Mechanisms of action of second-line agents and choice of drugs in combination therapy

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ABSTRACT

Second-line agents are used commonly for the treatment of rheumatoid arthritis (RA). They suppress inflammation and ameliorate symptoms but often fail to substantially improve long-term disease outcome. Their use in RA was discovered serendipitously and their modes of action were largely unknown. Recent researches have identified some of their mechanisms of action. Most of them have antiinflammatory properties and some are immunomodulators. Traditionally, second-line agents are used as monotherapy, but recent evidence suggests that combination treatment with two or more drugs may be more efficacious. However, the choice of agents in combination therapy is not based on their mechanisms of action. We review current knowledge on the modes of action of second-line agents and assess whether such understanding may offer a rational basis for combination therapy.

Introduction

Second-line or disease-modifying antirheumatic drugs (DMARDs) are standard treatment for rheumatoid arthritis (RA). Some, but not all, DMARDs retard joint damage (1). For all the current DMARDs, their use in RA has come about through serendipity rather than rational development. This, unfortunately, reflects our ignorance of the precise etiopathogenesis of RA. The exact mechanism of action of most DMARDs remains unknown.

Recent researches have increased our understanding of inflammation and pathogenesis of RA. This has been reviewed extensively elsewhere (2, 3). Briefly, RA is thought to be driven by unknown antigenic peptides presented in the groove of human leucocyte antigen (HLA)-DR molecules to CD4+ T lymphocytes. These antigen-specific CD4+ T cells release lymphokines such as interleukin-2 (IL-2) and interferon-γ (IFNγ) to activate the inflammatory cascade through the stimulation of IL-2 receptor (IL-2R) positive lymphocytes and monocytes. The latter release monokines, including IL-1, IL-6, and tumour necrosis factor α (TNFα) that stimulate mesenchymal cells such as fibroblasts, as well as endothelial cells. Activated fibroblasts, monocytes, and macrophages release matrix metalloproteinases, such as collagenases and stromelysin, that degrade connective tissues and result in tissue damage. Stimulated endothelial cells up-regulate surface vascular adhesion molecule expression. These include selectins and integrins such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). This leads to increased recruitment of leukocytes into the inflammatory site and augments the immune response.

The release of chemokines such as IL-8 further augments leucocyte trafficking into the inflamed joint. IL-6 stimulates hepatocytes to release acute phase reactants and is the main cytokine responsible for the raised erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in RA. Within this inflammatory milieu, there are factors, such as transforming growth factor β (TGFβ) and IL-10, which down-regulate inflammation. The degree of inflammation in RA rests on the balance between the pro-inflammatory and antiinflammatory mediators. The exact role of B cells in the pathogenesis of RA is unclear, but the presence of rheumatoid factor (RF) and hypergammaglobulinemia in RA patients suggests that they may have a role in the inflammatory process. A high titre of RF is associated especially with a poor prognosis, erosive disease, and extra-articular manifestations.

Clinical trials of DMARDs have confirmed their ability to reduce joint pain, swelling, and early morning stiffness, all of which are features of inflammation. Hence, most research efforts have focused on the effects of DMARDs on various inflammatory mediators. In or-

der to elucidate the mechanism by which a particular DMARD acts in RA, it is vital to obtain both *in vivo* and *in vitro* evidence. On its own, the former may reflect merely disease improvement or other factors rather than a direct action of the second-line drug. The results of *in vitro* studies vary greatly with the experimental model used, which may not truly reflect the complex *in vivo* situation. Furthermore, any *in vitro* effect must be demonstrable at doses that would be achieved in patients. This may be difficult to determine for some preparations. These caveats should be borne in mind in assessing many apparent conflicts in the current literature.

Gold

On the assumption that RA was caused by mycobacterial infection, Forestier *et al.* treated the first RA patient with gold in 1929 (4). The clinical efficacy of gold in RA was demonstrated by a multicentre trial in the UK published in 1960 (5). Gold salts are administered either orally as auranofin or intramuscularly as sodium aurothiomalate in the UK, and as sodium aurothioglucose in some European countries. Whilst intramuscular gold compounds appear to have similar mechanisms of action and pharmacokinetics, it is unclear to what extent auranofin shares these characteristics.

The primary therapeutic effect of gold salts appears to be on polymorphonuclear cells (PMN), monocytes, and endothelial cells, though they may also affect B cells and cytokines.

In vitro aurothiomalate and auranofin at pharmacologic doses inhibit PMN phagocytosis, aggregation, chemiluminescence, and the generation of superoxide (6). Interestingly, auranofin is a more potent inhibitor than aurothiomalate in these systems, whilst the latter is more effective clinically (7), suggesting that inhibition of PMN function is not a major therapeutic effect of aurothiomalate. Auranofin inhibits the depolarisation of stimulated PMN, while aurothiomalate stimulates the oxidative burst but has no effect on membrane depolarisation.

Another major effect of aurothiomalate is on monocytes. Synovial biopsies performed before and after aurothiomalate therapy showed that there was a reduction in the number of monocytes (8). Furthermore, *in vitro* aurothiomalate is a potent inhibitor of peripheral blood mononuclear cell proliferation induced by mitogen, antigen, or mixed allogeneic lymphocytes (9-12). This is, at least in part, a direct effect on monocytes, since addition of untreated monocytes could reverse the inhibition (10). Although such monocytes showed the decreased expression of major histocompatibility complex (MHC) class II molecules, this may not be the mechanism of action *in vivo* since a high dose of aurothiomalate was required *in vitro* to produce such effects (10).

Although aurothiomalate inhibits mitogen-stimulated peripheral blood mononuclear cell (PBMNC) proliferation and expression of IL-2 and IL-2R, hitherto there is no convincing direct evidence to suggest that aurothiomalate exerts its prime therapeutic effect through a direct action on lymphocytes (12). Interestingly, Verwilghen *et al.* showed that lymphocytes from patients who developed gold-induced skin rashes proliferated specifically to gold (13). Surprisingly, this response was directed against gold (III) rather than gold (I), which is the form actually found in aurothiomalate (13). It was therefore postulated that gold was oxidized from gold (I) to gold (III) *in vivo*, perhaps in the phagolysosomes of monocytes, macrophages, and neutrophils. Of particular interest, the patients who developed skin rashes as a result of chrysotherapy tended to be those who responded particularly well to treatment (14), suggesting that the induction of this gold (III)-specific T cell response might also be linked to a therapeutic mode of action of aurothiomalate.

Gold is known to affect endothelial cell proliferation and HLA expression (15). It has also been shown to affect leucocyte trafficking *in vivo* using the air pouch model (16). In support of this, Corkill *et al.* showed by synovial immunohistology in patients receiving aurothiomalate that there was a significant reduction in the expression of endothelial leucocyte adhesion molecule 1 (ELAM-1) on high endothelial venules (17) in the joint which would be expected to reduce cellular trafficking.

