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ANTIVIRAL ACTIVITY OF 5-THIOCYANATOPYRIMIDINE NUCLEOSIDES

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Abstract—The antiviral activity of the 5-thiocyanatopyrimidine nucleosides 5-NCSrU*, 5-NCSdU, 5-NCSaraU and tri-*O'*-acetyl-5-NCSrU has been evaluated in primary rabbit kidney (PRK) cell cultures challenged with either DNA (vaccinia, herpes simplex) or RNA (vesicular stomatitis) viruses. 5-NCSdU inhibited vaccinia virus multiplication at 10 μg/ml, and vaccinia and herpes simplex virus induced cytopathogenicity at 4 μg/ml. Tri-*O'*-acetyl-5-NCSrU inhibited vesicular stomatitis virus-induced cytopathogenicity at 1–10 μg/ml. None of the compounds had profound effects on host cell RNA or DNA synthesis, even at 200 μg/ml, as monitored by [^{3}H]uridine and [^{3}H]thymidine incorporation respectively, except 5-NCSdU, which brought about a 10–30-fold increase of [^{3}H]thymidine incorporation at 200 μg/ml. The inhibitory effect of 5-NCSdU on vaccinia virus replication and its stimulatory effect on [^{3}H]thymidine incorporation were almost completely reversed by thymidine at concentrations 100 times lower than that of the thiocyanato derivative. When treated with dithiothreitol, the 5-thiocyanatopyrimidine nucleosides also lost a significant part of their biological activity, presumably due to reduction to the corresponding 5-mercapto analogs.

A variety of 5-halogenopyrimidine nucleosides, more particularly 5-iodo-2'-deoxyuridine (5-IdU) and 5-bromo-2'-deoxyridine, exhibit a distinct antiviral activity in mammalian cell cultures and experimental animals, and 5-IdU has found wide application in topical treatment of herpes keratitis and cutaneous herpes lesions in man[1].

Structural considerations suggest that the biological acitivity of 5-mercaptopyrimidine nucleosides such as 5-mercapto-2'-deoxyuridine may be similar to that of 5-IdU. At physiologic pH, the SH group of 5-mercapto-2'-deoxyuridine is fully ionized (pK_a: 5·0) and the ionic radius of S^- (2·19 Å) closely resembles the van der Waals radius of the iodo group of 5-IdU (2·15 Å)[2]. 5-Mercapto-2'-deoxyuridine has been shown to be effective in inhibiting bacterial growth[2] and this inhibitory effect was attributed to an inhibition of thymidylate synthetase. Therefore, the mercapto derivative should be first phosphorylated in the cell by thymidine kinase to the corresponding 5'-monophosphate[3].

The cyanato derivatives of the 5-mercaptopyrimidine nucleosides have now been explored for their antiviral activity in mammalian (primary rabbit kidney) cell cultures. It was reasoned[4] that the thiocyanato derivatives would offer some advantages over the mercapto derivatives. Unlike the free sulfhydryl, the thiocyanato group should not be apt to autoxidation to disulfide. Furthermore, the neutral thiocyanato analogs might be more readily taken up by the cells than the ionized mercapto analogs. Within the cell the thiocyanato compounds might eventually be reduced to the biologically active mercapto species.

MATERIALS AND METHODS

The *synthesis* and *physico-chemical* characteristics of the 5-thiocyanatopyrimidine nucleosides have been described previously[4, 5]. The structure of these nucleosides is depicted in Fig. 1.

The techniques for evaluating the effects of the 5-thiocyanatopyrimidine nucleosides in PRK cells on (i) vaccinia virus, herpes simplex virus and vesicular stomatitis virus-induced cytopathogenicity, (ii) vaccinia virus growth, and (iii) DNA or RNA synthesis, as monitored by incorporation of [*methyl*-^{3}H]thymidine or [5-^{3}H]uridine, respectively, have all been described[6, 7]. For measuring the effects of the 5-thiocyanatopyrimidine nucleosides on vesicular stomatitis virus growth, a similar technique was employed as in measuring vaccinia virus growth, except that virus input was $10^{4.3}$ PFU/petri dish (instead of $10^{4.5}$) and that virus yields were determined in mouse L-929 cell monolayers (instead of PRK cells).

