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COMBINED ACTION OF ACIVICIN AND D-GALACTOSAMINE ON PYRIMIDINE NUCLEOTIDE METABOLISM IN HEPATOMA CELLS*

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Abstract—The glutamine antagonist acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, strongly reduced CTP and GTP contents in AS-30D rat hepatoma cells in suspension. UTP only dropped to 63% of the respective control after 4 hr; however, by combining acivicin with the uridylate-trapping sugar analogue D-galactosamine, a synergistic decrease in UTP contents to 7% of control was induced. Incorporation of $^{14}CO_2$ into purine and pyrimidine nucleotides followed by radio-high performance liquid chromatography showed marked inhibition of purine and pyrimidine biosynthesis *de novo;* the latter was reduced to 35% of control. The inhibitory potency of acivicin on glutamine-dependent carbamoyl-phosphate synthetase and consequently on *de novo* uracil nucleotide formation was also reflected by the complete suppression of the D-galactosamine-induced rise in total uridylate. Induction of UTP deficiency by interference with the first and rate-limiting step in pyrimidine biosynthesis *de novo* together with a trapping of uridylate by D-galactosamine may provide a promising approach to the chemotherapy of hepatocellular carcinoma.

The combination of pyrimidine antagonists can improve the selectivity and the efficiency in tumour chemotherapy. Acivicin‡ has been identified as a potent glutamine antagonist inhibiting glutamine-dependent carbamoyl-phosphate synthetase (CPS II; EC 6.3.5.5) from various cells and tissues [1–3]. This enzyme, part of the trifunctional protein *pyr 1–3*, catalyzes the first and rate-determining step in *de novo* pyrimidine biosynthesis [4, 5]. CPS II is rapidly inactivated by acivicin [2], a fermentation-derived antitumour antibiotic [1, 6, 7]. Inactivation through active site-directed alkylation of amidotransferases by acivicin is preceded by a reversible binding of the antagonist in a manner competitive with L-glutamine [2, 8]. In addition to CPS II, acivicin inhibits several glutamine-dependent amidotransferases involved in purine and pyrimidine synthesis, such as amidophosphoribosyltransferase (EC 2.4.2.14), phosphoribosylformylglycinamidine synthetase (EC 6.3.5.3), GMP synthetase (EC 6.3.5.2), and CTP synthetase (EC 6.3.4.2). This indicates that acivicin does not selectively inactivate the first enzyme involved in pyrimidine formation *de novo*. The effect on pyrimidine nucleotide metabolism can be intensified, however, by a trapping of uridylate associated with the metabolism of D-galactosamine (GalN). Lowering of UTP levels, resulting from the accumulation of UDP-amino sugars derived from GalN has been observed both in liver [9] and hepatoma cells [10]. Severe UTP deficiency and inhibition of hepatoma cell growth has been induced by the synergistic action of GalN with inhibitors of *de novo* pyrimidine synthetis including 6-azauridine [11, 12], *N*-(phosphonoacetyl)-L-aspartate [13] and pyrazofurin [14, 15].

In this paper, by employing hepatoma cells in suspension, we focus on the inhibition of *de novo* pyrimidine synthesis by acivicin and on the depletion of UTP pools intensified by GalN. *De novo* pyrimidine synthesis has been studied in the basal state as well as after its stimulation by GalN [12, 13]. The latter condition is a consequence of the lowered UTP levels and is associated with an expansion of the acid-soluble uracil nucleotide pool comprising mainly UDP-amino sugars [12]. Inhibition of CPS II by acivicin in the hepatoma cells suppressed both the GalN-elicited expansion of the uracil nucleotide pool and the incorporation of $^{14}CO_2$ into pyrimidine nucleotides. These changes were associated with the effects of acivicin on synthesis and contents of purine nucleotides.