Gold salts may have effects on B cells. Preincubating monocyte or B cell enriched PBMNC with aurothiomalate inhibited the mitogen stimulated production of immunoglobulin *in vitro* (18). This is confirmed by *in vivo* and *ex vivo* data showing that chrysotherapy reduces immunogobulin, immune complexes, and RF levels in serum (19, 20). Furthermore, the side effects of gold include hypogammaglobulinemia and selective IgE and IgA deficiency (21).

The effect of gold compounds on cytokine expression remains unclear. *In vivo* Madhok *et al.* found that after chrysotherapy there was a reduction in serum IL-6 which was correlated with disease activity, but this may be due to disease improvement rather than a direct effect of gold (22). Farahat *et al.* studied sequential synovial biopsies and showed that there was a significant decrease in the expression of IL-1α, IL-1β, IL-6, and TNFα after 12 weeks of chrysotherapy (8). *In vitro*, Harth *et al.* found that aurothiomalate inhibited the production of IFNγ from concanavalin A-stimulated peripheral blood mononuclear cells both in RA patients and normal controls (23). Danis *et al.* showed that aurothiomalate had a bimodal effect on IL-1 production by lipopolysaccharide-stimulated monocytes (24). At low concentrations, the production of IL-1 was enhanced, while higher concentrations had the opposite effect. These changes in cytokines could, of course, be secondary to the effect of aurothiomalate on monocytes. However, one intriguing mode of action of gold may be the direct inhibition of DNA binding by transcription factors (TF) (25). TF such as activator protein 1 (AP-1) and, to a lesser extent, AP-2, nuclear factor 1 (NF-1), and TFIID are involved in the production of cytokines and have been shown to be inhibited *in vitro* by gold (25).

Gold salts may also inhibit the proliferation of cultured synovial cells and the synthesis of collagen *in vitro*; this may be especially important in the inhibition of pannus formation (26).

Methotrexate

Methotrexate (MTX) is an inhibitor of folate metabolism that has been used traditionally primarily in the treatment of malignancies. At high doses, it suppress-

es proliferation of cells by inhibiting essential enzymes such as dihydrofolate reductase, thymidylate synthetase, and aminoamidazole carboxamide ribonucleotide transformylase in the folate metabolic pathway (27, 28). However, at low doses such as those used in the treatment of RA and psoriasis, its immunosuppressive and antiinflammatory effects are largely unrelated to these enzymes.

One of the most significant antiinflammatory actions of MTX is the inhibition of leucocyte trafficking. Following MTX treatment, there is a decrease in PMN infiltration in the skin and joints of patients with psoriatic arthritis (29) and RA (30), respectively. Recent *in vitro* work by Cronstein *et al.* supported this hypothesis. They showed that MTX enhanced the intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide and the release of adenosine from injured neutrophils, fibroblasts, and endothelial cells (31); adenosine is known to inhibit leucocyte migration. Subsequently, using a murine carrageenan inflamed air pouch model, they were able to confirm this finding *in vivo* (32). Furthermore, both MTX and adenosine were shown to inhibit leucocyte-endothelial cell adhesion induced by platelet-activating factor. The inhibition could be partially reversed by adenosine deaminase and the adenosine A2 receptor antagonist, but not the A1 receptor antagonist. Furthermore, MTX reduced PMN chemotaxis by suppressing leukotriene B4 production, decreasing neutral protease and serine protease activity (33), and inhibiting the production of superoxides (34).

Ex vivo MTX did not inhibit PBMNC proliferation in a high-folate medium, but in a low-folate medium RNA production decreased by more than 80% (35). Interestingly, Constantin *et al.* have shown that the PHA-stimulated PBMNC of MTX-treated RA patients have a higher IL-4 gene expression than patients with active RA (36). Recently, Genestier *et al.* have shown that MTX at 0.1 - 10 μM induced an apoptosis of activated T cells (37) that was independent of Fas and CD95L ligation.

Barrera *et al.* showed that *in vivo* MTX treatment is associated with decreases in serum IL-6, soluble IL-2, and p55 TNF receptors (38). In collagen-induced arthritis, MTX reduced spontaneous and IL-15-induced TNFα production by splenic T cells and macrophages (39). There is conflicting evidence as to whether MTX can inhibit IL-1β *in vitro* (40, 41). Seitz *et al.* showed that MTX induces monocytes to differentiate into macrophages *in vitro*, associated with an increase in IL-1Ra and sTNFR p75 release and a decrease in IL-1β (42). Others showed that MTX did not affect the production of IL-1 from murine macrophages and RA patients' PBMNC, but MTX seemed to inhibit IL-1ß activity in functional assay (43). One possible explanation suggested by Brody *et al.* is that MTX could inhibit the binding of IL-1β to the IL-1 receptor on PBMNC *in vitro* without decreasing the production of IL-1β (44). Although only 50% of patients receiving MTX showed a sharp decrease in serum (43) and synovial fluid IL-1β (30) *in vivo*, this was associated with a decrease in the number of painful joints.

MTX inhibited the spontaneous production of IgM-RF and IgA-RF *in vivo* (45), but clinical improvement was correlated only with decreased IgM-RF. This may be due to the inhibition of B cell proliferation and differentiation. However, since improvement with MTX is not limited to seropositive patients, this is unlikely to be the main therapeutic effect of MTX.

D-penicillamine

D-penicillamine (D-Pen)has been shown to be clinically effective in RA (46); its main anti-rheumatic action is thought to be mediated through the action of its thiol group. Since many DMARDs, including aurothiomalate, penicillin and levamisole, contain a thiol group, some authors have suggested that thiol itself may have disease-modifying properties. Thiols are metal chelators (47) and stabilize proteins, preventing their reacting with other moieties (48). In addition, T lymphocytes (49), NK cells (50), and monocytes (51) contain cell-surface sulphydryl groups that are important for their function. The thiol group can reduce disulphides by thiol-sulphide interchange, and hence can alter cell surface receptors and their function.

A second proposed mechanism of action of D-pen involves the formation of peroxides. Some investigators have suggested that the oxidation of D-pen in the presence of trace copper leads to the development of a reactive oxygen species, peroxide, which affects the function of T lymphocytes, endothelial cells, and fibroblasts (52-54). However, the exact relevance of this observation remains unclear, as the concentration of D-pen used in these experiments was greater than that achieved *in vivo*.

D-pen also inhibits leucocyte myeloperoxidases which catalyse the formation of hypochlorite (which may damage cartilage) from hydrogen peroxide and chloride (55). It also inhibits leukotriene D_4 dipeptidase *in vitro* (56), but has no effect on human neutrophil lipoxygenase (57).

D-pen-inhibited T cell proliferation to mitogenic stimulation is perhaps due to the generation of peroxide (58) or to the action of the thiol group (49), as previously discussed. D-pen is known to cause a number of autoimmune conditions including myasthenia gravis and systemic lupus erythematosus (SLE). O'Donnell *et al.* showed in a murine model that D-pen induced the development of D-pen-specific T cells which were CD4+ and restricted by MHC class II molecules (59). These T cells responded to drug-haptenated stimulator cells but not to unstimulated cells or free drug. Interestingly, generation of this D-pen-derived antigenic determinant for T cells did not require intracellular processing. This may be the mechanism through which D-pen induces an autoimmune disease such as myasthenia gravis. *In vitro*, D-pen causes DNA breaks (60), and in treated RA patients there are reported decreases in the lymphocyte number as well as reduced CD4:CD8 ratios.

