Biochemical Pharmacology, Vol. 43, No. 10, pp. 2053–2057, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
© 1992. Pergamon Press Ltd

A NOVEL ROUTE OF ATP SYNTHESIS

RYSZARD T. SMOLENSKI,* KRYSTYNA FABIANOWSKA-MAJEWSKA,† CELIA MONTERO,‡ JOHN A. DULEY, LYNETTE D. FAIRBANKS, MACIEJ MARLEWSKI* and H. ANNE SIMMONDS§

Purine Research Laboratory, Clinical Science Laboratories, UMDS Guy's Hospital, London Bridge, London SE1 9RT, U.K.

(*Received* 24 *October* 1991; *accepted* 5 *March* 1992)

Abstract—Incorporation of the adenine moiety of 2′-deoxyadenosine (dAdo) into ATP, consistently observed in human erythrocytes, is a phenomenon which cannot be explained by the operation of any known pathway. We reported previously that this effect was not observed in adenine phosphoribosyltransferase-deficient erythrocytes showing that adenine must be an obligatory intermediate. However, generation of adenine from dAdo was difficult to reconcile with the operation of any known process in human cells, and involvement of *S*-adenosylhomocysteine hydrolase (SAH-hydrolase) was postulated. The present studies with intact human erythrocytes demonstrate that nucleoside analogues which inhibit SAH-hydrolase caused substantial attenuation of adenine transfer from dAdo into ATP. It was confirmed that dAdo is not a substrate of 5′deoxy-5′methylthioadenosine (5′MT-adenosine) phosphorylase. Inhibition of the transfer of the adenine moiety of dAdo into ATP did not correlate with inhibition of 5′MT-adenosine phosphorylase by nucleoside analogues. This report provides further evidence that the pathway involving nucleoside (adenosine) analogue binding to SAH-hydrolase, release of base and subsequent phosphoribosylation can operate in intact cells. The metabolic significance of this process relates to the possible generation of free bases (adenine) in the human body, ATP synthesis and nucleoside drug interconversions.

Classical metabolic pathways involve the action of a sequence of enzymes specific with regard to substrate, catalysed reaction and metabolic products. *S*-Adenosylhomocysteine hydrolase (SAH-hydrolase‖; EC 3.3.1.1) is an enzyme which is involved in transmethylation processes and normally catalyses hydrolysis of SAH to adenosine and L-homocysteine [1]. But is this the only role of this enzyme in cellular metabolism?

Studies on purified SAH-hydrolase revealed that nucleosides, including some physiological metabolites, could release the base adenine as the result of interaction with this enzyme [2]. The mechanism invoked to explain this phenomenon included formation of a 3-ketonucleoside derivative with decreased stability of the glycoside bond. Spontaneous breaking of this bond would result in the release of the corresponding base. However, it was unclear whether this process could operate in intact cells.

We have reported that some nucleosides caused unexplained elevation of ATP concentrations in human erythrocytes [3–5]. This process was independent of adenosine kinase and did not operate in erythrocytes lacking adenine phosphoribosyltransferase (APRT), showing that the purine base must be released in this process before incorporation into ATP (Fig. 1). Involvement of SAH-hydrolase in base liberation was suggested as the only probable explanation, but definitive proof for this was lacking [3].

In this study, the effect of various nucleoside analogues on ATP synthesis from 2′-deoxyadenosine (dAdo) by intact erythrocytes was investigated and compared with their influence on SAH-hydrolase activity. We found a close relationship between these two effects. We suggest therefore that a pathway involving a process of nucleoside binding to SAH-hydrolase with subsequent base release could operate *in vivo*.

MATERIALS AND METHODS

Erythrocytes. Heparinized blood was obtained immediately before the experiment from healthy laboratory personnel and packed erythrocytes were washed twice with isotonic sodium chloride as described previously [4, 5].

Incorporation of the adenine moiety of dAdo into ATP in intact erythrocytes. Intact cell assays of metabolic pathways of dAdo in the erythrocyte were performed as described previously [3]. Briefly, freshly washed erythrocytes were added to the incubation medium—Earl's balanced salt solution

* On leave from the Department of Biochemistry, Academic Medical School Gdansk, Poland.

† On leave from the Department of General Chemistry, School of Medicine in Lodz, Poland.

