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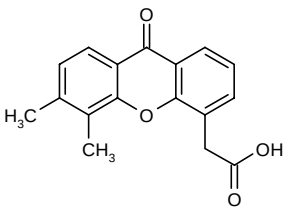
ASA404

Vascular-Disrupting Agent
Oncolytic

DMXAA
AS1404 (former code name)

5,6-Dimethyl-9-oxoxanthene-4-acetic acid

InChI=1/C17H14O4/c1-9-6-7-13-15(20)12-5-3-4-11(8-14(18)19)17(12)21-16(13)10(9)2/h3-7H,8H2,1-2H3,(H,18,19)



$C_{17}H_{14}O_4$
Mol wt: 282.2907

CAS: 117570-53-3

EN: 164510

Abstract

ASA404 (DMXAA, formerly AS1404) was originally synthesized at the Auckland Cancer Society Research Centre in an effort to identify more active analogues of flavone acetic acid (FAA). Phase I trials of ASA404 were based on its curative properties in murine transplantable tumors at 15-fold increased potency over FAA. Phase II studies showed activity for ASA404 in combination with standard chemotherapy in lung and prostate cancers, and phase III studies in lung cancers commenced in 2008. ASA404 exerts its antitumor effects partly through inhibition of tumor blood flow, but unlike other small-molecule vascular-targeting agents under clinical investigation, it does not act through modulation of the tubulin cytoskeleton of vascular endothelial cells. While the molecular target of ASA404 is unknown, a hallmark of its preclinical activity is its induction of cytokine production within the tumor tissue. These cytokines confer a multiplicity of indirect effects, including vascular collapse and enhanced immune response, making ASA404 one of the most promising agents of its class under clinical development.

Synthesis*

ASA404 can be synthesized as follows. The reaction of 2,3-dimethylaniline (I) with chloral hydrate (II) and hydroxylamine in hot aqueous HCl gives the isonitrosoacetanilide (III), which is cyclized in hot methanesulfonic acid or sulfuric acid to yield 6,7-dimethylisatin (IV). Oxidative cleavage of isatin (IV) by means of KOH and H_2O_2 affords 3,4-dimethylanthranilic acid (V), which is converted to 2-iodo-3,4-dimethylbenzoic acid (VI) by diazotization with $NaNO_2$ and H_2SO_4, followed by treatment with KI. Aryl iodide (VI) is then condensed with the disodium salt of 2-(2-hydroxyphenyl)acetic acid (VII) by means of tris[2-(2-methoxyethoxy)ethyl]amine (TDA) and CuCl in DMSO to afford the precursor 2-aryloxybenzoic acid (VIII), which is finally cyclized upon heating with concentrated sulfuric acid (Scheme 1) (1-3).

Aqueous and alcoholic solutions of the sodium salt of ASA404 (a form in which it is commonly formulated for *in vitro* and *in vivo* studies) are photochemically unstable, undergoing spontaneous decarboxylation to the corresponding methyl compound under ambient lighting conditions (Scheme 2) (4). This decarboxylation was complete in 3 h in bright sunlight, but could be prevented by handling the solutions in subdued light. An azide-containing analogue of ASA404 has recently been synthesized for use in photoaffinity labeling of the biological receptor(s) for this compound (5).

Background

The tumor vasculature is recognized as an attractive target for cancer therapy, as destruction of a single vessel has the potential for starving thousands of dependent