MATERIALS AND METHODS

Chemicals, enzymes and isotopes. D-Galactosamine–HCl (GalN) was purchased from C. Roth (Karlsruhe, F.R.G.); Dr. G. Neil (The Upjohn Co., Kalamazoo, Michigan) kindly supplied acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; NSC-163501). Powdered Swim's S-77 medium was from Grand Island Biological Co. (Grand Island, NY). Pluronic F 68 was a gift from BASF (Ludwigshafen, F.R.G.). Snake venom phosphodiesterase from *Crotalus durissus terrificus*

* Dedicated to Prof. E. Buddecke on the occasion of his 60th birthday.

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‡ Abbreviations: Acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, NSC-163501; GalN, D-galactosamine; CPS II, carbamoyl-phosphate synthetase (glutamine hydrolysing), EC 6.3.5.5; ΣUMP, ΣCMP, ΣAMP, ΣGMP, sum of total acid-soluble uracil, cytosine, adenine and guanine 5′-nucleotides, respectively.

was purchased from Boehringer Mannheim (Mannheim, F.R.G.) $NaH^{14}CO_3$ (57.8 Ci/mole) was from the Radiochemical Centre (Amersham, U.K.).

Ascites hepatoma cells. Female Sprague–Dawley rats (Voss, Tuttlingen, F.R.G.), 7–10 weeks of age, carried the transplantable AS-30D ascites hepatoma cell line [16]. At weekly intervals tumour cells were transplanted by i.p. injection of 0.2 ml of ascitic fluid collected under sterile conditions. Transplant generations 602–632 were used for the present experiments.

Incubation of hepatoma cells and extraction method. Tumour cells were collected, washed and suspended in the standard medium [10] modified to 2, 26, and 0.4 mmole/l for NaH_2PO_4, $NaHCO_3$ and L-glutamine, respectively. For $^{14}CO_2$-labelling experiments, the $NaHCO_3$ concentration was lowered to 20 mmole/l. The cell concentration in the suspension culture amounted to $2.1–3.0 \times 10^9$ cells/l. Cell suspensions were kept in closed Erlenmeyer flasks on a gyratory shaker (130 rpm) at 37° under CO_2/air (1/20) at a pH between 7.35 and 7.45. After a 40 min pre-incubation period, GalN (0.5 mmole/l), acivicin (75 μmole/l.), or the two combined, were added. These antipyrimidine concentrations were used throughout. Aliquots of 25 ml of the cell suspension were taken hourly up to 4 hr, centrifuged, frozen in liquid nitrogen and deproteinized in the cold by addition of approximately 5 volumes of $HClO_4$ (0.6 mole/l). After centrifugation, supernatants were adjusted to a pH near 6 by $KHCO_3$ and then either directly separated by HPLC or subjected to hydrolysis by snake venom phosphodiesterase [17] prior to HPLC analysis for total acid-soluble 5'-nucleoside monophosphates. Duplicate cell wet weight determinations were performed on 10 ml aliquots of the cell suspensions [10].

Metabolite analysis. Nucleoside 5'-phosphates as well as UDP-sugars were separated by anion-exchange high-performance liquid chromatography (HPLC), using a Partisil-10 SAX (Whatman, Clifton, NY) column with elution of the nucleotides by the flow and buffer gradients described recently [18]. The absorbance was measured at 262 nm; peak areas were integrated by a Spectra Physics SP-4100 (Darmstadt, F.R.G.) printer–plotter and quantified by referring to nucleotide standards that had been analyzed enzymatically [19]. The peak of the UDP-hexosamines (UDP-galactosamine and UDP-glucosamine) was superimposed on a peak that was similar in control and acivicin-treated cells and was therefore subtracted in samples from cells exposed to GalN. For separation of radioactively labelled nucleotides, samples were collected upon HPLC analysis every 20 sec and counted in a triton/toluene scintillation mixture at an efficiency of 90%.