Thiol-disulphide exchange inhibits the accessory cell function of monocytes (51). Handel *et al.* showed that D-pen is an inhibitor of AP-1 binding to DNA through the formation of sulphur-containing radicals (61). Since AP-1 is an important transcription activator of many cytokine and metalloproteinase genes, this may explain the anti-inflammatory properties of D-pen.

Other *in vitro* effects of D-pen include decreased production of immunoglobulin by B cells (62) and inhibition of endothelial-derived growth factor-stimulated neovascularisation (54). *In vivo*, the serum concentration of IgM and, to a lesser extent, IgG declines after treatment (63).

Sulphasalazine

Sulphasalazine (SASP) consists of sulphapyridine and 5-aminosalicylate. It is used extensively for the treatment of RA and inflammatory bowel diseases. However, the mechanisms of action in the two diseases are probably different, since 5-aminosalicylate alone is effective in inflammatory bowel disease but not in RA. Both sulphapyridine and 5-aminosalicylate are known to have antiinflammatory effects, and both are probably required for maximal effect in RA.

Some workers have suggested that the antibacterial effect of sulphapyridine may be important in the therapeutic action of SASP, as some antibiotics such as minocycline have been shown to reduce disease activity in RA (64). *In vivo* SASP treatment resulted in a reduction in *Clostridium perfringens* cultured from stool samples (65). However, other bowel flora did not appear to be affected and these changes in bowel flora were not correlated with clinical improvement. One interesting recent finding was that treatment with steroid together with SASP seemed to have a deleterious effect (66). The mechanism of this interaction is unclear, but corticosteroids may antagonise the antibacterial action of SASP.

One of the most potent immunomodulatory effects of SASP is on B cells (67). SASP inhibited B cell proliferation *in vitro* whilst *in vivo* it can lead to hypogammaglobulinaemia (68), decreased antibody production to orally administered antigen (69), and selective IgA deficiency. Samanta *et al.* showed that SASP inhibited peripheral blood mononuclear cell (PBMC) proliferation to PHA (70) *ex vivo*, an effect seen only in patients who responded clinically to the drug. SASP can also inhibit proliferation of synoviocytes and reduce release of IL-1β and IL-6 in a dose-dependent manner (71). SASP also increased the rate of neutrophil apoptosis which was abrogated by reactive oxygen species (72). Sulphapyridine inhibited NK cell activity *in vitro* (73), but the clinical significance of this finding is not known.

Danis *et al.* showed a decrease in *in vivo* serum levels of IL-1ß, IL-6, and TNFα following treatment with SASP. However, these changes may be effects of disease improvement rather than a direct action of SASP (74). Bissonnette *et al.* showed that SASP *in vitro* inhibited the release of TNFα from mast cells (75), while Wahl *et al.* showed that SASP inhibited the TNFα, LPS and phorbol ester-induced expression of NF-κβ (76). Since NF-κβ is a major transcription factor for a number of pro-inflammatory cytokines, SASP could inhibit inflammation through its anti-cytokine effect.

Recent evidence has suggested that an important mechanism of action of SASP is the inhibition of leucocyte trafficking. SASP, but not sulphapyridine, inhibited the activation-induced up-regulation of CD11b/CD18 (MAC-1), an important adhesion molecule, by granulocytes and monocytes (77). This could potentially reduce leucocyte trafficking into inflammatory sites. Furthermore, similar to MTX, SASP also increased extracellular adenosine levels and could thereby inhibit leucocyte trafficking (78).

Sharon *et al.* showed that SASP inhibited bovine endothelial cell proliferation *in vitro* (79), and this has been subsequently confirmed in human endothelial cells (80). Since angiogenesis is an important component of rheumatoid synovial hypertrophy, the inhibition of endothelial cell proliferation may limit disease. 5-aminosalicylate has been shown to scavenge oxygen and hypochlorite radicals *in vitro* (81). This is supported by a clinical study from Bradley *et al* in 19 RA patients treated with SASP. Superoxide levels fell in the responders but not in the non-responders (82). SASP inhibited IL-1β-induced prostaglandin E_2 and glycosaminoglycan release from rabbit chondrocytes *in vitro*, suggesting that SASP may affect joint damage (83).

Azathioprine

Azathioprine (AZA) is an oral purine analogue which interferes with the synthesis of adenosine and guanine. It is biologically inactive until metabolised, primarily in erythrocytes and in the liver, to 6-thioinosinic acid and 6-thioguanylic acid. As an anti-metabolite, it is toxic to cells in the S phase of the mitotic cycle. It acts mainly by inhibiting the function of T, B and NK cells.

AZA inhibits *in vitro* mixed lymphocyte culture only when it is added during the first 24 hours of culture (84). Furthermore, in animal models T cell-dependent immune responses, such as delayed hypersensitivity and graft rejection, are particularly sensitive to the action of the drug.

Abdou *et al.* showed that AZA inhibited B cell proliferation *in vitro* (85) and Levy *et al.* showed that the *in vivo* production of immunoglobulin was reduced after a few months of treatment (86). Interestingly, antibody responses directed against thymus-dependant antigens, which require the presence of T helper cells, are more sensitive to the effect of AZA than those directed against the thymus-independent antigens. This suggests that some of the suppressive effect on antibody production is mediated through T cell inhibition.

Cseuz *et al.* has shown that AZA inhibits the function of NK cells both *in vitro* and *ex vivo* (87, 88). However, treatment with AZA did not produce any consistent fall in the NK cell numbers *in vivo*. The decrease in NK cell function was not associated with changes in disease activity following treatment.

Unlike MTX, AZA did not affect the serum level of soluble cytokine receptors, although the serum IL-6 level was reduced (38). The decrease in IL-6 parallelled the improvement in RA disease activity, although this may be a consequence of disease improvement rather than a direct therapeutic action of AZA.

Antimalarials

Antimalarials, such as hydroxychloroquine and chloroquine, have been used extensively in the treatment of SLE and RA. Their exact mechanism of action remains unknown. Antimalarials accumulate avidly in acid-vesicle environments such as lysosomes and the Golgi apparatus (89, 90). They affect the pH of the organelles, thereby inhibiting the action of certain enzymes such as acid protease,

cathepsin B, and phospholipase A_2 (91). The inhibition of such enzymes would have various effects, depending on the cell type. Phagocytosis and the cleavage of peptides by enzymes such as cathepsin are vital for antigen processing. Thus, the accumulation of antimalarials in the lysosomes will result in defective antigen processing and presentation (89). Antimalarials have also been shown to inhibit *in vitro* antigen processing and presentation (90), as well as IL-1 release (91).

