TRANSFORMATION OF MOUSE FIBROBLASTS WITH THE ONCOGENES *H-ras* OR *trk* IS ASSOCIATED WITH PRONOUNCED CHANGES IN DRUG SENSITIVITY AND METABOLISM

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Malignant activation of oncogenes *ras* or *trk* is implicated in a number of solid tumors and leukemias. We determined the chemosensitivity profile of wild-type mouse NIH-3T3 fibroblasts, and that of NIH-3T3 lines transformed by the *H-ras* (S2-721) and *trk* (106-632) oncogenes, against 11 different drugs from various classes. Differences in sensitivity were related to drug accumulation and metabolism. Both *ras*- and *trk*-transformed cell lines were less sensitive to cisplatin (CDDP) and doxorubicin (DXR) than the wild type. NIH-3T3 transformants expressing *H-ras* were less sensitive than those expressing *trk* or the wild type to the indoloquinone EO9, methotrexate and arabino-furanosylcytosine. No clear difference in sensitivity was observed for vincristine, VP-16, or the new cytidine analog 2',2'-difluoro-deoxycytidine. In both *ras*- and *trk*-transformed cell lines sensitivity to 5FU was increased moderately, but sensitivity to 5'deoxy-5-fluorouridine (5'dFUR) was increased markedly. Only the *trk*-transformed line NIH-3T3 was more sensitive to 2'deoxy-5-fluorouridine. Expression of P-glycoprotein was not different between the 3 cell lines but DXR accumulation in both mutants was decreased, indicating a non-P-glycoprotein-associated difference in sensitivity. Conversion of 5'dFUR to 5FU (catalyzed by pyrimidine nucleoside phosphorylases) was 5–10 times higher in both mutants than in the wild type. The activity of the phosphoribosyl-transferase (direct conversion of 5FU to FUMP) was comparable, but the rate of conversion of 5FU to fluorouridine (FUR) was lower in the wild type, as well as that of 5FU to FUMP via FUR. In contrast, the activity of thymidylate synthase, the target enzyme for fluoropyrimidines, was higher in the wild-type cells. The concentrations of both purine and pyrimidine nucleotides were lower in cells expressing *trk*. In conclusion, transformation of cells with the *H-ras* or *trk* oncogenes can markedly influence sensitivity to several drugs and affect normal metabolism and that of several anti-cancer agents.

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Oncogenes play an important role in the transformation of normal cells into cancer cells. Over-expression or expression of mutated oncogenes has been demonstrated in human tumors as well as in cancer cell lines. The expression of malignantly activated oncogenes varies with tumor type and has been determined by measurements of number of gene copies and of expression of oncogene in RNA, or by cellular localization using antibodies against the gene products, the oncoproteins (Garrett, 1986; Field and Spandidos, 1990; Slamon *et al.*, 1984). Because of the high, but variable expression of oncogenes in cancer cells, a relation with therapy using anti-cancer agents was postulated (Sklar, 1988; Niimi *et al.*, 1991).

The effect of expression of an oncogene on drug sensitivity can be studied most easily by using defined cell lines in which one particular oncogene is over-expressed. Sklar (1988) investigated the sensitivity to cisplatin (CDDP) of murine fibroblasts transformed with different *ras*-oncogenes, and observed a higher resistance to CDDP. *Ras* oncogenes are expressed in most types of human malignancy (Slamon *et al.*, 1984; Field and Spandidos, 1990; Vogelstein *et al.*, 1989) and are an incompletely defined component in the signal transduction pathway. In the most common cancers world-wide (cancer of the stomach, lung, breast, colon, cervix and mouth and pharynx), the incidence of aberrant gene expression and genetic alterations of the *ras* gene family have been shown to

be important in one or more stages (initiation, promotion and progression) of carcinogenesis (Vogelstein *et al.*, 1989). Another oncogene of interest, *trk*, is a human transforming gene isolated from colon carcinoma (Mittra *et al.*, 1987). The oncoprotein is a fusion protein of tropomyosin and a truncated tyrosine kinase (Oskam *et al.*, 1988; Martin-Zanca *et al.*, 1989). The proto-*trk* gene encodes the receptor for a nerve growth factor (Klein *et al.*, 1991) and may frequently be involved in recombinational events in human neoplasia (Oskam *et al.*, 1988).

