Anti-tumor effects of AMT in the renal cell carcinoma model

MARCELLO CABALLERO1*, JÜRGEN SCHEELE3,4*, UTE ZIRRGIEBEL2, NORBERT ESSE2, CHRISTOPH SCHÄCHTELE2, JENS SOLTAU1, JOCHEN RENTSCHLER1, KLAUS DIERGARTEN3 and JOACHIM DREVS1

1Cancer Hospital Sanafontis; 2ProQinase GmbH; 3AURON Healthcare GmbH; 4Departments of Pharmacology I and Medicine I, University of Freiburg Medical Center, Freiburg, Germany

Received July 6, 2009; Accepted August 20, 2009

DOI: 10.3892/or_00000624

Abstract. Auron-Misheil-Therapy (AMT) is a defined but unique combination of approved pharmaceuticals. It consists of insulin, chlorpheniramine and an aqueous camomile extract, and it has been successfully applied clinically in late-stage cancer patients. The purpose of this study was to elucidate the anti-tumor efficacy of AMT in a validated murine renal cell carcinoma animal model (RENCA). There were two independent studies; each animal group consisted of 16 mice. During a 6-week pretreatment period, vehicle (group A) and AMT (1.6 mg/kg/d) (group B) were administered once daily in a 5 days/week schedule either intramuscularly or subcutaneously. Tumor challenge at day 0 was followed by a 3-week treatment period (either vehicle or AMT once daily intramuscularly for 21 days consecutively). In study 2 the AMT dosage was increased up to 4-fold by doubling individual doses and switching to a twice daily schedule. The injections were all intramuscular. With the exception of group D, a six-week pretreatment period preceded the tumor challenge at day 0. Tumor challenge was followed by a 3-week treatment period (vehicle, AMT at either 3.2 mg/kg/d (group A) or 6.4 mg/kg/d (group B), or AMT0, an AMT preparation which does not stimulate IL-6 secretion (6.4 mg/kg/d, group C) continuously for 21 days. AMT administration for group D (6.4 mg/kg/d) was limited to the treatment period from day 1 to 21. All mice were sacrificed 21 days after tumour transplantation. AMT administration was safe and well tolerated, and significantly reduced primary tumor volume in pretreated animals. The effective route of application was intramuscular, with dose escalation resulting in an improved anti-tumor effect. This is the first demonstration of a significant anti-tumorigenic effect of AMT in a validated tumor model.

Introduction

Auron-Misheil Therapy (AMT) is a defined combination of known, approved, and widely used pharmaceuticals. It consists of three active ingredients: insulin, chlorpheniramine and an aqueous camomile extract, and is administered intra-muscularly to support patients in various diseases, with a focus on late-stage cancer. The novelty of AMT is its unique combination of these pharmaceuticals; so far it has not been tested preclinically with respect to its anti-tumor potential.

Since 1989 AMT has been administered mainly in countries of the Middle East to late-stage cancer patients or patients suffering from other serious illnesses (1). Because the outcome of therapy was promising, ~50% of the patients experienced a clinical benefit response (CBR), a research and development program to investigate the pharmacological and toxicological properties of AMT was initiated. The focus of the present study was to investigate the possibility of a dose-dependent anti-tumor effect of AMT, to assess the effect of AMT on the immune system, and to validate the efficacy of intramuscular administration of AMT.

In AMT three well-known components, each with cancer-relevant characteristics, are combined (2,3). Cancer patients often experience deregulated glucose metabolism (4). Exogenous addition of insulin can help to normalize glucose levels (5) and, more importantly, insulin is regarded as a potent biologic response modifier when its role in the insulin potentiation therapy concept is discussed (6). Here it is proposed that insulin sensitises tumor cells by modifying cell membrane characteristics and increasing their rate of proliferation, which in turn renders tumor cells more vulnerable to the anti-proliferative effects of classical chemotherapeutics (7,8). Recently, the addition of insulin to methotrexate in the treatment of patients with metastatic breast cancer improved clinical outcome (9), and the combination of gemcitabine and apigenin enhanced anti-tumor efficacy in vitro and in vivo (10).