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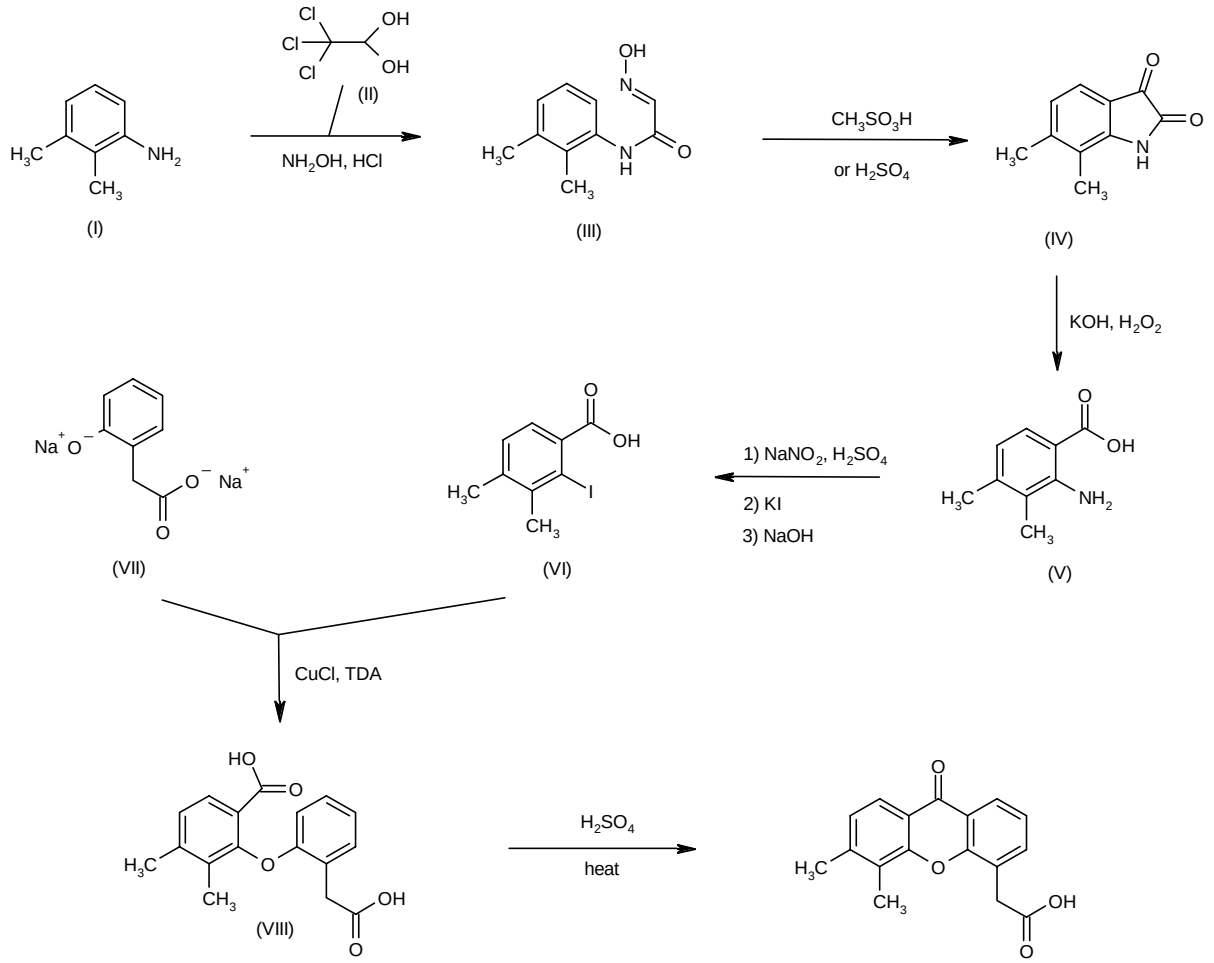
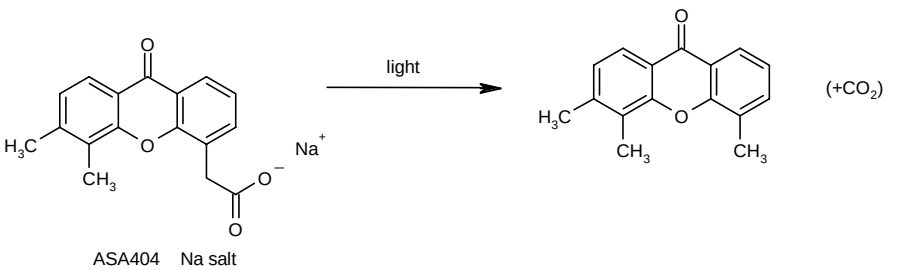
Scheme 1: Synthesis of ASA404