RESULTS

Nucleoside triphosphate contents in hepatoma cells exposed to acivicin

At the glutamine concentration of 400 μmole/l. an acivicin concentration of 75 μmole/l was required in the hepatoma cells in order to reach a maximal effect on pyrimidine nucleotide levels. The glutamine antagonist decreased both purine and pyrimidine nucleoside triphosphate contents (Fig. 1). The effect of acivicin was most pronounced on CTP and GTP contents, which were lowered to 23 ± 3 and 35 ± 12% (S.D.; $P < 0.001$) as compared to the respective control after a 3 hr incubation period. UTP was depressed only to 64 ± 19% (S.D.; $P < 0.01$) relative to the control after 4 hr of exposure to the glutamine antagonist. It is notable that the UTP content dropped with a delay of 2–3 hr in contrast to the other nucleoside triphosphates (Fig. 1).

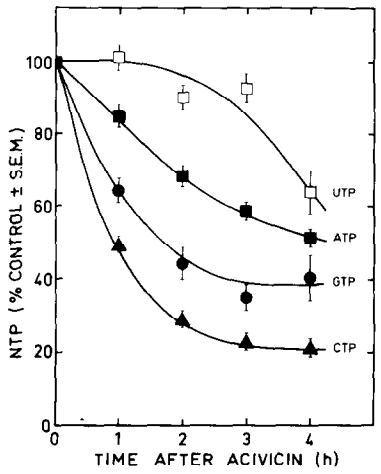

Fig. 1. Changes in nucleoside triphosphate contents induced by acivicin. Acivicin was added to AS-30D cell suspensions at a final concentration of 75 μmole/l, and the nucleotides were analyzed by HPLC. Mean values from 6–9 experiments ± S.E.M. are expressed as percent of the respective nucleoside triphosphate (NTP) content in control cells at the times indicated.

Synergistic action of D-*galactosamine and acivicin*

Acivicin, as an inhibitor of glutamine-dependent

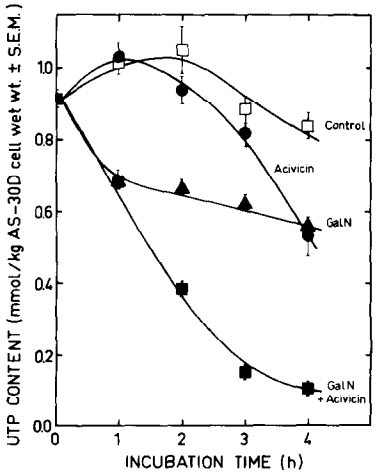

Fig. 2. Synergistic effect of galactosamine and acivicin on UTP contents of AS-30D cells. After exposing the hepatoma cells to galactosamine (GalN; 0.5 mmole/l) and/or acivicin (75 μmole/l), the cell supernatants were analyzed by anion-exchange HPLC. Mean values from 6–9 separate experiments ± S.E.M. are given as mmole/kg of AS-30D cell wet weight.

Table 1. Acivicin- and D-galactosamine- (GalN) induced changes in nucleotide contents of AS-30D hepatoma cells*

Nucleotide	Control	Acivicin	GalN	Acivicin + GalN
	(mmole/kg cell wet wt ± S.D.)			
UTP	0.84 ± 0.10	0.53 ± 0.16	0.56 ± 0.03	0.06 ± 0.03
CTP	0.54 ± 0.09	0.12 ± 0.03	0.25 ± 0.02	0.12 ± 0.04
ATP	3.45 ± 0.56	1.78 ± 0.22	2.41 ± 0.37	1.53 ± 0.19
GTP	0.51 ± 0.11	0.21 ± 0.10	0.36 ± 0.04	0.19 ± 0.04
UDPGlc + UDPGal	0.70 ± 0.10	0.38 ± 0.10	0.19 ± 0.05	0.06 ± 0.04
UDPGlcNAc + UDPGalNAc	0.54 ± 0.08	0.68 ± 0.13	0.82 ± 0.06	0.89 ± 0.06
UDPGlcN + UDPGalN	‡	‡	1.73 ± 0.52	0.76 ± 0.16

* Cells were exposed to acivicin and/or GalN for 4 hr. Mean values from 6–9 experiments.
‡ UDP-hexosamines are not formed without exposure of the cells to GalN.