Tumors potentially expressing either *H-ras* or *trk* oncogenes are being treated with a number of different drugs from various classes. A wide variation in response to these drugs has been observed. Therefore, we compared the drug-sensitivity profile of wild-type and *H-ras*- and *trk*-transformed murine NIH-3T3 fibroblasts. Eleven different drugs, both standard and investigational, were tested. These compounds belonged to various groups such as platinum, anthracyclines, Vinca alkaloids, anti-metabolites and indoloquinones. For several of these drugs the difference in sensitivity was related to known parameters of resistance, such as aberrant drug accumulation or differences in metabolism. The results show that transformation of cells with oncogenes can be associated with pronounced effects on drug sensitivity.

MATERIAL AND METHODS

Material

CDDP (Bristol Myers, Brussels, Belgium), doxorubicin (DXR; Farmitalia Carlo Erba, Nivelles, Belgium) and vincristine (VCR, Eli Lilly, Amsterdam, The Netherlands) were solubilized in 0.9% NaCl. EO9 (NDDO, Free University, Amsterdam, The Netherlands) was solubilized in absolute ethanol. 5-Fluorouracil (5FU; Sigma, St. Louis, MO), 5'deoxy-5-fluorouridine (5'dFUR; Hoffmann-La Roche, Basel, Switzerland), 2'-deoxy-5-fluorouridine (FUDR, Sigma), methotrexate (MTX; Sigma), arabinofuranosyl-cytosine (Ara-C; Sigma) and 2'2'-difluoro-deoxycytidine (dFdC; Eli Lilly, Indianapolis, IN) were solubilized in water, and VP16 (Eli Lilly, Amsterdam) was solubilized in DMSO. All solutions were stored at –20°C, except for CDDP which was stored in the dark at room temperature. Drugs were diluted in culture medium prior to addition to the cultures. Histostain SP-kit was obtained from Zymed (Burlingame, CA). The monoclonal antibodies JSB1 and CT6 were raised at the Free University Hospital (Scheper *et al.*, 1988) and C219 was obtained from Centocor, Tongeren, Belgium. [14-¹⁴C]-DXR, [6-¹⁴C]-FU and [6-³H]-dUMP were from Amersham, Aylesbury, UK. Sulforhodamine B (SRB), tetrazolium (MTT), nucleotides and tetra-hydrofolate were

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from Sigma. All other chemicals used were of the highest quality commercially available.

Cell culture

The cell lines NIH-3T3, S2-721 (H-*ras*-transformed NIH-3T3) and 106-632 (*trk*-transformed NIH-3T3) were a kind gift from Dr. M. Barbacid (Squibb Institute for Medical Research, Princeton, NJ). NIH-3T3 is an oligo-passage standard cell line, which has been subcultured for several years. New stocks from both the parent and the transformed cells were used after 20–30 passages in order to prevent eventual genotypic and phenotypic changes during subculture. No differences in chemosensitivity and enzymatic properties were observed between different stocks. S2-721 and 106-632 were generated in gene transfer assays by transfection of wild-type NIH-3T3 with H-*ras* or *trk* oncogene expression plasmids, using the calcium phosphate precipitation technique (Sukumar and Barbacid, 1990; Oskam *et al.*, 1988; Mitra *et al.*, 1987). Highly refractile foci were isolated with a sterile glass cloning cylinder. The cells were dispersed in 0.1% trypsin and replaced. Single cells were subsequently cloned in semi-solid agar, disaggregated and expanded. The cells were maintained at 37°C under an atmosphere of 5% CO₂ in culture flasks (Nunc, Glostrup, Denmark). The cell lines were subcultured in Dulbecco's medium (Flow, Irvine, UK) containing 10% heat inactivated FCS (GIBCO, Grand Island NY) and 1 mM L-glutamine. During the experiments, 50 µg/ml gentamycin were added to the culture medium and 5% instead of 10% FCS was used.

Tumorigenicity of the transformed cell lines was determined by injection of 7.5 and 15 × 10⁵ NIH-3T3, H-*ras*- and *trk*-transformed cells s.c. in the thoracic region of female BALB-c mice. Only the animals receiving transformed cells developed tumors.

In order to determine linearity in 96-well plates, cells in log-phase were harvested and counted with a hemacytometer. Subsequently, 3 different concentrations (in sextuplicate) of each cell line were plated in 96-well flat-bottomed microtiter plates (Greiner, Solingen, Germany) in a volume of 100 µl. These plates (one for each day) were incubated at 37°C for 1, 2, 3, 4 and 7 days. Cell growth was determined by measurement of the cellular protein content with the SRB assay as described previously (Keepers *et al.*, 1991). Prior to the SRB assay, 100 µl of medium were added to the wells. Blanks included wells with only culture medium. Linearity of growth in this time period was observed at initial cell concentrations of 5,000 to 30,000 cells/well.