Chlorpheniramine (also chlorphenamine, CPN) is a first-generation alkylamine antihistamine used in prevention of allergic conditions. Antihistamines are used...
as supportive cancer medication to alleviate cancer pain (11). It has also been shown that, like insulin, antihistamines have a chemotherapy sensitizing effect on tumor cells (12,13). In addition to this well-established function, it has been demonstrated that chlorpheniramine has anti-tumorigenic potential. Preclinical evidence for antiproliferative effects of chlorpheniramine on breast cancer cells (14), in addition to this well-established function, it has been demonstrated that chlorpheniramine has an anti-tumorigenic effect on tumor cells (12,13). In this model, primary kidney tumors are induced by subcapsular renal injection of RENCA cells with angiogenic potential (22). There are indications that the three herbal components, the experiment was designed to obtain insight into how essential this mode of application might be. In short, despite positive clinical experience with AMT and a number of publications supporting its promising role as an anti-cancer agent, there is still very limited in vivo data to establish AMT’s anti-tumor efficacy.

Materials and methods

Compounds. AMT (Auron-Misheil-Therapy) was provided as a 3 vial formulation by Auron GmbH (MCS Microcarrier Systems GmbH, Neuss, Germany). For study 1 (batch no.) AMT 2003 Cam (5228), AMT 2003 Vit (+) (5229) and AMT 2003 Ins (5230) were used. For study 2 AMT 2003 Cam (6091), AMT 2003 Vit (+) (6090), and AMT zero IL6 (AMT 0, 6089) were used. All components were stored at 4°C.

Preparation of compounds. AMT was prepared by mixing defined amounts of the 3 individually supplied components. For 10 ml of AMT, 5 ml AMT 2003 Cam H were transferred to each application.

Cell culture. For animal experiments, RENCA cells (Professor H. Pahl, University Freiburg, Germany) were originally generated from a tumor that arose spontaneously in the kidney of BALB/c mice. Monolayers of murine RENCA cells were grown in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml. RENCA cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Media were routinely changed every 3 days. Cells were released from the tissue flasks by treatment with 0.05% trypsin/EDTA. For the experiments, cells were collected during the logarithmic growth phase.

Animal experiments. All experiments were carried out in accordance with the guidelines of the Ethics Committee of the local authorities (Regierungspräsidium Freiburg, Germany AZ-Nr.9185.82/3/277). Female BALB/c mice were used at 6-8 weeks of age (approximate weight 20 g). After the animals were anaesthetized with 0.5-1.5 volume percent isoflurane with an oxygen flow of 1.5 l/min, the injection of 4x10^5 cells in 25 μl aliquots into the subcapsular space of the left kidney was performed through a flank incision.

Table I. Design of study 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose per day</th>
<th>Schedule*</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>Saline</td>
<td>day -2 to -1 daily, 5 days/week</td>
<td>i.m.</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>day 1 to 21 daily 7 days/week</td>
<td>i.m.</td>
</tr>
<tr>
<td>A AMT</td>
<td>1.6 mg/kg</td>
<td>day -2 to -1 daily 5 days/week</td>
<td>i.m.</td>
</tr>
<tr>
<td></td>
<td>1.6 mg/kg</td>
<td>day 1 to 21 daily 7 days/week</td>
<td>i.m.</td>
</tr>
<tr>
<td>B Contro</td>
<td>Saline</td>
<td>day -2 to -1 daily 5 days/week</td>
<td>s.c.</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>day 1 to 21 daily 7 days/week</td>
<td>i.m.</td>
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</tr>
<tr>
<td></td>
<td>1.6 mg/kg</td>
<td>day 1 to 21 daily 7 days/week</td>
<td>i.m.</td>
</tr>
</tbody>
</table>

*The day of tumor challenge was defined as day 0.
The tumor cell injection induced progressive development of a primary tumor mass in the left kidney. One week after implantation, the primary tumor is usually macroscopically visible; after 10 days, spontaneous metastases will develop in regional lymph nodes, the lung, the peritoneum and the liver. The mean survival time of untreated RENCA-bearing mice is ~28 days.

**Study design. Study 1:** the first study consisted of two groups, A and B, each consisting of 16 female BALB/c mice (Table I). Preceding the implantation of RENCA tumor cells (tumor challenge at day 0) was administered in both groups i.m.

Study 2. In this follow-up study the dosage of AMT was increased up to 4-fold compared with study 1. This was achieved by doubling individual doses and switching to a twice daily schedule (Table II). All doses were administered i.m. Again, with the exception of group D, a 6-week pre-treatment period preceded the tumor challenge at day 0. During the pretreatment period, vehicle and AMT were administered twice daily for 5 days/week (Fig. 2). As in study 1, tumor challenge at day 0 was followed by a 3-week treatment period during which all animals received either vehicle (two control groups for group A and groups B-D), AMT at either 3.2 mg/kg/d (group A) or 6.4 mg/kg/d (group B), or AMT<sub>0</sub>, an AMT preparation which does not stimulate IL-6 secretion (6.4 mg/kg/d, group C) continuously for 21 and 19 days respectively. AMT application for group D was limited to the treatment period from day 1 to 21.