CPS II, as well as the uridylate-trapping sugar analogue GalN each depressed cellular UTP contents to 60–70% of control within 4 hr ($P < 0.01$; Fig. 2). As opposed to the time course in acivicin-treated cells, GalN decreased UTP rapidly in 1 hr, but rather slowly after this time interval. The synergistic action of both antipyrimidines was reflected by a marked lowering of UTP contents to 37 ± 6 and 7 ± 4% (S.D.; $P < 0.001$) as compared to the controls after 2 and 4 hr of incubation, respectively. Table 1 summarizes the changes in nucleoside triphosphates and UDP-sugars under acivicin and/or GalN after 4 hr. The GalN-induced rise in UDP-glucosamine and UDP-galactosamine, metabolites not present in untreated cells, was suppressed to a major extent by acivicin.

Effect of acivicin on nucleotide biosyntheses de novo

In order to establish the inhibition of pyrimidine and purine biosynthesis *de novo* by the glutamine antagonist, $^{14}CO_2$ incorporation into acid-soluble uracil, cytosine, adenine and guanine nucleotides was determined in AS-30D hepatoma cells over a 2 hr period. After hydrolysis of the nucleotides yielding 5'-nucleoside monophosphates, the latter were separated and quantified by radio-HPLC (Fig. 3). The inhibitory action of acivicin was detectable by the marked reduction of $^{14}CO_2$ incorporation into each of the four nucleotide fractions with a predominant suppression of purine formation to 7 and 6% in adenine and guanine nucleotides, respectively (Fig. 4). *De novo* pyrimidine synthesis was reduced to 35% of control, even after stimulation of this pathway by a GalN-induced lowering of UTP levels. This uridylate-trapping amino sugar enhanced the labelling of uracil nucleotides 2-fold during the 2 hr incubation period (Fig. 4). Based on the specific activity of $^{14}CO_2$ in the medium, this corresponds to a rate of *de novo* uridylate synthesis of 0.59 as compared to 0.30 mmole/kg per hr. This elevation

