MTZ displayed considerable inhibition of endotoxin-stimulated TNF-α generation, at therapeutic levels than in a whole-blood assay system (Krehmeier et al. 2002). Based on these findings, immunosuppression by MTZ is evident in the macrophage function. In contrast, another study reported that MTZ increased the phagocytic and killing ability of peritoneal macrophages in rats and humans in a dose-dependent fashion up to a certain dose as measured by an *in vitro* assay technique. But in compromised host defenses the monocyte function was reduced (Rege and Dahanukar 1993).

3. Metronidazole and acquired immunity

3.1. Metronidazole and lymphocyte proliferation

The standard of the immune system of individuals is mainly determined in practice by estimating the number of lymphocyte subsets in the peripheral blood (Blum and Pabst 2007). A

number of scientists have contributed to determining the influence of MTZ on lymphocyte proliferation. MTZ has an immunopotentiating effect as it removes disturbances of the balance in some subpopulations of immunocompetent cells and so resets the percentage of cells governing immune response to a normal level (Shroit et al. 1986). MTZ in therapeutic doses has a possible immunopotentiating effect as indicated by extended lymphocyte proliferation kinetics in MTZ-treated patients (Elizondo et al. 1994). Similarly, MTZ treatment elevated human lymphocyte proliferation with inter-individual differences. MTZ and its hydroxyl metabolites elevated the mitogenic response to phytohemagglutinin (PHA) in a dose dependent manner. During competitive studies, MTZ and its hydroxyl metabolites blocked the inhibitory effect of histamine on lymphocyte proliferation in a dose dependent fashion by increasing the mitogenic response to PHA (Elizondo and Ostrosky-Wegman 1996). MTZ and its three analogues enhanced the third division metaphase of human lymphocyte culture after metabolic activation (Gomez-Arroyo et al. 2004). In contrast, the concentration-dependent inhibition by MTZ of human peripheral blood lymphocyte proliferation, indicates its immunosuppressive action (Fararjeh et al., 2008).

3.2. Metronidazole induced damage to lymphocytes

The proliferation of lymphocytes is inhibited with breaking of lymphocyte DNA, which in turn produces immunosuppression. MTZ also causes damage to the DNA of lymphocytes. MTZ treatment (at the recommended doses in patients with *Trichomonas vaginalis* infection) caused DNA single strand breaks in lymphocytes, which were recouped after seven days' therapy (Reitz et al. 1991a). MTZ exposure induced concentration- and time-dependent DNA single strand breaks in resting and proliferating human lymphocytes, proliferating lymphocytes presenting a higher number of DNA breaks than resting ones.(Reitz et al. 1991b). Breakage of DNA helix content, strand damage, and concomitant functional inhibition have been observed with reduced MTZ (Knight et al. 1978).

Addition of MTZ to human lymphocyte cultures which were excited by exposure to plant mitogens prolonged the blast transformation response (Miller et al. 1980). It was observed that 1-acetic acid-2-methyl-5-metronidazole repressed murine lymphocyte transformation while it was enhanced by MTZ and its hydroxy metabolite at concentrations corresponding to human serum levels after a normal dose of MTZ (Bahr and Ullmann 1983). MTZ induced considerable enhancement in the number of irregular anaphases and chromosome abnormalities *in vitro* (Mudry et al. 1994). Another study reported that a considerable increase in the fraction of cells displaying chromatid and isochromatid damage was observed in peripheral blood lymphocyte cultures after MTZ treatment (1500 mg per day for 10 days) (Elizondo et al. 1996). Human peripheral blood lymphocytes showed a great increase in single strand breaks following MTZ exposure (Rodriguez Ferreiro et al. 2002).

MTZ treatment of non-smoking women with *Trichomonas vaginalis* infection resulted in an enhancement in the rate of sister-chromatid exchange, a fall in the mitotic index, changes in cell proliferation kinetics and a fall in the replication index in lymphocyte cultures (Carballo et al. 2004). Genotoxicity was observed *in vitro* at all doses of MTZ after its incubation with feline peripheral blood mononuclear cells and feline T-cell lymphoma line, and also *in vivo* (cats) in peripheral blood mononuclear cells after oral administration (Sekis et al. 2009). The *in vitro* cytotoxic and genotoxic effect of MTZ at plasma concentrations on cultured human peripheral blood lymphocytes was somewhat higher than encountered therapeutically (Celik and Aras Ates 2006). Some MTZ metabolites, as compared to the parent drug, displayed a great increase in DNA strand breaks in lymphocytes of healthy individuals (Menendez et al. 2001). Proliferation was enhanced in a dose response fashion after MTZ treatment in all P53 functional cell lines. On the other hand, the hydroxy metabolite of MTZ did not affect cell proliferation (Menendez et al. 2002). In patients with Crohn's disease, no considerable enhancement of irregularities in the number or structure of chromosomes was reported after administration of MTZ or sulphasalazine alone, but, interestingly, a great increase in the number of chromatid and isochromatid damages was observed after combined therapy with MTZ and sulphasalazine (Mitelman et al. 1982). MTZ and dimetridazole caused DNA breakage in human lymphocytes (Re et al. 1997). Genotoxic and cytotoxic effects were reported for MTZ and ornidazole at therapeutic doses with decreased mitotic index, elevated sister chromatid exchange and chromosomal aberrations in human peripheral blood cultures (Lopez Nigro et al. 2003). MTZ incubated with V79-379A cells presented a marked clastogenic action in hypoxic, but not in oxygenated cells (Korbelik and Horvat 1980).