‡ On leave from Instituto de Investigaciones Biomedicas del CSIC, Madrid, Spain.

§ Corresponding author: Dr H. Anne Simmonds, Purine Research Laboratory, Clinical Science Laboratories, Guy's Hospital, London Bridge, London SE1 9RT, U.K. Tel. (071) 955 4024; FAX (071) 407 6689.

‖ Abbreviations: dCf, 2′-deoxycoformycin; Itu, 5′-iodotubercidin; APRT, adenine phosphoribosyltransferase; ADA, adenosine deaminase; AK, adenosine kinase; SAH-hydrolase, *S*-adenosylhomocysteine hydrolase; 5′MT-adenosine, 5′deoxy-5′methylthioadenosine; dAdo, 2′-deoxyadenosine; 9β-D-ade ara, 9β-D-arabinofuranosyladenine; 5′I-5′d-adenosine, 5′-iodo-5′-deoxyadenosine; 2Cl-adenosine, 2-chloroadenosine.

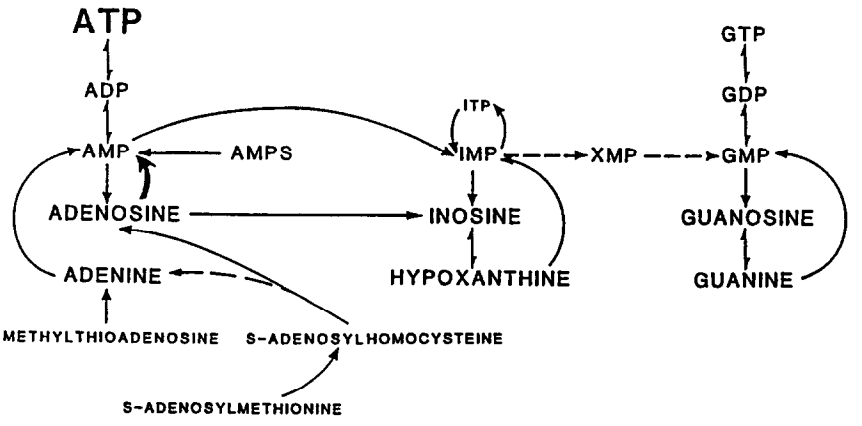

Fig. 1. Pathways of ATP formation in the human erythrocyte.

containing 5.6 mM glucose and 18 mM orthophosphate, plus 10 μM 2′-deoxycoformycin (dCf), an adenosine deaminase (ADA) inhibitor, and 20 μM 5′-iodotubercidin (Itu), an adenosine kinase (AK) inhibitor. Where indicated, the medium contained nucleoside analogues at 1.25 mM concentration. The inhibitors of ADA and AK were added to minimize the flux of both dAdo and adenosine analogues along the phosphorylation and deamination pathways. Relatively high concentration of nucleoside analogues was used to achieve excess in comparison with dAdo concentration. After 30 min preincubation at 37°, [8-^{14}C]dAdo (55 μCi/μmol) was added (10 μM final concentration) and incubation was continued for the next 40 min. Incubation was terminated by the addition of trichloracetic acid, the protein precipitate removed by centrifugation and the supernatant immediately extracted with water-saturated diethylether to remove the acid. Metabolism of dAdo was followed by analysis of the samples using either anion exchange HPLC [3] or reversed-phase HPLC [6], coupled to in-line radiodetection.

Incorporation of adenine into the nucleotide pool. Erythrocytes were prepared and incubation conditions were similar to the experiments with dAdo and as described previously [3] except that radioactive dAdo was replaced by [8-^{14}C]-labelled adenine (55 μCi/μmol) at 10 or 2 μM concentration. Deproteinized extracts were analysed using an anion exchange HPLC system with in-line radiodetection.