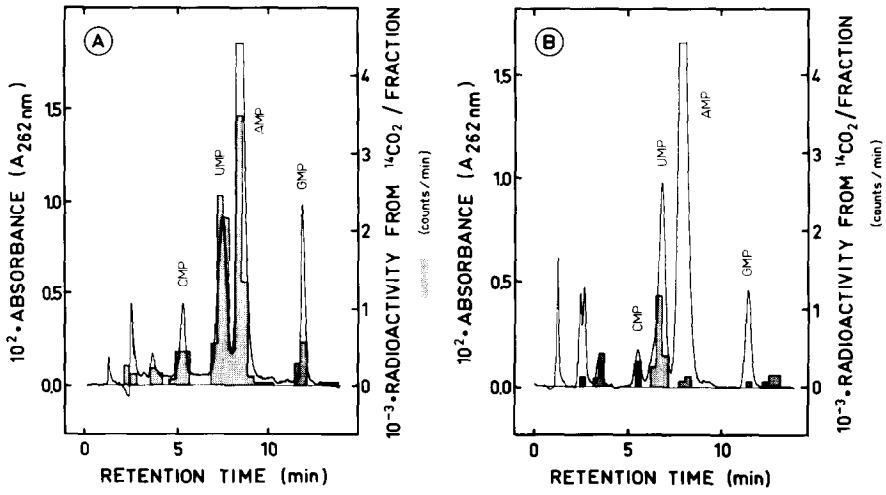

Fig. 3. Radioactivity pattern of $^{14}CO_2$ incorporation into acid-soluble nucleoside 5'-monophosphates. AS-30D cells were incubated in a final volume of 1 ml for 2 hr in a medium containing $NaH^{14}CO_3$ (150 mCi/l; 20 mmole/l); the gas volume was 2.3 ml. Acivicin was added 10 min prior to $NaH^{14}CO_3$. After deproteinization of the cell pellet (10 mg) with 250 µl of $HClO_4$ (0.6 mole/l), supernatants were treated with snake venom phosphodiesterase to yield acid-soluble 5'-nucleoside monophosphates [17] which were subsequently separated by anion-exchange HPLC, collecting samples for radioactivity counting every 20 sec. The radioactivity pattern with simultaneous registration of the absorbance at 262 nm is shown for control cells (A), and acivicin-treated cells (B).

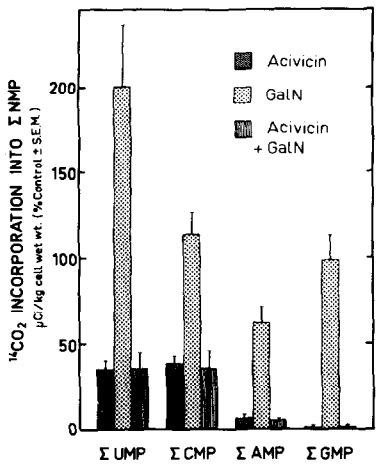

Fig. 4. $^{14}CO_2$ incorporation into the sum of acid-soluble nucleoside 5'-monophosphates (ΣNMP) after acivicin and/or galactosamine. AS-30D cells were incubated with acivicin and/or galactosamine for 130 min as described in the legend of Fig. 3. $NaH^{14}CO_3$ was added 10 min after the drugs. Columns represent the radioactivity (μCi/kg AS-30D cell wet weight) expressed as per cent of the respective control with the mean from 3–4 experiments ± S.E.M.

of *de novo* pyrimidine synthesis was reflected by the expansion of the acid-soluble uridylate pool (Fig. 5) that was mainly composed of UDP-amino sugars (Table 1). The rise in ΣUMP, most pronounced after a 2 hr lag phase, was completely suppressed when GalN was combined with acivicin (Fig. 5).

DISCUSSION

Acivicin has been shown to be a powerful inhibitor of carbamoyl-phosphate synthetase II (CPS II)

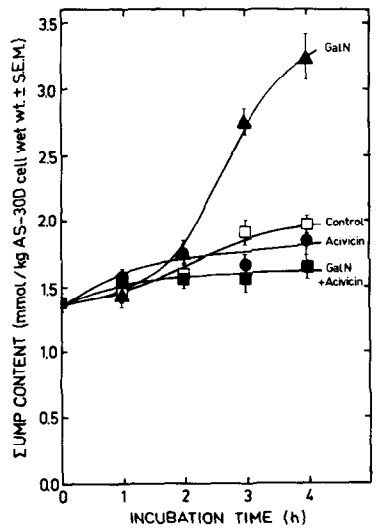

Fig. 5. Suppression of the galactosamine-induced increase in the sum of acid-soluble uracil 5'-nucleotides (ΣUMP) by acivicin. Hepatoma cells were incubated as described in Materials and Methods. Acid-soluble supernatants were treated with snake venom phosphodiesterase and assayed for UMP by HPLC. Points represent the mean from 6–9 experiments ± S.E.M.