Scheme 2: Decarboxylation of ASA404



cancer cells. Collectively known as tumor vascular-disrupting agents (VDAs), a number of synthetic small molecules with the ability to damage the tumor vasculature have entered clinical evaluation for the treatment of human cancers. Most VDAs disrupt the existing tumor vasculature through an interaction with the tubulin cytoskeleton, leading to altered endothelial cell shape and destruction of intercellular junctions (6). ASA404 (also known as DMXAA and formerly known as AS1404) was developed at the Auckland Cancer Society Research Centre (1) as a more active analogue of flavone acetic acid (FAA), a flavonoid that displayed excellent activity

against transplantable murine tumors but failed to demonstrate clinical efficacy (7).

ASA404 differs from the other small-molecule VDAs in clinical development in that tubulin does not appear to be the primary target. ASA404 inhibited blood flow in subcutaneous murine tumors by a process that involves partial dissolution of the actin cytoskeleton (8) and selective induction of apoptosis of tumor vascular endothelial cells within 30 min. At later time points, the indirect effects elicited by the cascade of cytokines induced by the agent supervene. Evidence of similar apoptotic endothelial cell induction was obtained in biopsies from 1 of 3 patients with breast cancers investigated in phase I trials (9). Moreover, DCE-MRI monitoring in one of the phase I trials indicated up to 66% reduction in tumor blood flow in 8 of 11 patients (10). Following promising phase II results in combination with taxanes and carboplatin for the treatment of non-small cell lung cancer (NSCLC) (11) and prostate cancer (12), phase III trials began in 2008, making ASA404 the most advanced of its class in clinical development. The preclinical and clinical data for ASA404 are summarized in the following sections.

Preclinical Pharmacology

As the antitumor actions of the flavonoids are not caused by direct cytotoxic effects on cancer cells, a histological screen of necrosis in subcutaneously implanted tumors in mice was used for determining the potency of the xanthenone derivatives synthesized at the Auckland Cancer Society Research Centre. ASA404 was shown to be the most potent in this screen, causing 100% necrosis of colon 38 tumors at its maximum tolerated dose (MTD) of 30 mg/kg, compared with 330 mg/kg for FAA, in BDF1 mice. Moreover, 80% of the tumors regressed compared with only 50% tumor regression in the FAA-treated group (1).

Evidence of vascular disruption by ASA404 was first obtained by quantifying the loss of functional vessels labeled using a fluorescent dye in colon 38 tumors. Significant reductions in functioning vessels were observed in tumors 30 min after treatment with ASA404, and reached a maximum reduction of 80% after 4 h (13). TUNEL staining as a determinant of apoptosis was also seen in vascular endothelial cells in colon 38 tumors as early as 30 min after ASA404 administration (9). The number of apoptotic vessels correlated with the loss of functioning vessels, as measured using Hoechst fluorescent dye staining, indicating that the vascular-disrupting effects of ASA404 are a consequence of induction of apoptosis of vascular endothelial cells. No apoptotic vessels were observed in the brain, liver, spleen or heart of mice treated with ASA404, demonstrating the selectivity of the agent for the tumor vasculature (14). The vascular-disrupting effects of ASA404 were subsequently confirmed using magnetic resonance imaging (MRI) techniques in a variety of murine and human tumor xenograft models in mice (15-18). In rats, small but prolonged decreases in tumor blood flow were observed in transplanted prolactinomas at the MTD of 350 mg/kg when assessed using dynamic contrast-enhanced MRI (19), but only transient effects were observed when intrinsic susceptibility MRI was used (20). In 4 of 5 rats bearing chemically induced primary mammary tumors, treatment with ASA404 at the MTD caused necrosis involving 3.7-41.2% of the area of the tumor section after 24 h, compared with no necrosis in 4 untreated controls (21).