SAH-hydrolase assay. The activity of SAH-hydrolase was analysed in hemolysates prepared by repeated freeze–thawing of packed erythrocytes diluted 6-fold with distilled water, followed by centrifugation. Lysate (25 μL) was added to 80 μL of incubation buffer containing, where indicated, various nucleoside analogues at 1.25 mM concentrations. This mixture was preincubated for 30 min at 37°. The reaction was started by addition of 20 μL of [8-^{14}C]-labelled adenosine and L-homocysteine solution and the incubation was continued for the next 40 min. Final concentrations in the incubation medium were as follows: 26 mM sodium phosphate buffer (pH 7.4), 0.8 mM dithiothreitol, 0.8 mM EDTA, 10 μM dCf, 20 μM Itu, 6 mM L-homocysteine and 0.5 mM [8-^{14}C]-adenosine. The reaction was terminated by the addition of 25 μL of 40% trichloroacetic acid. Protein precipitate was removed by centrifugation and the supernatant extracted with water-saturated diethyl ether. Conversion of adenosine into SAH was determined by reversed-phase HPLC [6] linked to in-line radiodetection.

5′MT-adenosine phosphorylase assay. The assay system for 5′MT-adenosine phosphorylase has been described previously [7] with the exception that the 5′MT-adenosine concentration was 0.1 mM, and that dilution of hemolysate was the same as in SAH-hydrolase assay. Various nucleoside analogues were present at 1.25 mM concentration during incubation. Trichloracetic acid extracts obtained after incubation were analysed by HPLC [6] and enzyme activity was evaluated by analysis of substrate consumption.

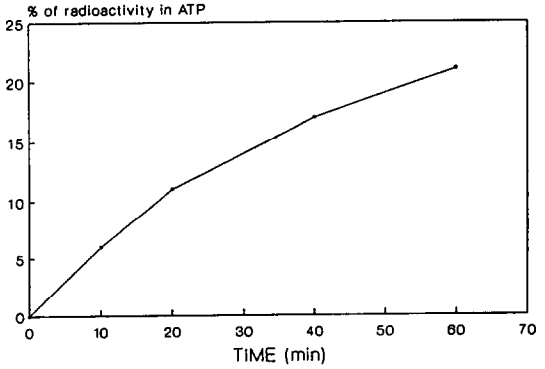

Fig. 2. Time-dependent formation of radioactive ATP from [8-^{14}C]dAdo in the human erythrocytes, expressed as the per cent of total radioactivity recovered. Cells were incubated at 37° in Earl's balanced salt solution containing 18 mM phosphate, 20 μM Itu and 10 μM dCf. dAdo was present at 10 μM concentration.