Thymidine was purchased from BDH Chemicals Ltd., Poole, England; [*methyl*-^{3}H]thymidine (12 Ci/m-mole), [5-^{3}H]deoxycytidine (23 Ci/m-mole), [8-^{3}H]deoxyadenosine (12 Ci/m-mole), [8-^{3}H]deoxyguanosine (5 Ci/m-mole), and [5-^{3}H]uridine (26 Ci/m-mole) were purchased from the C.E.N. Radioisotopes Department, Mol, Belgium. Dithiothreitol was obtained from Sigma Chemical Co.

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* Abbreviations: 5-NCSrU, 5-thiocyanatouridine; 5-NCSdU, 5-thiocyanato-2'-deoxyuridine; 5-NCSaraU, 5-thiocyanato uracil arabinoside or 1-β-D-arabinofuranosyl-5-thiocyanatouracil; tri-O'-acetyl-5-NCSrU, 2',3',5'-tri-*O*-acetyl-5-thiocyanatouridine; ara-C, cytosine arabinoside, 1-β-D-arabinofuranosylcytosine; 5-ethyl-dU, 5-ethyl-2'-deoxyuridine; dT, 2'-deoxythymidine; 5-IdU, 5-iodo-2'-deoxyuridine; $CCID_{50}$, cell culture infecting dose 50 (dose infecting 50% of the cell cultures); PFU, plaque forming units.

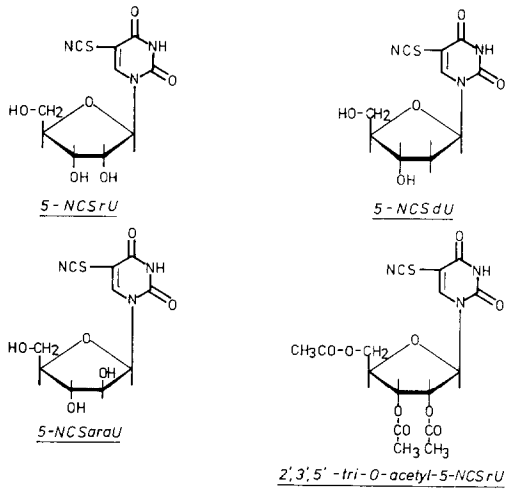

Fig. 1. Chemical structures of 5-NCSrU, 5-NCSdU, 5-NCSaraU and 2',3',5'-tri-*O*-acetyl-5-NCSrU.

RESULTS AND DISCUSSION

Antiviral activities. In accord with previous findings[4] 5-NCSdU inhibited the growth (Fig. 2) and cytopathogenicity (Table 1) of vaccinia virus at relatively low concentrations (10 and 4 μg/ml, respectively). At 100 μg/ml the inhibitory effect of 5-NCSdU on vaccinia virus replication did not markedly differ from that of 5-ethyl-dU, but at 10 μg/ml 5-ethyldU was definitely more active (Fig. 2). In similar conditions, ara-C and 5-IdU suppressed vaccinia virus multiplication almost completely[7]. 5-NCSdU was clearly more effective in inhibiting vaccinia virus-induced cytopathogenicity than either 5-NCSrU, 5-NCSaraU or 2',3',5'-tri-*O*-acetyl-5-NCSrU (Table 1). In conditions at which 4 μg/ml of 5-NCSdU was required to inhibit virus-induced cytopathogenicity, cytosine arabinoside, 5-IdU and 5-ethyl dU inhibited vaccinia virus cytopathogenicity at 0·04, 0·1 and 1 μg/ml, respectively[7]. Of all thiocyanato analogs tested, 2',3',5'-tri-*O*-acetyl-5-NCSrU was most effective in inhibiting the cytopathogenicity of vesicular stomatitis virus (minimal inhibitory concentration: 1–10 μg/ml) (Table 1).