Sustained stoppage of tumor blood flow is required for the development of hemorrhagic necrosis, since cell viability is maintained for over 1 h after the arrest of blood flow (22). The sustained inhibition of blood flow obtained in mice has been suggested to involve a number of other factors secondary to the direct antivascular effects. The initial vascular damage is likely to lead to subsequent activation of platelets and the release of serotonin (5-HT), which has effects on the vasculature in its own right (23). Indeed, elevated levels of 5-HT, or its principal metabolite 5-hydroxyindole acetic acid (5-HIAA), are detected following ASA404 administration to mice (24), rats (19) and patients in clinical trials (25). Another possible component of the vasoactive cascade produced by ASA404 is the production of nitric oxide (NO), which has been inferred from elevated levels of plasma nitrite and nitrate following ASA404 administration to mice (26). NO has dual effects on the vasculature, relaxing vascular smooth muscle and improving blood flow if released systemically, or increasing vessel permeability if released locally (27). It is not known if ASA404 induces local release of NO in the tumor microenvironment, but the antitumor activity of the flavonoid analogues correlated well with their ability to induce NO production in cultured murine macrophages (28).

Tumor necrosis factor α (TNF-α), a vasoactive cytokine which, by itself, can cause vascular occlusion and blood flow collapse in tumors, has been shown in a number of studies to be produced following ASA404 administration to mice (29-31). TNF-α synthesis was more sustained in the tumor, resulting in higher intratumoral concentrations than those in serum, spleen or liver (29). Studies with human melanoma and ovarian cancer xenografts in immunodeficient mice (29), as well as murine tumors implanted in TNF-α knockout mice (30), showed that both cancer cells and host stromal cells in the tumor expressed TNF-α in response to ASA404. The importance of TNF-α in enhancing and prolonging the initial direct effects on the tumor vasculature was supported by studies which showed marked attenuation of the antitumor activity of ASA404 in TNF receptor 1 (TNFR1) and TNF-α knockout mice (31). However, as these knockout mice can tolerate much higher doses of ASA404 than wild-type mice, significant growth delays and cures could be achieved using higher doses of ASA404 in the knockout strains. The results implied that while TNF-α played a part, other factors were also involved.

Indeed, several studies have shown that TNF-α is only one of a panel of cytokines and chemokines that are induced by ASA404 (32-35). In addition to TNF-α, 6 h after ASA404 administration, elevated levels of IP-10, IL-6, KC,

MIP2 (macrophage inflammatory protein 2), MCP-1 (monocyte chemotactic protein 1) and RANTES were detected in L1C2 murine bronchoalveolar carcinomas implanted into syngeneic BALB/c mice. The induced chemokines have been suggested to be responsible for the 10-fold influx of macrophages into murine Lewis lung carcinomas 24 h following ASA404 treatment (34). Dendritic cell activation in tumor-draining lymph nodes was also observed after 24 h in mice bearing EG7 thymomas treated with ASA404, followed by a marked increase in the number of tetramer-specific $CD8^+$ T lymphocytes in the spleen (36). An influx of $CD8^+$ T cells was seen in tumors 3 days after ASA404 administration, and these cells appear to be critical to the overall antitumor activity. Inhibition of the growth of L1C2 tumors was lost by depletion of $CD8^+$ T cells, and in perforin knockout mice but not in $CD4^+$-depleted mice (34). Thus, ASA404 is a VDA with immune-enhancing capabilities due to its induction of a panel of cytokines. Both of these actions appear to be important to the overall antitumor response in mice.

While there is significant overlap in the spectrum of cytokines induced by ASA404 and lipopolysaccharide (LPS), ASA404 is a much more potent stimulator of the interferons (32, 37). LPS stimulates cytokine production through binding with Toll-like receptors (TLRs) expressed on the cell surface, but cytokine induction by ASA404 does not appear to involve any of the known TLRs (36, 37). The receptor for ASA404 has not been identified and may be intracellular rather than on the cell surface. Since the transcription of numerous cytokine genes is under NF-κB regulation, it is not surprising that ASA404 was shown to activate NF-κB transcription factors in a number of studies both *in vitro* and *in vivo* (38-41). The addition of salicylate or parthenolide concomitantly inhibited NF-κB activation and the production of TNF-α and interferon gamma induced by ASA404 in primary murine splenocyte cultures (35), consistent with the involvement of the NF-κB signaling pathway. On the other hand, minimal involvement of NF-κB was necessary for ASA404-stimulated increases in mRNA for interferon gamma in primary murine macrophages, which could also be inhibited by salicylate. Interferon gamma gene transcription induced by ASA404 in this study was critically dependent on the TBK1-IRF-3 (interferon regulatory factor 3) signaling axis (37). Information to date would suggest that ASA404 activates multiple and different signaling pathways in various cell lineages.