In PMN, antimalarials inhibit chemotaxis and phagocytosis (92, 93). In addition, they inhibit phospholipase A_2 which is important in the production of arachidonate, the precursor of prostaglandins. Anti-malarials bind *in vitro* to DNA, affecting DNA and RNA polymerase as well as increasing chromosome breakage (94). This may explain its inhibitory action on mitogen-induced T cell proliferation both *in vitro* and *ex vivo* (95, 96). Antimalarials inhibit antibody production to the live rabies vaccine, though not to the killed typhoid vaccine (97). This may be secondary to their effect on antigen presentation. Antimalarial treatment has been shown to protect cartilage from damage by prostaglandins *in vitro* (98); however, there is no clear clinical evidence that they retard radiologic progression in RA.

Cyclosporin A

Cyclosporin A (CSA) is a cyclic endecapeptide isolated from the fungi *Tolypoclasium inflatum* and *Cylindrocarpon lucidium*. CSA has established itself as an important drug in transplantation and in the treatment of a number of autoimmune diseases, including RA (99). It is a potent immunomodulator which primarily inhibits T cells. *In vitro* CSA is known to inhibit IL-2 secretion *in vitro* when PBMC are stimulated either by mitogen or antigen (100,101). This effect is mediated through the inhibition of IL-2 gene transcription through an effect on the nuclear transcription factor of activated cells (NF-AT) (102). During T cell activation, extra-cellular signals lead to a sharp rise in intracellular calcium. This binds to calmodulin, which in turn binds to calcineurin; the activated calcineurin dephosphorylates the cytoplasmic subunit of NF-AT, resulting in its translocation from the cytoplasm into the nucleus to form a competent transcriptional activator (103).

NF-AT is an important transcription factor for the production of IL-2 (104). Recent work has shown that CSA binds to immunophilins, in particular cyclophilin (Cyp), which has enzymatic functions and regulates protein folding during protein synthesis. The Cyp/CSA complex can bind to calcineurin and calmodulin, thereby inhibiting its phosphatase activity. This prevents the translocation of transcription factor into the nucleus and inhibits the gene expression of IL-2 and other cytokines. Recently, Matsuda *et al.* have shown that CSA can also inhibit intracellular kinases, including MKK6 and MMK7 (105).

Although CyA is primarily a T cell-directed drug, some evidence has been reported for its effects on other cell types (106). In human monocytes and macrophages, CSA induced apoptosis and abolished the inositol 1,4,5-triphosphate-mediated release of calcium ions from intracellular stores. CSA inhibits nitric oxide synthesis in fibroblast cell lines *in vitro.* This effect is independent of calcineurin inhibition (107). In human gingival fibroblasts stimulated by LPS, CSA inhibited collagenase gene expression by suppressing the transcription activator AP-1 (108). Low-dose CSA inhibited endothelial cell proliferation, chemotaxis, and the release of metalloproteinases 2 and 9, both *in vitro* and *in vivo* (109).

One of the most important side effects of cyclosporin is nephrotoxicity. Recent evidence suggests that this is mediated through TGFβ, as anti-TGBβ antibody abrogated renal histopathologic changes in cyclosporin-treated animals (110).

Cyclophosphamide

Cyclophosphamide (CYC) is a powerful immunosuppressant widely used in the treatment of malignancy, SLE and vasculitides. In RA, it improves symptoms and retards radiologic progression (1). CYC not only inhibits cells in the pre-mitotic (G_2) phase, but also inhibits a number of metabolic pathways. Its major therapeutic action in RA, however, is likely to be its inhibition of B cell function. At low doses, CYC leads to a reduction in the circulating level of autoantibodies and immune complexes (111), which renders this drug particularly useful in treating the antibody-mediated systemic complications of RA such as vasculitis. At high doses CYC also causes a reduction in lymphocyte numbers, especially of CD8+ cells. This is due to the binding of CYC covalently to DNA, which prevents replication (112). The toxicity of CYC, involving late malignancies, limits its clinical use in RA only to patients with life-threatening vasculitis and other severe extra-articular manifestations of disease

Leflunomide

Leflunomide is a isoxazol derivative and a new immunomodulator which has been used in the treatment of RA. Approximately 50% of leflunomide-treated patients achieved the ACR 20% criteria of clinical improvement in a double-blind placebo-controlled trial in RA, in which it was as efficacious as SASP (113). Leflunomide is metabolised by the liver to A771726, which is the active compound (114). The main *in vitro* action of leflunomide is the inhibition of T cell proliferation stimulated by either mitogen or antigens (115,116). Leflunomide inhibits T cell proliferation by slowing cell cycling by binding to dihydro-orotate dehydrogenase, an enzyme involved in *de novo* pyrimidine synthesis (117, 118). This latter pathway is particularly vital for activated rather than resting lymphocytes. Hence the claimed favourable therapeutic/toxicity window of leflunomide.

Combination DMARD therapy

It is difficult to make rational decisions regarding combination therapies for the treatment of RA. The evidence for the modes of action of the various drugs available is fragmentary and bedevilled by methodological problems, not the least being the difficulty of extrapolating from *in vitro* phenomena to *in vivo* responses. For example, it is difficult to account for the fact that intramuscular gold salts take several weeks to have an effect, while the *in vitro* experiments purporting to analyse their mode of action are short term. Table I shows a summary of the main actions of DMARDs.

Table I. Summary of the main actions of disease-modifying antirheumatic drugs (DMARDs).

Drug	Mechanism of action
Antimalarials	Inhibits antigen processing
Intramuscular gold salts	Inhibits monocyte function Down-regulates endothelial cell selectins
D-penicillamine	Inhibits immunocompetent cells
Sulphasalazine	Decreases immunoglobulin production, especially IgA
Methotrexate	Inhibits cell migration
Leflunomide	Inhibits T cells
Cyclosporin A	Inhibits T cells
Azathioprine	Inhibits T cells
Cyclophosphamide	Inhibits B cells

Thus, antimalarials and intramuscular gold should demonstrate some additive effect, and this has indeed been shown to be the case (119). However, although the combination of antimalarials with D-pen should have additive or synergistic effects, clinical experience is that the combination is not effective and shows increased toxicity (120). By contrast, O'Dell and colleagues have clearly shown that triple therapy with antimalarials, SASP and MTX is effective and without increased toxicity (121). Could this outcome have been predicted from the known properties of the drugs? The antimalarial, by inhibiting antigen processing, would have inhibited T cell activation, while the MTX would have inhibited cytokine release and cell migration into the synovium. The SASP would have inhibited immunoglobulin production and, presumably, the generation of immune complexes. An animal model in which these combinations could first be tried out is clearly needed.

With the rapid growth of molecular genetics and, in particular, pharmacogenetics, increasing knowledge will remove much of the guesswork from this predictive process. Thus SASP has been shown recently to inhibit thiopurine methyltransferase, which catabolises the s-methylation of thiopurines (122). Therefore, SASP may interfere with the metabolism of AZA. Combination therapy of SASP with AZA should therefore be approached with caution.

Conclusion

Although much uncertainty remains concerning the mechanism of action of DMARDs, recent advances in the understanding of the pathogenesis of RA have helped in the investigation of many new possibilities. Perhaps the greatest potential of research into the modes of action of DMARDs is to develop better and less toxic treatments directed at the same targets.