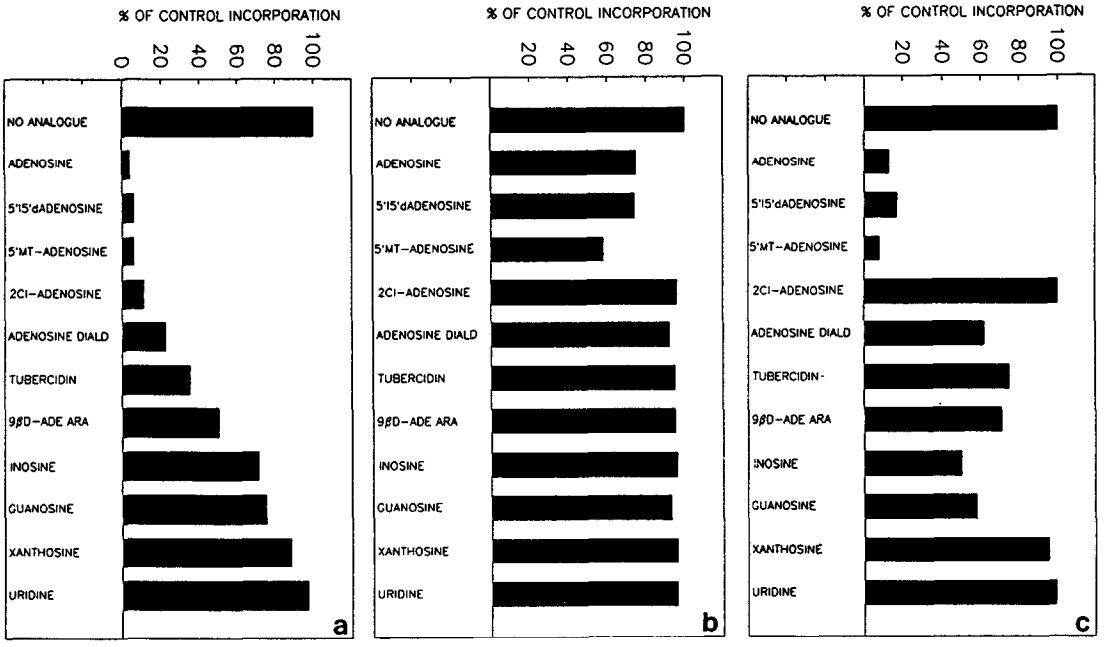

Fig. 3. (a) Influence of various nucleosides on the formation of radioactive ATP from [8-^{14}C]dAdo in the intact erythrocytes. Cells were incubated as described in Fig. 2 for 40 min in the presence of nucleoside analogues at 1.25 mM concentration. In the absence of analogues, about 15% of total radioactivity was found as ATP. Results are the average from four experiments. The differences between separate experiments were less than 20%. (b) Influence of nucleoside analogues on 2 μM [8-^{14}C]adenine incorporation into the adenylate pool in intact erythrocytes. Cells were incubated for 5 min in the presence of nucleoside analogues as described in Figs 2 and (a). In the absence of analogues about 60% of total radioactivity was found as ATP. Results are the average from three experiments. The differences between experiments were less than 35%. (c) Influence of nucleoside analogues on adenine incorporation into the adenylate pool in intact erythrocytes. Cells were incubated as described in (b) in the presence of nucleoside analogues, but for 40 min and at 10 μM [8-^{14}C]adenine. In the absence of analogues, about 95% of total radioactivity was found as ATP.

Chemicals. [8-^{14}C]-Labelled adenosine, dAdo and adenine were obtained from the Sigma Chemical Co. (Poole, U.K.) or Amersham International (Amersham, U.K.). Sources of other chemicals were described previously [3–5].

RESULTS

Effects of nucleoside analogues on ATP synthesis from dAdo

A progressive formation of radioactive ATP was observed with time in intact human erythrocytes incubated in the presence of adenine-labelled dAdo (Fig. 2). Figure 3a shows that several nucleoside analogues exerted significant inhibitory activity on this effect. The most potent analogues were adenosine, 5'MT-adenosine and 5'I-5'd-adenosine. Moderate activity was exerted by 2Cl-adenosine, adenosine dialdehyde, tubercidin and 9β-D-ade ara, while guanosine, inosine, uridine and xanthosine were only slightly inhibitory.

Effects of nucleoside analogues on adenine incorporation into ATP

The possible influence of nucleosides on the phosphoribosylation of adenine by APRT in intact erythrocytes was also analysed. This was studied both at the concentration of adenine which could be generated from dAdo under conditions described above and at a higher concentration (Fig. 3b and c, respectively). At the low adenine concentration, sensitive detection of any influence on adenine incorporation was achieved by using a very short (5 min) incubation time.

The results showed that only adenosine, 5'I-5'd-adenosine and 5'MT-adenosine substantially inhibited adenine incorporation into ATP in intact erythrocytes. However, since some adenine may be generated from these compounds by 5'MT-adenosine phosphorylase during the incubation, the observed effect was most likely the result of dilution of radiolabelled adenine. For all remaining nucleoside analogues studied, direct inhibition of APRT in intact cells can be excluded.

Effects of nucleoside analogues on SAH-hydrolase and 5'MT-adenosine phosphorylase activity

Figure 4a presents the results of experiments evaluating the influence of nucleosides on SAH-hydrolase activity in erythrocyte lysates. Most of the nucleoside analogues exerted strong inhibition with the exception of the moderate effect of 5'I-5'd-