The utility of ASA404, and indeed of most antivascular agents, is likely to lie in combination with other treatment modalities, and ASA404 has been shown in a number of preclinical studies to combine productively with a variety of established and experimental treatments that include gene therapy (42), hyperthermia (43), photodynamic therapy (44) and radiation (45). This section summarizes preclinical studies on ASA404 in combination with established drugs in the clinic.

Greater than additive growth inhibition of subcutaneously implanted murine MDAH-MCA-4 tumors was obtained with combined treatment of ASA404 and melphalan (46). Maximal synergy was obtained when melphalan was given 2 h after ASA404. Plasma pharmacokinetics of melphalan were unchanged, and although a small increase in weight loss was noted, no increase in host mortality due to melphalan was observed in ASA404-pretreated animals. ASA404 pretreatment resulted in a 33% increase in the AUC for melphalan in the tumor, providing a basis for the increased antitumor response. The increase in tumor AUC, according to the authors, was consistent with decreasing tumor blood flow, which shows a lag of 2 h and 70-75% inhibition of flow by 4 h after ASA404 (47), causing entrapment of the cytotoxic agent.

The same tumor model was used to survey combinations of ASA404 with nine other chemotherapeutic agents. Combination with paclitaxel gave the greatest therapeutic gain, but greater mortality was encountered for unknown reasons and the dose of both agents had to be significantly reduced below their respective MTDs. In contrast to melphalan, combination with paclitaxel resulted in significantly reduced AUCs of paclitaxel in the tumor, and the greatest synergy was obtained when paclitaxel was administered prior to ASA404 to avoid compromised delivery of paclitaxel to the tumor due to the vascular shutdown. In the same study, improved antitumor responses were obtained with ASA404 combined with vincristine, cisplatin, doxorubicin, cyclophosphamide, carboplatin and etoposide (in decreasing order of efficacy), while no gains were obtained with 5-fluorouracil (48). The results of this study with MDAH-MCA-4 tumors were consistent with those of an earlier study using KHT sarcomas, where ASA404 enhanced cisplatin-induced tumor cell kill when administered 1-3 h after the cytotoxic agent (49).

ASA404 also combines synergistically with nonsteroidal antiinflammatory drugs (NSAIDs), including diclofenac (50), pentoxifylline (51) and thalidomide (52). When thalidomide (100 mg/kg) was coadministered with ASA404 at its MTD to BDF1 mice with subcutaneous colon 38 tumors, 100% of the mice remained tumor-free 3 months after treatment, compared with 67% in the group treated with ASA404 alone and no cures in the group treated with thalidomide alone (52). The greatest synergy was achieved when the two agents were administered at the same time, and the therapeutic gain was lost when thalidomide was given more than 24 h before or after ASA404 (51). Mice treated with both agents had lower body temperatures and higher hematocrits than those treated with each of the agents alone, but there were no increases in mortality. Uptake of Evans blue into the tissues was shown to be transiently increased by ASA404 alone in colon 38 tumors, was minimally affected by thalidomide alone and was significantly increased for over 12 h in the group treated with the combination, suggesting an increase in macromolecular vascular permeability. In contrast, Evans blue uptake in liver was decreased with either monotherapy, with no additive effect observed when ASA404 and thalidomide were combined (53). In the same tumor model, coadministra-

tion of thalidomide increased the intratumoral concentrations of TNF-α and decreased the serum levels compared with ASA404 alone (54). The combined treatment also resulted in a pharmacokinetic interaction that slowed the rate of clearance of both ASA404 (50) and thalidomide (55) from the plasma, tumor, liver and spleen. This interaction was TNF-α-dependent, as no alteration of pharmacokinetics was detected in TNF-α knockout mice. The decreased rates of clearance in wild-type mice resulted in a 2.4- and a 3.0-fold increase in the AUC of thalidomide (55) and ASA404, respectively, in colon 38 tumors compared with those treated with the agents alone. It is not clear if the increased AUCs obtained with the combined treatment provide the basis for the improved antitumor activity, and the other altered pharmacodynamic properties. Whether these interactions also occur as part of the improved antitumor responses when ASA404 is combined with other NSAIDs has yet to be established.