References

1. IANNUZZI L, DAWSON N, ZEIN N, KUSHNER I: Does drug therapy slow radiographic deterioration in rheumatoid arthritis ? *N Engl J Med* 1983; 309: 1023-8.
2. PANAYI GS, LANCHBURY JS, KINGSLEY GH: The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum* 1992; 35: 729-35.
3. FELDMANN M, BRENNAN FM, MAINI RN: Rheumatoid arthritis. *Cell* 1996; 85: 307-10.
4. FORESTIER J: Rheumatoid arthritis and its treatment by gold salts. The results of six years' experience. *J Clin Lab Med* 1935; 20: 827-40.
5. EMPIRE RHEUMATISM COUNCIL RS: Gold therapy in rheumatoid arthritis. Report of a multi-centre controlled trial. *Ann Rheum Dis* 1960; 19: 95-119.
6. RUDKOWSKI R, ZIEGLER JB, GRAHAM GG, JOULIANOS G: Gold complexes and activation of human polymorphonuclear leukocytes. Dissociation of changes in membrane potential and oxidative burst. *Biochem Pharmacol* 1992; 44: 1091-8.
7. FELSON DT, ANDERSON JJ, MEENAN RF: The comparative efficacy and toxicity of second-line drugs in rheumatoid arthritis. Results of two metaanalyses. *Arthritis Rheum* 1990; 33: 1449-61.
8. FARAHAT MN, YANNI G, POSTON R, PANAYI GS: Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis* 1993; 52: 870-5.
9. HARTH M, STILLER CR, ST. C, SINCLAIR NR, EVANS J, MCGIRR D, ZUBERI R: Effects of a gold salt on lymphocyte responses. *Clin Exp Immunol* 1977; 27: 357-64.
10. LIPSKY PE, ZIFF MA: Inhibition of mitogen and antigen-induced human lymphocyte proliferation by gold compounds. *J Clin Invest* 1977; 59: 455-66.
11. LIES RB, CARDIN C, PAULUS HE: Inhibition by gold of human lymphocyte stimulation. An *in vitro* study. *Ann Rheum Dis* 1977; 36: 216-8.
12. SFIKAKIS PP, SOULIOTIS VL, PANAYIOTIDIS PP: Suppression of interleukin-2 and interleukin-2 receptor biosynthesis by gold compounds in *in vitro* activated human peripheral blood mononuclear cells. *Arthritis Rheum* 1993; 36: 208-12.
13. VERWILGHEN J, KINGSLEY GH, GAMBLING L, PANAYI GS: Activation of gold-reactive T lymphocytes in rheumatoid arthritis patients treated with gold. *Arthritis Rheum* 1992; 35: 1413-8.
14. MENARD HA, BEAUDET F, DAVIS P, HARTH M, PERCY JS, RUSSELL AS, THOMPSON JM: Gold therapy in rheumatoid arthritis. Interim report of the Canadian multicenter prospective trial comparing sodium aurothiomalate and auranofin. *J Rheumatol* 1982; 8 (Suppl.): 179-83.
15. KAWAKAMI A, EGUCHI K, MIGITA K *et al*.: Inhibitory effects of gold sodium thiomalate on the proliferation and interferon-gamma induced HLA-DR expression in human endothelial cells. *J Rheumatol* 1990; 17: 430-5.
16. SIN YM, WONG MK: Effect of sodium aurothiomalate on carrageenan induced inflammation of the air pouch in mice. *Ann Rheum Dis* 1992; 51: 112-6.
17. CORKILL MM, KIRKHAM BW, HASKARD DO, BARBATIS C, GIBSON T, PANAYI GS: Gold treatment of rheumatoid arthritis decreases synovial expression of the endothelial leukocyte adhesion receptor ELAM-1. *J Rheumatol* 1991; 18: 1453-60.
18. ROSENBERG SA, LIPSKY PE: Inhibition of pokeweed mitogen-induced immunoglobulin production in humans by gold compounds. *J Rheumatol* 1979; 5 (Suppl.): 107-11.
19. OLSEN N, ZIFF M, JASIN HE: Spontaneous synthesis of IgM rheumatoid factor by blood mononuclear cells from patients with rheumatoid arthritis: Effect of treatment with gold salts or D-penicillamine. *J Rheumatol* 1984; 11: 17-21.
20. HIGHTON J, PANAYI GS, SHEPHERD P, FAITH A, GRIFFIN J, GIBSON TG: Fall in immune complex levels during gold treatment of rheumatoid arthritis. *Ann Rheum Dis* 1981; 40: 575-9.
21. OSTUNI PA, SIMIONI M, MARSON P, TRAVAGLIA P, VOLANTE D, GAMBARI PF: Serum IgA and gold toxicity in rheumatoid arthritis: Lack of predicting value. *Clin Exp Rheumatol* 1986; 4: 359-62.
22. MADHOK R, CRILLY A, MURPHY E, SMITH J, WATSON J, CAPELL HA: Gold therapy lowers serum interleukin 6 levels in rheumatoid arthritis. *J Rheumatol* 1993; 20: 630-3.
23. HARTH M, COUSIN K, MCCAIN GA: *In vitro* effects of two gold compounds, and D-penicillamine on the production of interferon gamma. *Immunopharmacol Immunotoxicol* 1990; 12: 39-60.
24. DANIS VA, KULESZ AJ, NELSON DS, BROOKS PM: The effect of gold sodium thiomalate and auranofin on lipopolysaccharide-induced interleukin-1 production by blood monocytes *in vitro*: Variation in healthy subjects and patients

with arthritis. *Clin Exp Immunol* 1990; 79: 335-40.