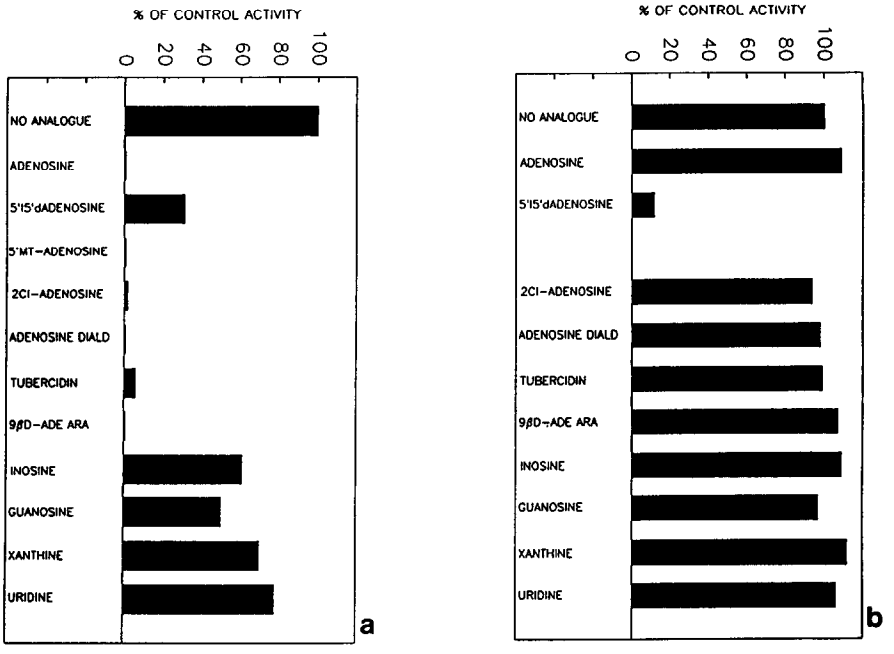

Fig. 4. (a) SAH-hydrolase activity of the erythrocyte lysates in the presence of various nucleosides at 1.25 mM concentration as described in Materials and Methods. Results are the average from three experiments. The differences between experiments were less than 30%. (b) 5'MT-adenosine phosphorylase activity of the erythrocyte lysates in the presence of nucleoside analogues at 1.25 mM concentration as described in Materials and Methods. Results are the average from two experiments. The differences between these determinations were less than 15%.

adenosine. The influence of guanosine, inosine, uridine and xanthosine was small.

Amongst all the nucleosides studied only 5'I-5'd-adenosine caused inhibition of 5'MT-adenosine phosphorylase, all other compounds being without effect (Fig. 4b). We could not detect formation of adenine from dAdo under conditions optimal for 5'MT-adenosine phosphorylase using the sensitive HPLC-linked assay system.

DISCUSSION

This study demonstrated that nucleoside analogues which inhibit SAH-hydrolase cause significant attenuation of the flux of radiolabelled adenine from dAdo into ATP, while those analogues which do not inhibit this enzyme are ineffective. This observation cannot be attributed to the inhibition of APRT. We suggest therefore that interaction of dAdo with SAH-hydrolase and release of adenine must be a necessary step in this flux, similar to the observations reported with the isolated enzyme [1, 2].

An alternative explanation involved the operation of a pathway which could generate adenine from dAdo by the action of 5'MT-adenosine phosphorylase [7]. However, the present results exclude this possibility, since the nucleosides which did not inhibit 5'MT-adenosine phosphorylase, such as 2Cl-adenosine, adenosine dialdehyde, tubercidin and 9β-D-ade ara (Fig. 4), were still effective in inhibiting adenine formation from dAdo by intact cells.

These findings are in accordance with the report showing that 5'MT-adenosine phosphorylase isolated from human liver was also inactive with dAdo as a substrate [8]; they are supported by our earlier studies of dAdo metabolism in a 5'MT-adenosine phosphorylase-deficient human malignant T cell-line, which efficiently formed ATP from dAdo when ADA was inhibited completely [9].

Another point which requires consideration is the substantial difference in the effect of various SAH-hydrolase inhibitors on adenine transfer from dAdo into ATP. It is known that SAH-hydrolase is a complex enzyme and inhibition of its physiological catalytic activity may result from interaction either with the active or the allosteric centre [1]. This interaction may not abolish completely the possibility of dAdo binding to a centre which is not occupied by the inhibitor.

Another observation which requires discussion is the time curve of ATP formation from dAdo (Fig. 2). The progressive nature observed is difficult to reconcile with the mechanism of adenine formation by SAH-hydrolase evoked from isolated enzyme studies [10, 11]. This mechanism involves suicide inactivation of the enzyme which means that for every mole of adenine formed a mole of SAH-hydrolase is inactivated irreversibly. This suggests that ATP formation from dAdo should proceed only up to the stage at which all SAH-hydrolase is inactivated. However, the intracellular environment in the intact cells may promote reactivation of the enzyme, in contrast with purified protein studies,

as has been observed by other authors [12]. Consequently, the continuous liberation of adenine suggested by the results in Fig. 2 may be a consequence of sequential inactivation and reactivation of SAH-hydrolase.