Pharmacokinetics and Metabolism

The maximum tolerated i.p. dose of ASA404 in mice is generally between 18 and 30 mg/kg and is highly dependent on the strain of mouse. In non-tumor-bearing female C57BL/6 mice at a dose of 25 mg/kg, the plasma half-life of the drug was 2.7 h following i.p. administration, with a C_{max} of 570 μmol/l and an AUC of 1715 μmol.h/l (56). These values were affected little by i.v. dosing, but following oral dosing at 30 mg/kg the plasma half-life increased to 9.2 h and the C_{max} decreased to 358 μmol/l. The AUC was reduced by 12% to 1392 μmol.h/l and the oral bioavailability was calculated to be 73%. Similar effects were observed upon i.p. and p.o. administration of ASA404 to colon 38 tumor-bearing mice, but in these cases the C_{max} values were significantly lower in tumor-bearing mice than in non-tumor-bearing animals. The antitumor activity of orally administered ASA404 at the MTD of 32.5 mg/kg was significantly lower than that following i.p dosing at the MTD of 27.5 mg/kg. However, tumor tissue exposure to ASA404 could be significantly increased following oral dosing by appropriate dose scheduling and adjustments, resulting in an impressive increase in cure rate (from 0% to 90%) (57). The MTDs of ASA404 in rats and rabbits were approximately 280 and 90 mg/kg, respectively (58). Following administration of ASA404 at the respective MTDs to rats and rabbits, the C_{max} values were 2200 and 1708 μM, respectively, and the AUCs were 19,000 and 2400 μM.h, respectively. In the same study, the pharmacokinetic parameters for ASA404 in BDF1 mice after a dose of 25.5 mg/kg were 600 μM and 2400 μM.h, respectively. In female rats, following a dose of 30 mg/kg of ASA404 the AUC was 2413 μM.h, the C_{max} was 1236 μM and the elimination half-life was 2.4 h. These values were 60%, 73% and 55% greater, respectively, in female rats compared with male rats, an effect that was found to correlate with an increased rate of metabolism of the drug by male rats (59).

ASA404 was found to be extensively protein-bound in plasma from four species studied. The unbound fraction determined at the concentration of 500 μM ASA404 was 4.6% in murine, 2.6% in rat, 2.0% in rabbit and 2.1% in human plasma (60).

ASA404 is extensively metabolized, undergoing β-glucuronidation at the acetic acid side-chain and hydroxylation at the 6-methyl group in mice, rats and humans (Fig. 1) (58, 61). In humans, the hydroxylated product M2 undergoes further glucuronidation to give M3, and all three metabolites are excreted in both bile and