In contrast, a group of researchers believed that no damage to the DNA of lymphocytes was caused by MTZ, reporting that MTZ and its metabolites [2-methyl-5-nitroimidazole-1-yl acetic acid and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole] did not exert a direct genotoxic action on human lymphocytes *in vitro* (Lambert et al. 1979). MTZ in cultured human lymphocytes did not induce any enhancement in sister chromatid exchanges. Crohn's disease patients on long-term treatment with MTZ showed no increase in the frequency of chromosomal abnormalities (Roe 1983). Chromosomal aberrations were absent in the circulating lymphocytes of patients with Crohn's disease taking MTZ long-term (Roe 1985).

In vitro chromosomal aberration frequency in human lymphocytes was not increased with MTZ treatment, and neither was any increase observed *in vivo* in lymphocytes of vaginal trichomoniasis infected patients after receiving MTZ treatment (Hartley-Asp 1981). MTZ is a non-genotoxic carcinogen as there was no induction of DNA strand breaks in lymphocytes of MTZ treated patients (Fahrig and Engelke 1997). MTZ treatment of patients having *Trichomonas vaginalis* infection, did not result in any considerable difference in sister-chromatid exchange frequency (Akyol et al. 2000).

3.3. Metronidazole and delayed type hypersensitivity

Delayed type hypersensitivity (DTH) is a major mechanism of defense against various intracellular pathogens, occurring in transplant rejection and tumor immunity. Apparently, MTZ selectively suppresses some aspects of the cell-mediated immune system. Delayed footpad reactions to soluble antigen ova were blocked by doses of 20 mg/kg daily, but 200 mg/kg on alternate days was unable to suppress skin allograft rejection (Grove et al. 1977). Imidazoles (nitroimidazoles) block steps of cell-mediated immunity in humans as well as in animals. Single and fractionated MTZ or misonidazole treatments were found to suppress DTH reactions to 2,4-dinitro-1-fluorobenzene in mice (Rockwell et al. 1983). It has been reported that MTZ (100 and 200 mg/kg) and cimetidine (200 mg/kg) markedly depressed the DTH reaction in mice as measured by the footpad thickness method. It also significantly blocked the migration of leukocytes in response to antigen (Sen et al. 1991). MTZ suppressed the DTH response 24 h after secondary injection of antigen in mice (Fararjeh et al. 2008). In the light of these reports, we can conclude that MTZ causes suppression of DTH, clearly indicating possible immunosuppression.