25. HANDEL ML, SIVERTSEN S, WATTS CK, DAY RO, SUTHERLAND RL: Comparative effects of gold on the interactions of transcription factors with DNA. *Agents Actions* 1993; 44 (Suppl.): 219-23.
26. GOLDBERG RL, KAPLAN SR, FULLER GC: Effect of heavy metals on human rheumatoid synovial cell proliferation and collagen synthesis. *Biochem Pharmacol* 1983; 32: 2763-6.
27. HAURANI FI, HERMAN GM, ABBOUD EM: Thymidylate synthetase of human lymphocytes augmented *in vitro* by methotrexate. *Cancer Biochemistry Biophysics* 1985; 8: 29-33.
28. ALLEGRA CJ, DRAKE JC, JOLIVET J, CHABNER BA: Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci USA* 1985; 82: 4881-5.
29. VAN DE KERKHOF PC, BAUER FW, MAASSEN DE, GROOD RM: Methotrexate inhibits the leukotriene B4 induced intraepidermal accumulation of polymorphonuclear leukocytes. *Br J Dermatol* 1985; 113: 251a-5a.
30. THOMAS R, CARROLL GJ: Reduction of leukocyte and interleukin-1 beta concentrations in the synovial fluid of rheumatoid arthritis patients treated with methotrexate. *Arthritis Rheum* 1993; 36: 1244-52.
31. CRONSTEIN BN, EBERLE MA, GRUBER HE, LEVIN RI: Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc Natl Acad Sci USA* 1991; 88: 2441-5.
32. CRONSTEIN BN, NAIME D, OSTAD E: The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an *in vivo* model of inflammation. *J Clin Invest* 1993; 92: 2675-82.
33. SPERLING RI, BENINCASO AI, ANDERSON RJ, COBLYN JS, AUSTEN KF, WEINBLATT ME: Acute and chronic suppression of leukotriene B4 synthesis *ex vivo* in neutrophils from patients with rheumatoid arthritis beginning treatment with methotrexate. *Arthritis Rheum* 1992; 35: 376-84.
34. AL BALLA S, JOHNSTON C, DAVIS P: The *in vivo* effect of nonsteroidal anti-inflammatory drugs, gold sodium thiomalate and methotrexate on neutrophil superoxide radical generation. *Clin Exp Rheumatol* 1990; 8: 41-5.
35. OLSEN NJ, MURRAY LM: Antiproliferative effects of methotrexate on peripheral blood mononuclear cells. *Arthritis Rheum* 1989; 32: 378-85.
36. CONSTANTIN A, LOUBET-LESCOULIE P, LAMBERT N *et al.*: Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis: Evidence of increased interleukin-4 and interleukin-10 gene expression demonstrated *in vitro* by competitive reverse transcriptase-polymerase chain reaction. *Arthritis Rheum* 1998; 41: 48-57.
37. GENESTIER L, PAILLOT R, FOURNEL S, FERRARO C, MIOSSEC P, REVILLARD JP: Immunosuppressive properties of methotrexate: Apoptosis and clonal deletion of activated peripheral T cells. *J Clin Invest* 1998; 102: 322-8.
38. BARRERA P, BOERBOOMS AM, JANSSEN EM *et al.*: Circulating soluble tumor necrosis factor receptors, interleukin-2 receptors, tumor necrosis factor alpha, and interleukin-6 levels in rheumatoid arthritis. Longitudinal evaluation during methotrexate and azathioprine therapy. *Arthritis Rheum* 1993; 36: 1070-9.
39. NEURATH MF, HILDNER K, BECKER C *et al.*: Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collagen-induced arthritis (CIA): A mechanism for methotrexate-mediated immunosuppression. *Clin Exp Immunol* 1999; 115: 42-55.
40. MEYER FA, YARON I, MASHIAH V, YARON M: Methotrexate inhibits proliferation but not interleukin 1 stimulated secretory activities of cultured human synovial fibroblasts. *J Rheumatol* 1993; 20: 238-42.
41. SEGAL R, MOZES E, YARON M, TARTAKOVSKY B: The effects of methotrexate on the production and activity of interleukin-1. *Arthritis Rheum* 1989; 32: 370-7.
42. SEITZ M, ZWICKER M, LOETSCHER P: Effects of methotrexate on differentiation of monocytes and production of cytokine inhibitors by monocytes. *Arthritis Rheum* 1998; 41: 2032-8.
43. CHANG DM, WEINBLATT ME, SCHUR PH: The effects of methotrexate on interleukin 1 in patients with rheumatoid arthritis. *J Rheumatol* 1992; 19: 1678-82.
44. BRODY M, BOHM I, BAUER R: Mechanism of action of methotrexate: Experimental evidence that methotrexate blocks the binding of interleukin 1 beta to the interleukin 1 receptor on target cells. *Eur J Clin Chem Clin Biochem* 1993; 31: 667-74.
45. OLSEN NJ, CALLAHAN LF, PINCUS T: Immunologic studies of rheumatoid arthritis patients treated with methotrexate. *Arthritis Rheum* 1987; 30: 481-8.
46. ANONYMOUS: Controlled trial of D(-)penicillamine in severe rheumatoid arthritis. *Lancet* 1973; 1: 275-80.
47. BACON PA, SALMON M: Modes of action of second-line agents. *Scand J Rheumatol* 1987; 64 (Suppl.): 17-24.
48. GERBER DA: Copper-catalyzed thermal aggregation of human gamma-globulin. Inhibition by histidine, gold thiomalate, and penicillamine. *Arthritis Rheum* 1974; 17: 85-91.
49. THORNE KJ, FREE J, FRANKS D: Role of sulphydryl groups in T lymphocyte-mediated cytotoxicity. *Clin Exp Immunol* 1982; 50: 644-50.
50. RISTOW SS, STARKEY JR, STANFORD DR, DAVIS WC, BROOKS CG: Cell surface thiols, but not intracellular glutathione, are essential for cytolysis by a cloned murine natural killer cell line. *Immunol Invest* 1985; 14: 401-14.
51. MCKEOWN MJ, HALL ND, CORVALAN JR: Defective monocyte accessory function due to surface sulphydryl (SH) oxidation in rheumatoid arthritis. *Clin Exp Immunol* 1984; 56: 607-13.
52. LIPSKY PE, ZIFF M: The effect of D-penicillamine on mitogen-induced human lymphocyte proliferation: synergistic inhibition by D-penicillamine and copper salts. *J Immunol* 1978; 120: 1006-13.
53. MATSUBARA T, HIROHATA K: Suppression of human fibroblast proliferation by D-penicillamine and copper sulfate *in vitro*. *Arthritis Rheum* 1988; 31: 964-72.
54. MATSUBARA T, SAURA R, HIROHATA K, ZIFF MA: Inhibition of human endothelial cell proliferation *in vitro* and neovasculisation *in vivo* by d-penicillamine. *J Clin Invest* 1989; 83: 158-67.
55. CUPERUS RA, HOOGLAND H, WEVER R, MUIJSERS AO: The effect of D-penicillamine on myeloperoxidase: Formation of compound III and inhibition of the chlorinating activity. *Biochim Biophys Acta* 1987; 912: 124-31.
56. HUBER M, KEPPLER D: Inhibition of leukotriene D4 catabolism by D-penicillamine. *Eur J Biochem* 1987; 167: 73-9.
57. NIELSEN OH, AHNFELT-RONNE I, ELMGREEN J: A comparison of the effect of timegadine, levamisole, and D-penicillamine on human neutrophil metabolism of endogenous arachidonic acid and chemotaxis. *Pharmacol Toxicol* 1988; 62: 322-5.
58. MITA S, MATSUNAGA K: Differences in the effects of the antirheumatic drugs, bucillamine and D-penicillamine, on mitogen-induced proliferation of mouse spleen cells. *Agents Actions* 1990; 30: 363-8.
59. O'DONNELL CA, COLEMAN JW: A T-cell response to the anti-arthritic drug penicillamine in the mouse: requirements for generation of the drug-derived antigen. *Immunology* 1992; 76: 604-9.
60. YAMANAKA H, HAKODA M, KAMATANI N, KASHIWAZAKI S, CARSON DA: Formation of DNA strand breaks by D-penicillamine and bucillamine in human lymphocytes. *Immunopharmacology* 1993; 26: 113-8.
61. HANDEL ML, WATTS CK, SIVERTSEN S, DAY RO, SUTHERLAND RL: D-penicillamine causes free radical-dependent inactivation of activator protein-1 DNA binding. *Mol Pharmacol* 1996; 50: 501-5.
62. BAIER-BITTERLICH G, SCHUMACHER W, SCHULZ, TF, WACHTER H, DIERICH MP: Effects of D-penicillamine on human cell lines. *Arzneimittel-Forschung* 1993; 43: 395-8.
63. WERNICK R, MERRYMAN P, JAFFE I, ZIFF M: IgG and IgM rheumatoid factors in rheumatoid arthritis. Quantitative response to penicillamine therapy and relationship to disease activity. *Arthritis Rheum* 1983; 26: 593-8.
64. KLOPPENBURG M, BREEDVELD FC, MILTENBURG AM, DIJKMANS BA: Antibiotics as disease modifiers in arthritis. *Clin Exp Rheumatol* 1993; 11 (Suppl. 8): S113-5.
65. BRADLEY SM, NEUMANN VC, BARR K *et al.*: Sequential study of bacterial antibody levels and faecal flora in rheumatoid arthritis patients taking sulphasalazine. *Br J Rheumatol* 1993; 32: 683-8.
66. GOUGH A, SHEERAN T, ARTHUR V, PANAYI G, EMERY P: Adverse interaction between intramuscular methylprednisolone and sulphasalazine in patients with early rheumatoid arthritis. A pilot study. *Scand J Rheumatol* 1994; 23: 46-8.
67. IMAI F, SUZUKI T, ISHIBASHI T, DOHI Y: Effect of sulfasalazine on B cells. *Clin Exp Rheu-*