Immune response monitoring. Spleens of animals from selected experimental groups were collected under sterile conditions and transferred immediately to FOCUS, Heidelberg for further investigation.

**Statistics.** Each animal group consisted of 16 mice, with a vehicle control group for every treatment schedule. Data analysis was performed using the unpaired t-test.

**Results**

Using the orthotopic RENCA model we investigated the anti-tumoral potential of AMT, a three-component mixture,
known to successfully support late stage cancer patients. Due to the immune modulatory characteristics of AMT both studies presented here consisted of a 6-week pretreatment period before challenging the mice by injecting RENCA tumor cells into the left kidney. The route of application is of special interest, since clinical experience with AMT is based mainly on intramuscular administration. Therefore, we included a group of animals which received AMT during pretreatment subcutaneously.

Animal weights were monitored every other day and did not decline throughout the treatment phases of the studies. In general, during both studies no significant differences between control groups and treatment groups were observed. This finding was independent of the route of administration.

After three weeks of continuous therapy with AMT (1.6 mg/kg/d) its potential to interfere with tumor formation was determined. We analysed AMT effects with respect to the primary tumor volume and found a significant decrease when AMT was administered intramuscularly during the pretreatment as well as the treatment period (Fig. 3, group A). By contrast, tumor volumes of group B, with subcutaneously administered AMT during pretreatment, showed no difference compared to vehicle treated animals (Fig. 3, group B).

Since AMT was well tolerated in study 1 the promising anti-tumor efficacy of AMT was further investigated in study 2 implementing a dose-dense design: dose was doubled by applying AMT as in study 1, but twice daily [group A, (3.2 mg/kg/d)]. In addition, a 4-fold dose increase compared to study 1 was achieved by administering 3.2 mg/kg AMT twice daily [groups B, C and D, (6.4 mg/kg/d)].

Again, primary tumor volume was assessed, and while group A did not show a significant decrease, with group B (6.4 mg/kg/d AMT) the initial results of study 1 were confirmed (Fig. 4). To obtain more detailed experimental information on AMT mechanism, study 2 was varied by introducing AMT0, an AMT preparation that lacks the property of stimulating IL-6 release when tested in the relevant cell culture model. In group C, application of AMT0 with the identical dose and scheduling as the regular AMT (group B) did not interfere with primary RENCA tumor formation. With group D another experimental setting was introduced to examine whether AMT pretreatment is a general prerequisite for its anti-tumor activity. Regular AMT was applied with the increased dose of 6.4 mg/kg/d, but initiated after tumor challenge. Here, a trend to lower primary tumor volumes were observed, but the difference did not reach significance.

Using the RENCA tumor model it is possible to get insight into the anti-metastatic potential of the tested compounds. Table III summarizes the results of both studies with respect to tumor volume as shown in Figs. 3 and 4 and the onset of lung metastases and animal losses in each experimental group.

Efficacy of AMT to reduce formation of lung metastases was achieved solely in group B of study 2 with increased twice daily dosing up to 6.4 mg/kg/d. AMT had no effect on animal weight in any group, a fact that indicates a general tolerability; this finding was independent of the route of application. Neither did we observe any signs of irritation at the site of injection, even after numerous applications. However, in experimental groups B, C and D more animals in the treatment arms had to be sacrificed for ethical reasons.

Immune response monitored by assessing cytokine release was performed by preparing full spleen cell cultures and keeping them overnight either unstimulated or after stimulation with CD3/28. Firstly, the chosen RENCA tumor model displayed an immune suppressive effect: neither of the animals carrying a tumor reached the level of IL-2 secretion seen in control animals (Fig. 5). In addition, there is a clear, inverse relationship between tumor size and immune cell activity if animals are analyzed individually. Animals with large tumors responded with low IL-2 secretion whereas animals with small tumors had high levels of IL-2 in the supernatant (data not shown). However, this correlation was independent of the treatment with AMT.
In summary, AMT administered intra-muscularly during the pretreatment and treatment phases resulted in a significant reduction of primary tumor volume. But in spite of evidence supporting the positive effect of pretreatment, and the negative result obtained by using AMT0 which lacked IL-6 stimulating activity, the biological test system chosen to detect the effect of AMT on tumor-related immune suppression did not provide further mechanistic insight.