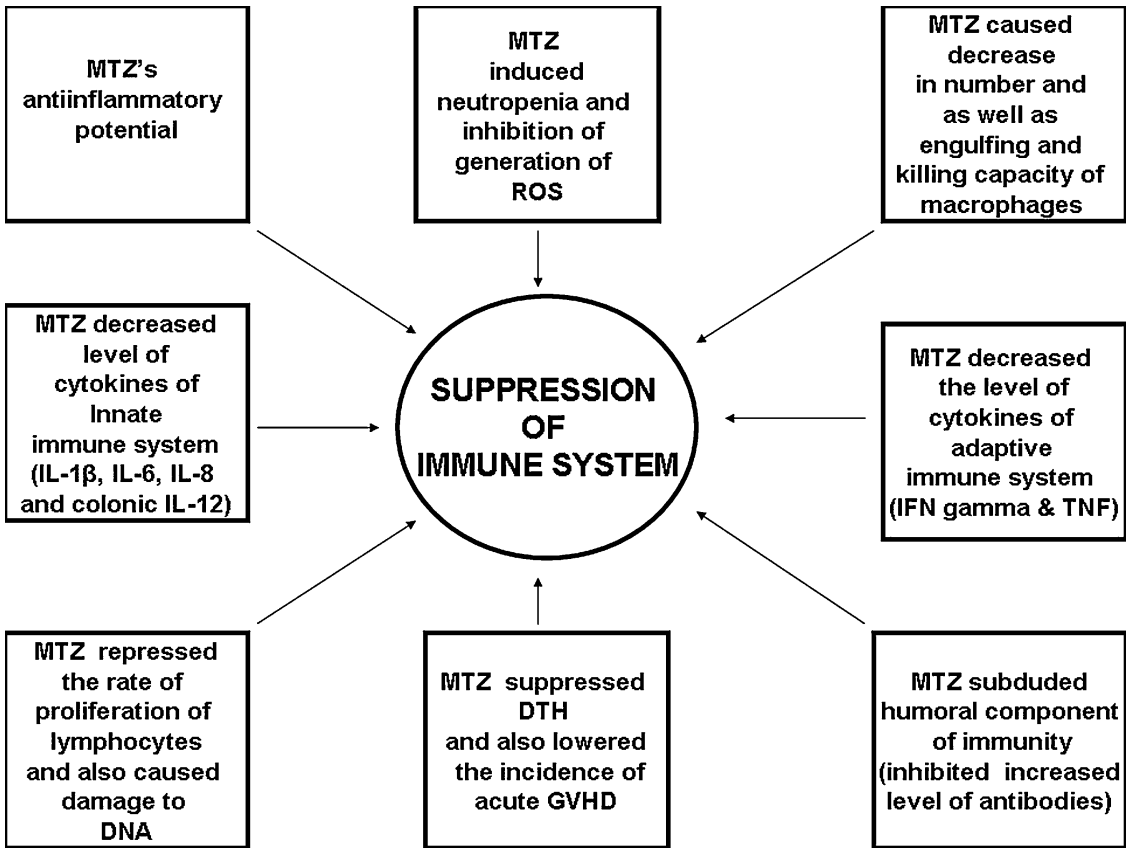

Fig.: Role of metronidazole (MTZ) in immunosuppression. MTZ can produce immunosuppression by down-regulating various cytokines of both innate and acquired immune system. MTZ induces anti-inflammatory effect, neutropenia, inhibition of generation of reactive oxygen species (ROS) and reduction in the macrophage number and functions which may produce immunosuppression. In addition, MTZ can produce immunosuppression by inhibiting delayed type hypersensitivity (DTH) and lymphocyte proliferation as well as increasing antibody levels and reducing incidence of graft-versus-host disease (GVHD)

Levels of the cytokines involved in cell-mediated immunity - IL-2 and IFN-γ (Th1 response), IL-4 and IL-5 (Th2 response) (Belardelli and Ferrantini 2002) - are generally affected after MTZ therapy; for example, MTZ and ciprofloxacin given orally in a SAMP1/YitFc mouse model of ileitis caused a decreased number of CD4(+)/CD45RB lymphocytes in mesenteric lymph nodes, and reduced production of IFN-gamma and TNF. In addition, the number of activated lamina propria lymphocytes was also reduced after treatment (Bamias et al. 2002). This result supports MTZ-induced immunosuppression, but in contrast, it was reported that MTZ and ceftriaxone induced an increase in the level of cytokines, especially IL-5, in the supernatant of a lymphocyte proliferation analysis (Yawalkar et al. 1999).

3.4. Metronidazole and graft-versus-host disease (GVHD)

Graft-versus-host disease (GVHD) is a serious complication following allogeneic bone marrow transplantation, resulting in morbidity and mortality (Theobald 1995). MTZ and ciprofloxacin markedly reduced the strength of acute GVHD in marrow transplant recipients as determined in a single center, open-label, prospective, randomized study (Beelen et al. 1999). Enteral MTZ (20 mg/kg/day/TID) seemed to be effective in reducing the risk of grade II–IV GVHD in pediatric allogeneic bone marrow transplant recipients (Guthery et al. 2004).

3.5. Metronidazole and humoral immunity

Humoral immunity is that aspect of the immune system which is mediated by secreted antibodies, mostly produced in cells of the B lymphocyte lineage. Antibodies identify and neutralize foreign objects. Antibody production is significantly inhibited by MTZ administration, indicating the expected immunosuppression. Metronidazole and mebendazole pretreatment provoked suppression of the intestinal and serum antiamebic antibody response in mice immunized with GFT (glutaraldehyde-fixed trophozoites). However, it remains to be determined which of the two drugs exerts the immunosuppressive effect, or whether only the combined treatment provokes suppression of the antibody response (Moreno-Fierros et al. 2000). MTZ and tetracycline blocked an increase in the level of anti-PG antibody (anti-PG IgA, IgG, and IgM levels) in rats with jejunal self-filling blind loops (Lichtman et al. 1991). Similarly, MTZ repressed the humoral immune response as evidenced by suppression of antibody production in response to secondary injection of antigen in mice (Fararjeh et al., 2008).

4. Conclusions and perspectives

MTZ shows multiple actions on the immune system and induces immunosuppression by exerting different effects on immune cells (Fig.). It can down-regulate various cytokines of both the innate and acquired immune system. It can also produce anti-inflammatory effects, neutropenia and a reduction in the number and functions of macrophages. In addition, it also has the ability to stimulate/inhibit lymphocyte proliferation as well as inhibiting the rise in the level of antibodies. Furthermore, MTZ can inhibit DTH and reduces the incidence of GVHD. The immunological effects of MTZ stimulate renewed interest in the development of antiprotozoal and antibacterial agents. The results of ongoing investigations will undoubtedly reveal the potential role of MTZ beyond that as an antiprotozoal and antibacterial agent, and may provide hope for improvement both in survival without deterioration in quality of life and also in the benefit to risk ratio during chemoimmunotherapy.

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