matol 1991; 9: 259-64.
68. FARR M, KITAS GD, TUNN EJ, BACON PA: Immunodeficiencies associated with sulphasalazine therapy in inflammatory arthritis. *Br J Rheumatol* 1991; 30: 413-7.
69. SHELDON P, PELL P: Comparison of the effects of sulphasalazine, 5-aminosalicylic acid and sulphapyridine on the humoral response to antigen *in vivo*. *Adv Exp Med Biol* 1995; 371B: 905-8.
70. SAMANTA A, WEBB C, GRINDULIS KA, FLEMING J, SHELDON PJ: Sulphasalazine therapy in rheumatoid arthritis: Qualitative changes in lymphocytes and correlation with clinical response. *Br J Rheumatol* 1992; 31: 259-63.
71. AONO H, HASUNUMA T, FUJISAWA K, NAKAJIMA T, YAMAMOTO K, MITA S, NISHIOKA K: Direct suppression of human synovial cell proliferation *in vitro* by salazosulfapyridine and bucillamine. *J Rheumatol* 1996; 23: 65-70.
72. AKAHOSHI T, NAMAI R, SEKIYAMA N, TANAKA S, HOSAKA S, KONDO H: Rapid induction of neutrophil apoptosis by sulfasalazine: implications of reactive oxygen species in the apoptotic process. *J Leukocyte Biol* 1997; 62: 817-26.
73. GIBSON PR, JEWELL DP: Sulphasalazine and derivatives, natural killer activity and ulcerative colitis. *Clin Sci* 1985; 69: 177-84.
74. DANIS VA, FRANIC GM, RATHJEN DA, LAURENT RM, BROOKS PM: Circulating cytokine levels in patients with rheumatoid arthritis: Results of a double blind trial with sulphasalazine. *Ann Rheum Dis* 1992; 51: 946-50.
75. BISSONNETTE EY, ENCISO JA, BEFUS AD: Inhibitory effects of sulfasalazine and its metabolites on histamine release and TNF-alpha production by mast cells. *J Immunol* 1996; 156: 218-23.
76. WAHL C, LIPTAY S, ADLER G, SCHMID RM: Sulfasalazine: A potent and specific inhibitor of nuclear factor kappa B. *J Clin Invest* 1998; 101: 1163-74.
77. GREENFIELD SM, HAMBLIN AS, SHAKOOR ZS, TEARE JP, PUNCHARD NA, THOMPSON RP: Inhibition of leucocyte adhesion molecule upregulation by tumor necrosis factor alpha: A novel mechanism of action of sulphasalazine. *Gut* 1993; 34: 252-6.
78. GADANGI P, LONGAKER M, NAIME D *et al.*: The anti-inflammatory mechanism of sulfasalazine is related to adenosine release at inflamed sites. *J Immunol* 1996; 156: 1937-41.
79. SHARON P, DRAB EA, LINDER JS, WEIDMAN SW, SABESIN SM, RUBIN DB: The effect of sulfasalazine on bovine endothelial cell proliferation and cell cycle phase distribution. Comparison with olsalazine, 5-aminosalicylic acid, and sulfapyridine. *J Lab Clin Med* 1992; 119: 99-107.
80. MADHOK R, WIJELATH E, SMITH J, WATSON J, STURROCK RD, CAPELL HA: Is the beneficial effect of sulfasalazine due to inhibition of synovial neovascularization ? *J Rheumatol* 1991; 18: 199-202.
81. WILLIAMS JG, HALLETT MB: Effect of sulphasalazine and its active metabolite, 5-aminosalicylic acid, on toxic oxygen metabolite production by neutrophils. *Gut* 1989; 30: 1581-7.
82. BRADLEY SM, LE GALLEZ P, THROUGHTON PR, GOOI HC, ASTBURY C, BIRD HA: The effect of sulphasalazine on neutrophil superoxide generation in rheumatoid arthritis. *Br J Rheumatol* 1997; 36: 530-4.
83. NOSE M, SASANO M, KAWASHIMA Y: Salazosulfapyridine suppresses chondrocyte mediated degradation induced by interleukin 1beta. *J Rheumatol* 1997; 24: 550-4.
84. AL-SAFI SA, MADDOCKS JL: Effects of azathioprine on the human mixed lymphocyte reaction (MLR). *Br J Clin Pharmacol* 1983; 15: 203-9.
85. ABDOU NI, ZWEIMAN B, CASELLA SR: Effects of azathioprine therapy on bone marrow-dependent and thymus-dependent cells in man. *Clin Exp Immuno* 1973; 13: 55-64.
86. LEVY J, BARNETT EV, MACDONALD NS, KLINENBERG JR, PEARSON CM: The effect of azathioprine on gammaglobulin synthesis in man. *J Clin Invest* 1972; 51: 2233-8.
87. CSEUZ R, PANAYI GS: The inhibition of NK cell function by azathioprine during the treatment of patients with rheumatoid arthritis. *Br J Rheumatol* 1990; 29: 358-62.
88. CSEUZ R, BARNES P, PANAYI GS: Natural killer cells in the blood of patients with rheumatoid arthritis treated with azathioprine. *Br J Rheumatol* 1990; 29: 284-7.
89. KROGSTAD DJ, SCHLESINGER PH: Acid-vesicle function, intracellular pathogens, and the action of chloroquine against *Plasmodium falciparum*. [1]. *N Engl J Med* 1987; 317: 542-9.
90. MACINTYRE AC, CUTLER DJ: Role of lysosomes in hepatic accumulation of chloroquine. *J Pharm Sci* 1988; 77: 196-9.
91. GINSBURG H, GEARY TG: Current concepts and new ideas on the mechanism of action of quinoline-containing antimalarials. [1]. *Biochem Pharmacol* 1987; 36: 1567-76.
92. WARD PA: The chemosuppression of chemotaxis. *J Exp Med* 1966; 124: 209-26.
93. RHODES JM, MCLAUGHLIN JE, BROWN DJ, NUTTALL LA, JEWELL DP: Inhibition of leucocyte motility and prevention of immune-complex experimental colitis by hydroxychloroquine. *Gut* 1982; 23: 181-7.
94. PANAYI GS, NEILL WA, DUTHIE JJR, MCCORMICK JN: The action of chloroquine phosphate in rheumatoid arthritis. 11 Chromosome damaging effect. *Ann Rheum Dis* 1973; 32: 547.
95. TRIST DG, WEATHERALL M: Inhibition of lymphocyte transformation by mepacrine and chloroquine. *J Pharm Pharmacol* 1981; 33: 434-8.
96. PANAYI GS, NEILL WA, DUTHIE JJR, MCCORMICK JN: Action of chloroquine phosphate in rheumatoid arthritis. 1. Immunosuppressive effect. *Ann Rheum Dis* 1973; 32: 316-8.
97. THOMPSON GR, BARTHOLOMEW L: The effect of chloroquine on antibody production. *University of Michigan Medical Center Journal* 1964; 30: 227-30.
98. MANKU MS, HORROBIN DF: Chloroquine, quinine, procaine, quinidine, tricyclic antidepressants, and methylxanthines as prostaglandin agonists and antagonists. *Lancet* 1976; 2: 1115-7.
99. PANAYI GS, TUGWELL P: The use of cyclosporin A in rheumatoid arthritis: Proceedings of an international consensus meeting. *Br J Rheumatol* 1993; 32 (Suppl. 1): 1-3.
100. KRONKE M, LEONARD WJ, DEPPER JM *et al.*: Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc Natl Acad Sci USA* 1984; 81: 5214-8.
101. GRANELLI PIPERNO A, ANDRUS L, STEINMAN RM: Lymphokine and non-lymphokine mRNA levels in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin. *J Exp Med* 1986; 163: 922-37.
102. WIEDERRECHT G, LAM E, HUNG S, MARTIN M, SIGAL N: The mechanism of action of FK-506 and cyclosporin A. *Ann NY Acad Sci* 1993; 696: 9-19.
103. BRAM RJ, HUNG DT, MARTIN PK, SCHREIBER SL, CRABTREE GR: Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: Roles of calcineurin binding and cellular location. *Mol Cell Biol* 1993; 13: 4760-9.
104. MCCAFFREY PG, PERRINO BA, SODERLING TR, RAO A: NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J Biol Chem* 1993; 268: 3747-52.
105. MATSUDA S, MORIGUCHI T, KOYASU S, NISHIDA E: T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J Biol Chem* 1998; 273: 12378-82.
106. YOCUM D: Immunological actions of cyclosporin A in rheumatoid arthritis. *Br J Rheumatol* 1993; 32 (Suppl. 1): 38-41.
107. TRAJKOVIC V, BADOVINAC V, JANKOVIC V, SAMARDZIC T, MAKSIMOVIC D, POPADIC D: Cyclosporin A suppresses the induction of nitric oxide synthesis in interferon-gamma-treated L929 fibroblasts. *Scand J Immunol* 1999; 49: 126-30.
108. SUGANO N, ITO K, MURAI S: Cyclosporin A inhibits collagenase gene expression via AP-1 and JNK suppression in human gingival fibroblasts. *J Periodont Res* 1998; 33:448-52.
109. IURLARO M, VACCA A, MINISCHETTI M *et al.*: Antiangiogenesis by cyclosporine. *Exp Hematol* 1998; 26: 1215-22.
110. KHANNA AK, CAIRNS VR, BECKER CG, HOSENPUD JD: Transforming growth factor (TGF)-beta mimics and anti-TGF-beta antibody abrogates the *in vivo* effects of cyclosporine: Demonstration of a direct role of TGF-beta in immunosuppression and nephrotoxicity of cyclosporine. *Transplantation* 1999; 67: 882-9.
111. SCOTT DG, BACON PA, ALLEN C, ELSON CJ, WALLINGTON T: IgG rheumatoid factor, complement and immune complexes in rheumatoid synovitis and vasculitis: Comparative and serial studies during cytotoxic therapy. *Clin Exp Immunol* 1981; 43: 54-63.
112. BAST RC, BAST BS: Critical review of previously reported animal studies of tumor immunotherapy with nonspecific immunostimulants. [2]. *Ann NY Acad Sci* 1976; 277: 60-93.