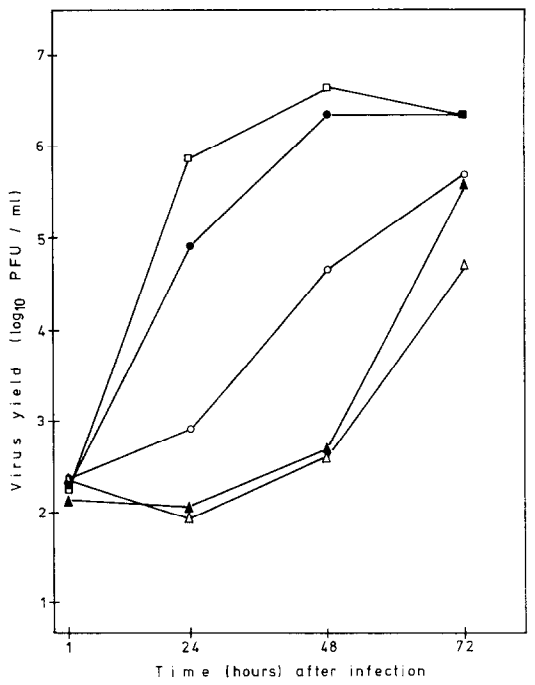

Fig. 2. Effect of 5-NCSdU and 5-ethyl-dU on vaccinia virus growth in PRK cells. Virus input: 4·5 $\log_{10}$ PFU per petri dish. Compounds added at either 100 or 10 μg/ml, immediately after virus adsorption: control (—□—), 5-NCSdU at 100 μg/ml (—▲—), 5-ethyl-dU at 100 μg/ml (—△—), 5-NCSdU at 10 μg/ml (—●—), 5-ethyl-dU at 10 μg/ml (—○—).

Table 1. Effect of 5-NCSrU, 5-NCSdU, 5-NCSaraU and 2',3',5'-tri-*O*-acetyl-5-NCSrU on virus-induced cytopathogenicity in PRK cells

Compounds†	Minimum inhibitory concentration* (μg/ml)		
	Vaccinia virus‡	Herpes simplex virus‡	Vesicular stomatitis virus‡
5-NCSrU	40	40	100
5-NCSdU	4	4	200
5-NCSaraU	40	4	40
2',3',5'-tri-*O*-acetyl-5-NCSrU	100	40	1–10
Dithiothreitol	10		>100
5-NCSdU (in the presence of dithiothreitol at 10 μg/ml)	4		
2',3',5'-tri-*O*-acetyl-5-NCSrU (in the presence of dithiothreitol at 10 μg/ml)			100

* Required to inhibit viral cytopathogenicity at 50%.
† Compounds added immediately after virus adsorption.
‡ Virus input: 100 $CCID_{50}$ per tube.

To establish whether 5-thiocyanato nucleosides offer some advantages over the corresponding 5-mercapto analogs, the antiviral activity of 5-NCSdU and 2',3',5'-tri-*O*-acetyl-5-NCSrU was assayed in the absence and presence of dithiothreitol. The 5-thiocyanatopyrimidine nucleosides are readily reduced to 5-mercaptopyrimidine nucleosides by dithiothreitol, glutathione, etc.,[4]. As shown in Table 1, dithiothreitol annihilated the inhibitory effect of 2',3',5'-tri-*O*-acetyl-5-NCSrU on vesicular stomatitis virus-induced cytopathogenicity. Its effect on the inhibition of vaccinia virus-induced cytopathogenicity by 5-NCSdU could not be assessed since dithiothreitol itself inhibited vaccinia cytopathogenicity at about the same concentration as 5-NCSdU (Table 1). The results obtained with dithiothreitol (Table 1) also eliminate the role of free cyanide ions in the antiviral activity of 5-NCSdU and 2',3',5'-tri-*O*-acetyl-5-NCSrU. If the release of free CN^- were responsible for the antiviral activity of the latter, one should expect a rise in antiviral potency upon dithiothreitol treatment.

How would thymidine affect the antiviral activity

of 5-NCSdU? In a previous study [4], dT was found to completely reverse the inhibitory effect of 5-NCSdU on the growth of L 5178 Y cells. As shown in Fig. 3, dT nearly completely suppressed the inhibitory effect of 5-NCSdU on vaccinia virus growth, even at a concentration 100-fold lower than that of 5-NCSdU. In the experiments presented in Fig. 3 virus titers were measured 24 hr after infection. However, similar results were obtained if virus yield was determined 48 hr after infection.

Antimetabolic activities. Could 5-NCSdU and 2′,3′,5′-tri-*O*-acetyl-5-NCSrU be considered as 'specific' antiviral agents, or are their inhibitory effects on virus multiplication merely the consequence of an inhibition of host cell DNA or RNA synthesis? Therefore, DNA and RNA synthesis as monitored by incorporation of [*methyl*-^{3}H]thymidine and [5-^{3}H]uridine respectively, were determined in PRK cells which had been exposed to either 5-NCSrU, 5-NCSdU or 2′,3′,5′-tri-*O*-acetyl-5-NCSrU.