Discussion

We describe for the first time a significant effect of AMT on primary tumor growth and a trend to lower rates of lung metastases using the RENCA tumor model. A clear supportive function of AMT was already evident prior to tumor challenge, and this improved with the introduction of a dose-intense regimen: by increasing the AMT dose up to 6.4 mg/kg/d a higher significance of the initially observed anti-tumor efficacy was reached. Moreover, with this increased AMT dose, applied solely during the treatment phase, a remarkable though not significant 30% reduction of primary tumor volume was observed. The onset of lung metastases was assessed, but the analysis was impeded by the high degree of variability of this parameter and the data did not reach significance. Notably, 30% fewer animals in group B of study 2 developed lung metastases. Since the renal cell carcinoma develops as a highly vascularized tumor the RENCA model is the suitable animal model to demonstrate an anti-angiogenic potential. In line with our findings, apigenin, the flavonoid found in camomile extracts, was found to suppress VEGF expression in A549 lung cancer cells (22).

Furthermore, the results presented here support intra-muscular administration as the effective route for AMT. Pretreating the animals subcutaneously diminished the AMT effect on tumor volume completely, while a systematic difference (e.g. stress response) of these application modes can be excluded (27). Apparently, with the dosing in study 2 the maximal tolerated dose (MTD) of AMT was reached, which is most likely due to the effect of the relatively high levels of insulin that have not been adjusted with respect to physiological murine levels. Still, recent data confirm the anti-tumor potential of insulin. It has been shown that IGF-1 plasma levels correlate with delayed recurrence of colorectal lesions (28). The notion that high insulin levels are correlated with tumor progression in general (29) is partly disproved when analysing IGF-1 levels in postmenopausal breast cancer patients (30). We believe that the CBR seen after AMT application in the clinical setting is most likely correlated with long-term influence on the glucose metabolism (31), an aspect that cannot be assessed with the applied experimental model.

The components of AMT as well as the low-dose regimen that has been used successfully to treat a variety of indications led to the hypothesis that AMT function is caused by a general immunostimulatory effect. We complemented our regular experimental setting by a thorough analysis of a possible alleviation of the immune suppression caused by the RENCA tumor formation in the animals. The test system readily detected immune suppressive activity of RENCA tumor cells grown in vivo, which correlated with tumor size when analyzed in individual animals. However, we did not observe an improved cytokine release after AMT treatment. With AMT0 we tested an AMT preparation lacking the ability to stimulate IL-6, to assess whether this aspect of AMT action is inevitable for the overall effect. In the event, no anti-tumor efficacy was detected when AMT0 was applied. Therefore, the mechanism by which AMT exerts its immunostimulatory effect remains unclear and is still under investigation.

Table III. Results of study 1 and 2 with T/C values for tumor volumes, lung metastases (onset and number) and information on animal losses.

<table>
<thead>
<tr>
<th>Daily dose (mg/kg/d)</th>
<th>Pretreatment</th>
<th>Route</th>
<th>Efficacy T/C (%)</th>
<th>Lung metastases T/C (%)</th>
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<td>Yes</td>
<td>im/im</td>
<td>56</td>
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<td>Group B AMT</td>
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<td>Yes</td>
<td>sc/im</td>
<td>99</td>
<td>&gt;0.3</td>
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<tr>
<td>Study 2</td>
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<td></td>
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<tr>
<td>Group A AMT</td>
<td>3.2</td>
<td>Yes</td>
<td>im/im</td>
<td>112</td>
<td>&gt;0.3</td>
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<tr>
<td>Group B AMT</td>
<td>6.4</td>
<td>Yes</td>
<td>im/im</td>
<td>64</td>
<td>0.017</td>
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<tr>
<td>Group C AMT0</td>
<td>6.4</td>
<td>Yes</td>
<td>im/im</td>
<td>96</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>Group D AMT</td>
<td>6.4</td>
<td>No</td>
<td>---/im</td>
<td>67</td>
<td>0.12</td>
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</table>

![Figure 5. Cultured spleen cells of either non-tumor bearing control animals or generated from animals with developed RENCA tumors. After stimulation with CD3/28 IL-2 levels were determined in the supernatants. While in control cells this stimulation is followed by a robust IL-2 secretion, this response is suppressed in RENCA tumor cells.](205-210.png)
Collectively, anti-tumor efficacy of AMT was demonstrated in two independent experiments, while its administration was safe, as previously shown in preclinical toxicological studies. With 6.4 mg/kg/d the MTD for AMT in mice was reached, presumably because of the insulin levels involved.

Acknowledgements

We thank B. Giessen and S. Moor for performing the animal work and F. Zuehl for preparing the initial study.

References