113. SMOLEN JS, KALDEN JR, SCOTT DL *et al.*: Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: A double-blind, randomised, multicentre trial. European Leflunomide Study Group. *Lancet* 1999; 353: 259-66.
114. MIRMOHAMMADSADEGH A, HOMEY B, ABTS HF, KOHRER K, RUZICKA T, MICHEL G: Differential modulation of pro- and anti-inflammatory cytokine receptors by N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide (A77 1726), the physiologically active metabolite of the novel immunomodulator leflunomide. *Biochem Pharmacol* 1998; 55: 1523-9.
115. CHONG AS, FINNEGAN A, JIANG X, GEBEL H, SANKARY HN, FOSTER P, WILLIAMS JW: Leflunomide, a novel immunosuppressive agent. The mechanism of inhibition of T cell proliferation. *Transplantation* 1993; 55: 1361-6.
116. BARTLETT RR: Immunopharmacological profile of HWA 486, a novel isoxazol derivative. II. *In vivo* immunomodulating effects differ from those of cyclophosphamide, prednisolone, or cyclosporin A. *Int J Immunopharmacol* 1986; 8: 199-204.
117. CHERWINSKI HM, COHN RG, CHEUNG P *et al.*: The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. *J Pharmacol Exp Ther* 1995; 275: 1043-9.
118. BRUNEAU JM, YEA CM, SPINELLA-JAEGLE S *et al.*: Purification of human dihydro-orotate dehydrogenase and its inhibition by A77 1726, the active metabolite of leflunomide. *Biochem J* 1998; 336: 299-303.
119. SCOTT DL, DAWES PT, TUNN E *et al.*: Combination therapy with gold and hydroxychloroquine in rheumatoid arthritis: A prospective, randomized, placebo-controlled study. *Br J Rheumatol* 1989; 28: 128-33.
120. GIBSON TG, EMERY P, ARMSTRONG RD, CRISP AJ, PANAYI GS: Combined D-penicillamine and chloroquine treatment - A comparative study. *Br J Rheumatol* 1987; 26: 279-84.
121. O'DELL JR, HAIRE CE, ERIKSON N *et al.*: Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. *N Engl J Med* 1996; 334: 1287-91.
122. SZUMLANSKI CL, WEINSHILBOUM RM : Sulphasalazine inhibition of thiopurine methyltransferase: A possible mechanism for interaction with 6-mercaptopurine and azathioprine. *Br J Clin Pharmacol* 1995; 39: 456-9.