[1–3]. Exposure for 4 hr of hepatoma cells to acivicin led to a decrease in UTP contents by 36% (Fig. 1). An analogous effect of the glutamine antagonist on UTP has been observed recently in Lewis lung carcinoma in mice [3]. Acivicin lowered the $^{14}CO_2$ incorporation into total uridylate to 35% of control (Fig. 4). This corresponds to a decreased rate of pyrimidine synthesis *de novo*, as calculated from the specific activity of $^{14}CO_2$, of 0.10 mmole/hr per kg cells as compared to untreated controls (0.30 mmole/hr per kg cells). The inhibition of pyrimidine biosynthesis *de novo*, demonstrated by this direct measurement of newly formed uracil nucleotides, is in accordance with previous studies that have shown the reduction of the pyrazofurin-provoked orotate and orotidine accumulation by acivicin [20]. Furthermore, acivicin completely suppressed the expansion of the acid-soluble uridylate pool elicited by D-galactosamine (GalN) (Fig. 5), which represents another approach of demonstrating inhibition of pyrimidine synthesis *de novo* [12, 13]. The rate of $^{14}CO_2$ incorporation into total uridylate under the combined action of GalN and acivicin even dropped below control; relative to the stimulated rate in the presence of GalN, the incorporation was reduced to 18% (Fig. 4). Combination of the inhibitor of CPS II with the uridylate-trapping amino sugar resulted in a synergistic depletion of UTP pools (Fig. 2). This observation is in line with previous studies combining GalN with inhibitors of aspartate carbamoyl-transferase or orotidylate decarboxylase [11–15]. Acivicin lowered CTP, GTP, and ATP contents in hepatoma cells more than the UTP levels (Fig. 1). This corresponds to the sequential inhibition of two glutamine-dependent enzymes involved in the *de novo* synthesis of CTP and ATP, and of three reactions in the case of GTP, whereas only one step in the *de novo* synthesis of UTP can be blocked by acivicin. Synergistic effects of the glutamine antagonist with GalN were not evident for CTP, GTP, or ATP contents (data not shown) as the amino sugar rather selectively interferes with uracil nucleotide metabolism.

Most studies on UTP contents in tissues and cells exposed to acivicin alone yielded minor effects [21–23] or even an elevation of UTP [23, 24], depending on the experimental conditions. In hepatoma cells, we observed only a late and limited decrease of UTP levels (Fig. 1). Possible explanations for the apparent discrepancy between pyrimidine synthesis inhibiton (Figs. 4 and 5) and limited UTP depression (Fig. 1) include an increased salvage of uridine as a result of uridine kinase activation by lowering of CTP levels (Fig. 1) [25], as well as the decreased utilization of UTP for CTP synthesis. UTP accumulation can be promoted by a stronger inhibition by acivicin of CTP synthetase relative to CPS II [22, 23]. This is consistent with the UTP and CTP contents observed 1 hr after acivicin (Fig. 1). Furthermore, acivicin elevates PRPP pool sizes 3 to 10-fold [3, 26, 27], thus increasing allosteric activation of carbamoyl-phosphate synthetase II [4] as well as providing additional substrate for orotate phosphoribosyltransferase. Depression of GTP (Fig. 1), another inhibitor of CPS II [4], can also cause increases in uracil nucleotide contents [28, 29].

The induction of a severe UTP deficiency by two antipyrimidines represents a valuable approach to selective chemotherapy of hepatocellular carcinoma, if GalN as a uridylate-trapping sugar analogue acting on hepatoma and liver [11, 30] is combined with acivicin, thereby inhibiting the rate-determining enzyme of *de novo* pyrimidine synthesis in hepatoma more than in liver [23]. The extent of UTP depletion observed in the AS-30D cells in the presence of acivicin plus GalN (Table 1, Fig. 2) causes severe inhibition of hepatoma cell proliferation in suspension culture [12]. In addition, the marked reduction of UTP contents can be used for an enhanced uptake of 5-fluorouridine [30, 31], thus intensifying the antineoplastic action of these antimetabolites. Our preliminary studies on the sequential administration of acivicin, GalN and 5-fluorouridine in doses of 110, 300, and 15 μmole/kg, respectively, to rats bearing the AS-30D ascites hepatoma have demonstrated the chemotherapeutic effectiveness of this antimetabolite combination.

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