5-NCSrU and 2′,3′,5′-tri-*O*-acetyl-5-NCSrU did not affect [^{3}H]thymidine incorporation even at concentrations as high as 200 μg/ml (Fig. 4). However, 5-NCSdU caused a dramatic increase of [^{3}H]thymidine incorporation (10–30-fold at a concentration of 200 μg/ml). A similar, but quantitatively less pronounced, enhancement of [^{3}H]thymidine incorporation has previously [7] been noted in PRK cell cultures exposed to varying concentrations of 5-ethyl-dU. In the same cell cultures, ara-C and 5-IdU caused a marked inhibition of thymidine incorporation (>50% at concentrations of >0·4 and >40 μg/ml, respectively) [7]. None of the compounds tested had a significant influence on [5-^{3}H]uridine incorporation, except 5-NCSrU which caused a small but reproducible rise in uridine incorporation (30% at 200 μg/ml) (Table 2). No microscopically discernible alteration of normal cell morphology was observed with either 5-NCSdU, 5-NCSrU or 2′,3′,5′-tri-*O*-acetyl-5-NCSrU, except the appearance of some floating cells at the highest concentrations tested (200 μg/ml).

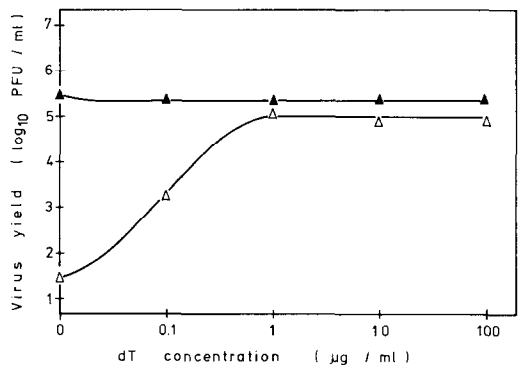

Fig. 3. Effect of dT on the inhibitory effect of 5-NCSdU on vaccinia virus growth in PRK cells: mixtures of 5-NCSdU (100 μg/ml) and dT (at the concentrations indicated in the abscissa) (—△—), dT alone (at the concentrations indicated in the abscissa) (—▲). Virus input: 4·5 $\log_{10}$ PFU per petri dish. Compounds added immediately after virus adsorption. Virus yield determined 24 hr after infection.

5-NCSdU enhanced the incorporation of [*methyl*-^{3}H]thymidine and, to a lesser extent, that of [5-^{3}H]deoxycytidine (Table 3). The incorporation

Table 2. Effect of 5-NCSrU, 5-NCSdU and 2′,3′,5′-tri-*O*-acetyl-5-NCSrU on RNA synthesis* in PRK cells as monitored by [5-^{3}H]uridine incorporation into RNA

Compounds	Dose (μg/ml)	[5-^{3}H]uridine incorporated into RNA: Gross counts (cpm/petri dish)	% (compared to control)
5-NCSrU	200	16427	130·5
	40	9193	73·0
	4	10771	85·6
5-NCSdU	200	8415	66·8
	40	9505	75·5
	4	9507	75·5
2′,3′,5′-tri-*O*-acetyl-5-NCSrU	200	8709	69·2
	40	9353	74·3
	4	10522	83·6
Control		12589	—

* Incorporation of [5-^{3}H]uridine into RNA was measured after the cells had been exposed to the compounds at the concentrations indicated for 24 hr. Data represent mean values for 4 petri dishes.