Chemosensitivity testing

For chemosensitivity testing, cell concentrations of 10,000/well for the transformed lines and of 16,000/well for the wild type were used. For this purpose cells in log-phase were harvested, counted and plated in 96-well microtiter plates in a volume of 100 µl. The plates were incubated for 1 day at 37°C to allow cell attachment and thereafter 100 µl of the drug were added in 8 concentrations. All drug concentrations were tested in 3 replicate wells. Blanks included wells with culture medium only and controls included wells with cells only. The SRB assay was used to determine the density of the cells when the drug was added (zero-point; OD-zero) and after 3 days of culture without drug (OD control) or with continuous exposure to the drugs (OD-treated). For some purposes the MTT assay was used. The method of plating and incubation was identical to the SRB-assay (Keepers *et al.*, 1991). The relative growth inhibition was calculated as follows:

$$\frac{[(\text{OD treated}/\text{OD-zero})-1]/[(\text{OD control}/\text{OD-zero})-1]}{\times 100\%}$$

The optical density (OD) was read at 540 or 510 nm using a Titertek microplate reader (Titertek Multiskan MCC/340, Flow) (Skehan *et al.*, 1990; Keepers *et al.*, 1991).

Statistical evaluation was performed using Student's *t*-test.

P-glycoprotein staining

P-glycoprotein expressed in the cell membrane was stained using the Histostain SP-kit. Cytospin slides (3 × 10⁴ cells per slide) were incubated with the monoclonal antibodies JSB1 and C219 in order to recognize intracellular epitopes of P-glycoprotein (Scheper *et al.*, 1988), at dilutions of 1:50 and 1:10, respectively, in a serum blocking solution. CT6, a negative control, was diluted 1:50. Incubations lasted for 1 hr at room temperature.

DXR accumulation

DXR accumulation in cells was performed essentially as described (Schuurhuis *et al.*, 1987). Briefly, at day one, 35 × 10⁴ cells (in 2 ml culture medium) were plated in 6-well tissue-culture plates (Costar, Cambridge, MA). At day two, the medium was removed and 1 ml of medium A (Dulbecco without sodium bicarbonate and phenol red sodium salt) containing 10% FCS was added. Subsequently, cells were incubated for 2 hr at 37°C with 25 µl 14-¹⁴C-labelled DXR (one part of 14-¹⁴C-labelled DXR and 2 parts of non-radioactive DXR solubilized in 0.9% NaCl; the concentration of the mixed solution was 20.5 µM; the final incubation concentration was 0.5 µM). After the 2-hr incubation period, cells were washed with PBS containing 2% FCS (washing solution, pH 7.4) on ice 3 times, then harvested. Cell harvest was followed by 2 wash steps with 950 µl washing solution. A volume of 15 ml liquid scintillation fluid (Packard, Groningen, The Netherlands) was added to the cells and the 14-¹⁴C-labelled DXR was counted for 5 min. The experiments were done in triplicate; 3 wells for cell count and 3 wells for drug addition. Two backgrounds were used; one included 2 ml culture medium that was incubated with the DXR combination for 2 hr and the other included cells which were incubated with the DXR combination for only a few seconds.

Enzyme assays

Cells were washed with Hanks' balanced salt solution (HBSS) containing EDTA, trypsinized, washed with Dulbecco's medium containing 5% FCS and centrifuged at 300 g for 10 min. The pellet was resuspended in Tris-EDTA buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) at a concentration of 20 × 10⁶ cells/ml. For the thymidylate synthase assays, cell pellets were suspended in another Tris-buffer (200 mM Tris-HCl, 100 mM NaF, 15 mM CMP, 20 mM 2-mercaptoethanol, pH 7.4). Cells were disrupted by sonication for 3 cycles of 5 sec at maximal output with intervals of 10 sec at 4°C (Peters *et al.*, 1986). The extract was subsequently centrifuged for 5 min in a minicentrifuge at 4°C at 7,000 g. The assays for enzymes from pyrimidine metabolism were carried out essentially as described previously (Peters *et al.*, 1986). Briefly, the orotate phosphoribosyl-transferase (OPRT) assay was carried out in a total volume of 90 µl containing 50 µl enzyme extract in the Tris-EDTA buffer (equivalent to 10⁶ cells), 10 µl glycerol-2-P (150 mM), 10 µl PRPP (24 mM), 10 µl MgCl₂ (50 mM) and 10 µl mixed 5FU (1 part 6-¹⁴C-FU, 55 µCi/µmol, and 4 parts 2.5 mM FU), at 37°C for 30 or 60 min.

