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 research has 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 anti-rheumatic 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 anti-inflammatory 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-
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 aurothioglucone 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 (PBMC) 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 PBMC 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 immunoglobulin, 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 TFHID 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 aminoimidazole-carboxamidole 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-aminomidazole-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 PBMC 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 PBMC 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’ PBMC, but MTX seemed to inhibit IL-1β 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 PBMC 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 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 D₄ 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).

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**Sulphasalazine (SASP)**

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 hypogammaglobulinemia (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 synovocytes 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-1β, 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-κB (76).

Since NF-κB 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 E2 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-thioguanine 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 paralleled 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,
A potent 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.
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 methyl transferase, which catabolises the s-methylthiol 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.

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Mechanisms of action of second-line agents / E. Choy & G. Panayi

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Mechanisms of action of second-line agents / E. Choy & G. Panayi


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