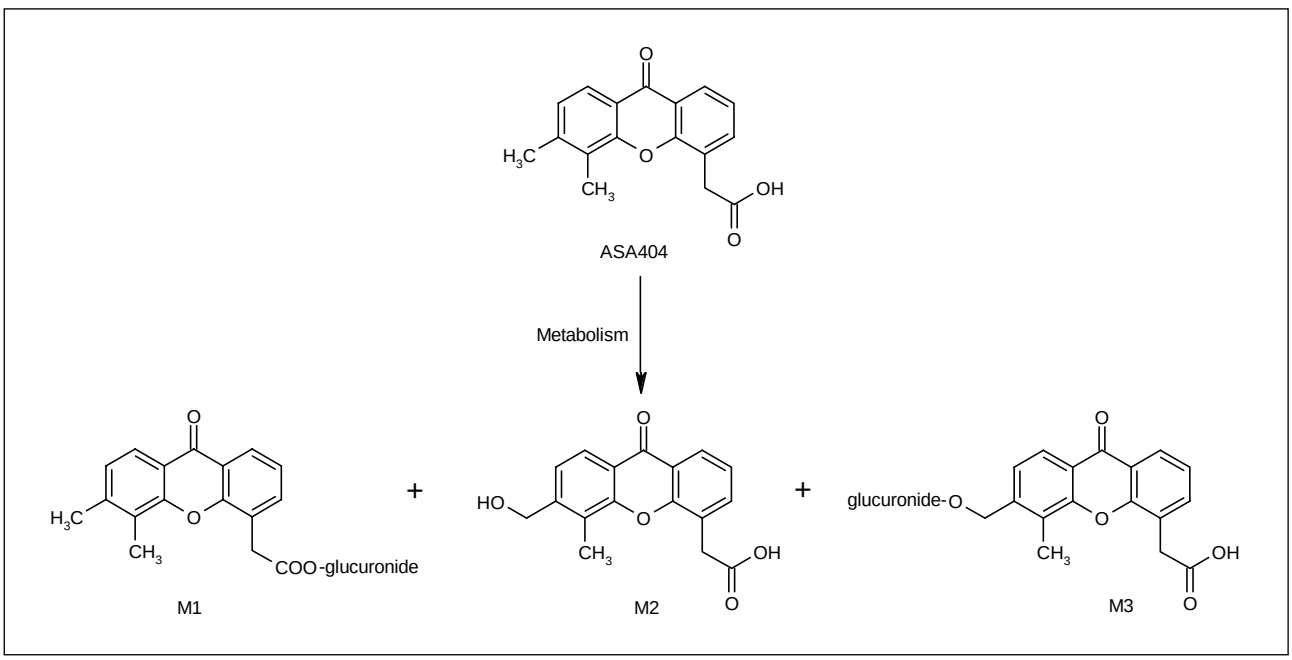


Fig. 1. Metabolism of ASA404.

urine. In humans, up to 60% of the total dose of ASA404 was excreted as M1, 5.5% as M2 and 4.5% as M3. The acyl glucuronide M1 is unstable both *in vitro* and *in vivo*, undergoing hydrolysis and covalent binding to plasma proteins (61). ASA404 glucuronidation at the acetic acid side-chain is catalyzed by uridine diphosphate glucuronosyltransferases (UGTs) (62) and hydroxylation of the 6-methyl group is catalyzed by the CYP1A isoform of cytochrome P-450 (63).

Clinical Studies

In a phase I clinical trial of ASA404 involving 63 patients with a range of advanced cancers, the drug was administered as a 20-min i.v. infusion every 3 weeks (64). The dose was escalated over 19 levels from 6 to 4900 mg/m^2 and 3700 mg/m^2 was established as the MTD. Over the dose range from 6 to 4900 mg/m^2 the clearance decreased in a nonlinear manner from 16.1 to 1.42 l/h.m^2 and the AUC increased accordingly from 1.29 to 12,400 μM.h. The increase in C_{max} from 2.17 to 1910 μM over this dose range was approximately linear. The terminal plasma half-life of the drug was 8.1 h. ASA404 was found to be highly bound to albumin (> 99% at doses up to 320 mg/m^2) until saturation occurred at higher doses, when the free fraction of drug increased up to a value of approximately 20%. The drug was well tolerated at lower doses, while at the highest dose studied (4900 mg/m^2), reversible toxicities included confusion, tremor, slurred speech, visual disturbances, anxiety, urinary incontinence and possible left ventricular failure. Transient prolongation of the cardiac Q-T interval was seen in 13 patients beginning at a dose of 2000 mg/m^2. One patient with metastatic cervical carcinoma achieved an unconfirmed partial response at 1100 mg/m^2, which progressed after eight courses of treatment.