The uridine phosphorylase (FUP) assay was carried out in a total mixture of 80 µl containing 50 µl enzyme extract in the same Tris-EDTA buffer (equivalent to 0.125–1.0 × 10⁶ cells), 10 µl MgCl₂ (50 mM), 10 µl ribose-1-P (20 mM) and the above-mentioned substrate mixture, at 37°C for 15 or 30 min.

The "channelled" uridine kinase (FUP-UK) assay was carried out in the same mixture as the FUP assay with the addition of 10 µl ATP (20 mM, pH 7). The number of cells and

the incubation times were twice as high as in the FUP assay (equivalent to $0.25\text{--}2.00 \times 10^6$ cells and 30 or 60 min).

All reactions were stopped by heating for 3 min at 95°C in an Eppendorf heater followed by chilling on ice and addition of $20\text{ }\mu\text{l}$ excess carrier FU/UR/UMP (each 5 mM) or carrier FU/UR (5/5 mM). The denatured protein was centrifuged for 5 min at 12,000 g. Separation of products and substrates was performed on polyethylene-imine (PEI) cellulose thin-layer plates with distilled water as developing agent. The spots were visualized under a UV lamp and the separate spots were counted using PPO-POPOP in toluene as liquid scintillation fluid.

The conversion assay of 5'dFUR to 5FU was carried out in the Tris/EDTA buffer in a total volume of $200\text{ }\mu\text{l}$, containing 25 or $50\text{ }\mu\text{l}$ of the enzyme extract (equivalent to $0.25\text{--}0.50 \times 10^6$ cells), $10\text{ }\mu\text{l}$ potassium phosphate (800 mM) and $10\text{ }\mu\text{l}$ of $10\text{--}20\text{ mM}$ 5'dFUR or $20\text{ }\mu\text{l}$ of 20 mM 5'dFUR, at 37°C for 30 min. Reactions were stopped by boiling for 3 min followed by chilling on ice, the protein was centrifuged for 5 min at 12,000 g and the supernatants were analyzed using high-pressure liquid chromatography (HPLC) (Laurense *et al.*, 1988), on a LiChrosorb 5-RP-18 column ($150 \times 4.6\text{ mm}$, length $\times$ ID, particle size of $5\text{ }\mu\text{m}$) using isocratic elution of 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ plus 2.5% methanol (pH 6.5) at a flow of 1.5 ml/min and UV detection at 254 nm .

HPLC assay for nucleotides

The nucleotides ADP, UTP, CTP, ATP and GTP were measured by HPLC essentially as described (Peters *et al.*, 1990). Briefly, cells were trypsinized, resuspended in PBS and precipitated in TCA at a final concentration of 10%, then the supernatant was neutralized with triethylamine/1,1,2-trichlorotrifluoroethane (1:4). The nucleotides were separated using a Whatman (Whatman, Maidstone, UK) Partisphere column ($110 \times 4.7\text{ mm}$, length $\times$ ID, particle size $5\text{ }\mu\text{m}$) with isocratic elution of 0.25 M $\text{KH}_2\text{PO}_4/0.5\text{ M}$ KCl (pH 4.5) at a flow of 1.5 ml/min and were determined at 254 and 280 nm .

Cell-cycle distribution

Cells were trypsinized, resuspended in culture medium, washed twice with PBS and fixed for at least 16 hr in 100% methanol at 4°C . After washing with ice-cold PBS, cells (2×10^6 /sample) were resuspended in RNase A (0.5 mg/ml TRIS-buffer) and incubated for 30 min at 37°C . Thereafter an equal volume of pepsin solution (1 mg pepsin/ml 0.4% HCl) was added and incubated for 5 min at 37°C . Cells were stained by addition of ethidium bromide and Hoechst 33218 diluted in PBS containing 1% BSA (pH 9). The distribution of cellular DNA was measured by flow cytometry using a fluorescence-activated cell sorter (FACSstar^{plus}, Becton-Dickinson, Etten-Leur, The Netherlands).