Table 3. Effect of 5-NCSdU on DNA synthesis* in PRK cells as monitored by [*methyl*-^{3}H]thymidine, [5-^{3}H]deoxycytidine, [8-^{3}H]deoxyadenosine or [8-^{3}H]deoxyguanosine incorporation into DNA

5-NCSdU Concentration (μg/ml)	Gross counts (cpm/petri dish)	% (compared to control)
	[*methyl*-^{3}H]thymidine incorporation	
200	82432	1172·7
40	56106	798·2
4	8323	118·4
Control	7029	—
	[5-^{3}H]deoxycytidine incorporation	
200	2082	128·6
40	3513	216·9
4	1460	90·2
Control	1619	—
	[8-^{3}H]deoxyadenosine incorporation	
200	18012	66·4
40	19620	72·4
4	23142	85·4
Control	27111	—
	[8-^{3}H]deoxyguanosine incorporation	
200	19654	73·9
40	20541	77·2
4	29486	110·9
Control	26595	—
	[*methyl*-^{3}H]thymidine incorporation	
200 all in the	60227	561·5
40 presence of	16348	152·4
4 10 μg/ml of	11465	106·9
Control dithiothreitol	10726	—

* Incorporation of the radiolabelled nucleosides into DNA was measured after the cells had been exposed to 5-NCSdU for 24 hr. Data represent mean values for 2 petri dishes.

of [8-^{3}H]deoxyadenosine and [8-^{3}H]deoxyguanosine was slightly depressed (Table 3). However, for the purine deoxyribonucleosides the measurements do not truly reflect incorporation into DNA, since cleavage of the *N*-glycosidic bond occurs with subsequent reutilization of the bases by the ribonucleotide pathways[8].

The mechanism of the stimulatory effect of 5-NCSdU (Fig. 4) on DNA synthesis in PRK cells is not immediately clear. It cannot be ascribed to an

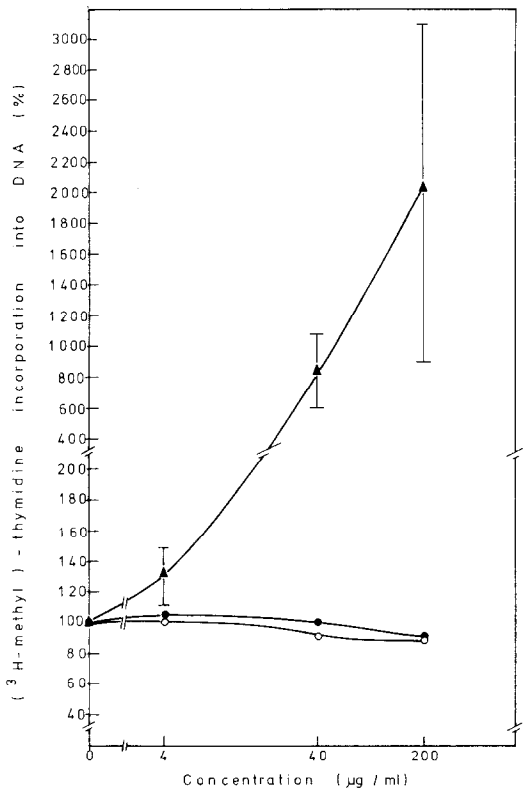

Fig. 4. Effect of 5-NCSrU (—○—), 5-NCSdU (—▲—) and 2′,3′,5′-tri-*O*-acetyl-5-NCSrU (—●—) on [*methyl*-^{3}H]thymidine incorporation into DNA of PRK cells. [*methyl*-^{3}H]thymidine incorporation was measured after the cells had been exposed to different concentrations of the compounds (indicated in the abscissa) for 24 hr. Data represent means ± S.D.

aspecific effect of the cyanide ion since 5-NCSrU and 2′,3′,5′-tri-*O*-acetyl-5-NCSrU failed to cause such stimulation. Furthermore, reduction of 5-NCSdU to its 5-mercapto counterpart by dithiothreitol led to a decrease of the stimulatory effect of 5-NCSdU on DNA synthesis (Table 3). An increase should be anticipated if free cyanide ions were responsible for the DNA synthesis-stimulating effect of 5-NCSdU. The effect of dithiothreitol was most clearly seen if 5-NCSdU was applied at 40 µg/ml and dithiothreitol at 10 µg/ml (Table 3). Higher concentrations of dithiothreitol could not be applied since dithiothreitol itself inhibited [*methyl*-^{3}H]thymidine incorporation at 100 µg/ml and up.

As noted previously with 5-ethyl-dU[7], the enhanced [^{3}H]thymidine incorporation observed in PRK cells exposed to 40 µg/ml of 5-NCSdU was completely reversed if (cold) thymidine was applied to the cells together with 5-NCSdU (Fig. 5). Thymidine was effective in abrogating the stimulatory effect of 5-NCSdU on DNA synthesis at a concentration 100-fold lower than that of 5-NCSdU.