Similar pharmacokinetic parameters were obtained in a concurrent second phase I trial that included 46 patients who were treated weekly rather than triweekly with ASA404 over the same dose range (65). Similar toxicities were noted at the higher doses and the MTD was confirmed to be 3700 mg/m^2. In this study, there was also 1 unconfirmed partial response at 1300 mg/m^2. From these two phase I trials, a dose of 1200 mg/m^2 was selected for further phase II evaluations, chosen partly as a result of the 2 unconfirmed clinical responses seen at around this dose level and because the drug was very well tolerated at this dose. In a small phase I study in 15 patients, at a dose of 1200 mg/m^2, which was chosen for further clinical evaluation, the C_{max} for total drug was 1116 μM and the AUC was 2465 μM.h. (66).

Phase II combination studies of ASA404 were carried out in specific tumor types to compare standard therapy with and without the addition of ASA404, since preclinical studies had established that the agent was synergistic with a range of clinical anticancer agents, including carboplatin and the taxanes (48). Three randomized phase II trials have combined ASA404 with taxane and carboplatin regimens in lung, prostate and ovarian cancers. In a phase Ib/II study of ASA404 in combination with carboplatin and paclitaxel in non-small cell lung cancer (NSCLC; squamous and nonsquamous, locally advanced or metastatic), 37 randomized patients received carboplatin (AUC 6 mg/ml.min as a 3-h i.v. infusion), followed by paclitaxel (175 mg/m^2 as a 30-min i.v. infusion), then ASA404 (1200 mg/m^2 as a 20-min i.v. infusion) (67). The drugs were administered sequentially on the same day once every 3 weeks for up to 6 cycles. Results were compared with those for 36 patients who received a standard therapy of carboplatin and paclitaxel alone. The addition of ASA404 was well tolerated, resulting in no significant additional toxicity. The inclusion of ASA404 to the regimen resulted in an increase in median survival of 5.2 months (from 8.8 to 14.0 months), of particular note for this refractory tumor type. The time to tumor progression was 5.4 months in the ASA404-treated group and 4.4 months in the standard therapy group. The relative response rates were 31.2% in the ASA404 group and 22.2% in the standard group.

A single-arm study tested a higher dose of ASA404 at 1800 mg/m^2 in combination with carboplatin and paclitaxel in NSCLC (11). This dose of ASA404 was also well tolerated, and the overall response rate by investigator's assessment was 37.9%. The time to tumor progression was 5.5 months and the median survival time was 14.9 months.

ASA404 has been evaluated in combination with docetaxel in hormone-refractory metastatic prostate cancer (HRPC) (12). In a randomized phase II study, 34 men received docetaxel (75 mg/m^2 as a 1-h infusion) followed by ASA404 (1200 mg/m^2 as a 20-min infusion) once every 3 weeks for up to 10 cycles; 40 men received docetaxel alone in the control arm. Interim results indicated that 57% of patients in the ASA404 arm showed a reduction in their prostate-specific antigen (PSA) levels (defined as a 50% or greater fall from baseline) compared with 35% of patients in the docetaxel arm. Seventeen percent of patients had an increase in their PSA levels in the combination arm compared with 29% in the docetaxel cohort. The data suggest that ASA404 may have activity in HRPC when used in combination with docetaxel.

In a randomized phase II study of ASA404 in combination with carboplatin and paclitaxel for recurrent ovarian cancer, 37 women received this combination of drugs and 38 received a standard therapy of carboplatin and paclitaxel. The doses and the scheduling were the same as those used in the phase II trials in NSCLC described above using 1200 mg/m^2 of ASA404. Interim results showed response rates of 63.9% in the ASA404 arm and 48.6% in the standard arm (68).

A phase III trial was recently commenced evaluating ASA404 in combination with paclitaxel and carboplatin as first-line treatment of patients with stage IIIb/IV NSCLC (69). A phase I trial is under way in Japanese patients with NSCLC in combination with paclitaxel and carboplatin (70), and a phase II study is evaluating ASA404 in combination with docetaxel in patients with HRPC (71).

Sources

Originally developed at the Auckland Cancer Society Research Centre, University of Auckland, ASA404 was licensed to Antisoma plc for clinical development, which subsequently licensed the compound to Novartis.

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