The metabolic significance of our observation needs also to be addressed. This includes not only a potential new route of ATP generation in the cell, but also the possibility of the unexpected conversion of nucleosides used in chemotherapy. In the case of dAdo, owing to its normally very low concentration, it is difficult to predict the physiological significance of this process but it could become significant within cells with a high rate of deoxynucleotide turnover. The rate of adenine flux via this route may be estimated as 0.1 nmol/mL erythrocytes/min according to data given in Figs 2 and 3a. The process appears to be more important in the case of inherited ADA deficiency where the dAdo concentration is greatly elevated [3]. A similar increase in dAdo concentration could occur during treatment of leukemia with deoxycoformycin [13].

In summary, a new pathway involving the interaction of dAdo with SAH-hydrolase followed by base release and subsequent incorporation into ATP appears to be operative in human cells. The unusual enzyme–ligand interaction invoked may be important also in other metabolic processes not related to nucleotide metabolism.

Acknowledgements—These studies were supported by the Special Trustees of Guy's Hospital. R.T.S. is the recipient of a Fellowship from the Smith and Nephew Foundation.

REFERENCES

1. Ueland PM, Interaction of adenosine with adenosine binding protein, *S*-adenosylhomocysteine hydrolase. In: *Regulatory Functions of Adenone* (Eds. Berne RR, Rall TW and Rubio R), pp. 157–170. Martinus Nijhof, The Hague, 1983.
2. Chiang PK, Guranowski A and Segal JE, Irreversible inhibition of *S*-adenosylhomocysteine hydrolase by nucleoside analogues. *Arch Biochem Biophys* **207**: 175–184, 1981.
3. Simmonds HA, Fairbanks LD, Duley JA and Morris GS, ATP formation from deoxyadenosine in human erythrocytes: a hitherto unidentified route involving adenine and *S*-adenosylhomocysteine hydrolase. *Biosci Rep* **9**: 75–85, 1989.
4. Montero C, Smolenski RT, Duley JA and Simmonds HA, *S*-Adenosylmethionine increases erythrocyte ATP *in vitro* by a route independent of adenosine kinase. *Biochem Pharmacol* **40**: 2617–2623, 1990.
5. Smolenski RT, Montero C, Duley JA and Simmonds HA, Effects of adenosine analogues on ATP concentration. Further evidence for a route independent of adenosine kinase. *Biochem Pharmacol* **42**: 1767–1773, 1991.
6. Smolenski RT, Lachno DR, Ledingham SJM and Yacoub MH, Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. *J Chromatogr* **527**: 414–420, 1990.
7. Sahota A, Webster DR, Potter CF, Simmonds HA, Rodgers VA and Gibson T, Methylthoadenosine phosphorylase activity in human erythrocytes. *Clin Chim Acta* **128**: 283–290, 1983.
8. Toorchein D and Miller RL, Purification and characterization of 5′-deoxy 5′-methylthioadenosine (MTA) phosphorylase from human liver. *Biochem Pharmacol* **41**: 2023–2030, 1991.
9. Simmonds HA, Goday A, Morris GS and Brolsma MFJ, Metabolism of deoxynucleosides by lymphocytes in long-term culture deficient in different purine enzymes. *Biochem Pharmacol* **33**: 763–770, 1984.
10. Hershfield MS, Apparent suicide inactivation of human lymphoblast *S*-adenosylhomopcystine hydrolase by 2′-deoxyadenosine and adenine arabinoside. *J Biol Chem* **254**: 22–25, 1979.
11. Abeles RH, Tashjian AH Jr and Fish S, The mechanism of inactivation of *S*-adenosylhomocysteine by 2′-deoxyadenosine. *Biochem Biophys Res Commun* **95**: 612–617, 1980.
12. Helland S and Ueland PM, Reactivation of *S*-adenosylhomocysteine hydrolase activity in cells exposed to 9-β-D-arabinofuranosyladenine. *Cancer Res* **42**: 2861–2866, 1982.
13. Bontemps F and Van den Berghe G, Mechanism of adenosine triphosphate catabolism induced by deoxyadenosine and by nucleoside analogues in adenosine deaminase-inhibited human erythrocytes. *Cancer Res* **49**: 4982–4989, 1989.