The striking similarities in the competitive effects of thymidine on both the virus-inhibiting and DNA synthesis-stimulating properties of 5-NCSdU (Figs. 3 and 5) point to the existence of at least one common link in the biochemical pathways underlying both events: e.g. 5-NCSdU may competitively inhibit some of the enzymes involved in thymidine metabolism (thymidylate synthetase, thymidine kinase, etc.). The 5′-monophosphate of 5-mercapto-2′-deoxyuridine is a potent inhibitor of thymidylate synthetase[3]. Its *S*-methyl analog is not [3]. Therefore, 5-NCSdU could be expected to inhibit thymidylate synthetase, only after it has been reduced to the 5-mercapto and phosphorylated to the 5′-monophosphate derivative. Whether this conversion does actually occur in PRK cells remains to be established. If it were proven that 5-NCSdU (after reduction and phosphorylation) inhibits thymidylate synthetase in PRK cells, it would be hard to visualize how this inhibition might lead to an increased [^{3}H]thymidine incorporation into DNA (Fig. 4).

Some observations suggest that the increased [^{3}H]thymidine incorporation observed in PRK cells exposed to 5-NCSdU (Fig. 4, this report) or 5-ethyl-dU (Fig. 3, reference 7) does not reflect regular but rather 'unscheduled' DNA synthesis (or DNA repair):

(i) there was no concomitant increase of RNA synthesis as monitored by [^{3}H]uridine incorpora-

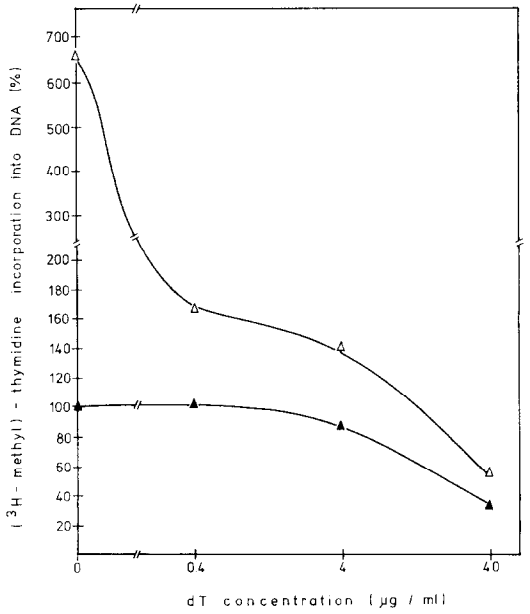

Fig. 5. Effect of dT on the stimulatory effect of 5-NCSdU on [*methyl*-^{3}H]thymidine incorporation into DNA of PRK cells: mixture of 5-NCSdU (40 µg/ml) and dT (at the concentrations indicated in the abscissa) (—△—), dT alone (at the concentrations indicated in the abscissa) (—▲—). [*methyl*-^{3}H]thymidine incorporation was measured after the cells had been exposed to 5-NCSdU and/or dT for 24 hr.

tion (Table 2, this report and Table 3, ref. 7);

(ii) only [^{3}H]thymidine, and, to a lesser extent, [^{3}H]deoxycytidine incorporation was increased; there was no increase of the incorporation of [^{3}H] deoxyguanosine or [^{3}H]deoxyadenosine (Table 3, this report and Table 2, ref. 7);

(iii) no increase in DNA synthesis as monitored by [^{3}H]thymidine incorporation was detected in human lymphocytes exposed to 5-NCSdU or 5-ethyl-dU; in fact, 5-NCSdU and 5-ethyl-dU suppressed phytohemagglutinin-stimulated DNA synthesis in lymphocytes[9] at concentrations (40–200 μg/ml) which were found to increase DNA synthesis in PRK cell cultures (Fig. 4, this report; Fig. 3, ref. 7).

The increase in DNA synthesis observed upon exposure of PRK cells to 5-NCSdU and 5-ethyl-dU might seem related to the unbalanced growth prior to death of HeLa cells exposed to 6-mercaptopurine[10]. This unbalanced cell growth was accompanied by an increase in cell size and DNA content (not DNA synthesis). It remains to be established whether the effects of 5-NCSdU noted herein and those of 6-mercaptopurine noted before[10] are based on the same mechanism(s).

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