RESULTS

Chemosensitivity profile

In NIH-3T3 cells the characteristic pattern of fibroblast growth—a monolayer of stretched cells—was observed. This typical fibroblast pattern was lost in the transformed cell lines, in which cells formed multilayered foci. Doubling times of the cell lines were about 25 hr for the H-ras-transformed NIH-3T3 (S2-721), about 22 hr for the trk-transformed NIH-3T3 (106-632) and about 38 hr for the wild-type NIH-3T3. The long doubling time of NIH-3T3 was related to a relatively low growth fraction of these cells (3% of cells in S phase, 8.3% of cells in G_2/M) compared to the ras-transformed cells (7.5% in S phase, 18.5% in G_2/M) and trk-transformed cells (8.9% in S phase, 16.5% in G_2/M).

Large differences were observed between the cell lines concerning sensitivity to various drugs (Table I). All 3 lines were least sensitive to the pyrimidine analog 5'dFUR, but most

TABLE I—SENSITIVITY OF NIH-3T3 CELLS AND THE H-ras- AND trk-TRANSFORMED VARIANTS TO 11 DIFFERENT DRUGS

	NIH-3T3	H-ras ^{1,2}	trk ¹⁻⁴
5FU	1.22 ± 0.25	0.68 ± 0.12^2	0.35 ± 0.04^1
5'dFUR	97 ± 16	7.33 ± 1.45^1	3.5 ± 0.5^1
2'FUdR	0.0015 ± 0.0002	0.0014 ± 0.0002	$0.00068 \pm 0.00008^{2,3}$
Ara-C	0.58 ± 0.09	0.92 ± 0.15	0.37 ± 0.02^4
dFdC	0.032 ± 0.010	0.033 ± 0.010	0.024 ± 0.001
MTX	0.054 ± 0.010	0.13 ± 0.03	0.056 ± 0.008
CDDP	0.32 ± 0.16	4.17 ± 0.44^1	$2.10 \pm 0.31^{1,4}$
DXR	0.05 ± 0.02	0.22 ± 0.05^1	0.21 ± 0.03^1
EO9	0.03 ± 0.01	0.06 ± 0.02	0.02 ± 0.01^3
VCR	0.10 ± 0.04	0.09 ± 0.05	0.08 ± 0.04
VP-16	0.70 ± 0.16	0.93 ± 0.19	0.88 ± 0.09

IC₅₀ values (in μM) are means $\pm$ SEM of 3–7 separate experiments.^{1,2}Significantly different from NIH-3T3 at the level $p < 0.01$, $^{2,3}0.01 < p < 0.05$.^{3,4}Significantly different from H-ras at the level $^3p < 0.01$, $^40.01 < p < 0.05$.

sensitive to the other fluoropyrimidine 2'FUdR. The wild type appeared to be more resistant to 5FU than the 2 transformed cell lines. For its pro-drug 5'dFUR, the wild type was 15–25 times more resistant than the 2 transformed cell lines. The 3T3/trk was significantly more sensitive to 2'FUdR and the cytidine analog, Ara-C, than the wild type and the 3T3/H-ras. However, the difference for the other cytidine analog dFdC was less pronounced. A different pattern was found for the antifolate MTX for which the lowest sensitivity was observed in the 3T3/H-ras. The transformed cell lines were 4–5 times more resistant to DXR than the wild type. The same was found for CDDP. A marked difference in chemosensitivity between the 2 transformed cell lines was observed for the indoloquinone EO9; the IC₅₀ value of EO9 for the H-ras-transformed line was about 3 times higher than for the trk-transformed line.

No significant differences in sensitivity between the 3 cell lines were found for VCR and VP-16. The OD values of VCR-treated cells increased with drug concentrations higher than 10^{-7} M (data not shown). This might be inherent to the SRB assay, higher OD values resulting from higher protein content. The MTT assay, based on the capacity of living mitochondrial enzyme systems to reduce the MTT, is independent of protein content. However, the same effect was observed as when the SRB assay was used.

Drug accumulation and metabolism

The differences in sensitivity to each drug can be due to a number of factors. For several of the drugs with the most pronounced differences, various resistance factors were investigated. Resistance to DXR has been related to an overexpression of P-glycoprotein, resulting in decreased drug accumulation. The degree of DXR resistance did not correlate with the amount of P-glycoprotein. Negative staining of the cell membranes was found for the 3 cell lines. The cytoplasm of the cells was slightly stained. However, there was a significant difference in DXR accumulation between NIH-3T3 and the transformed cell lines (Fig. 1). Both transformed lines accumulated less DXR than the wild type (unpaired Student's *t*-test, $0.001 < p < 0.01$ and $p < 0.001$, respectively). No significant difference in DXR accumulation was found between the 2 transformed cell lines ($p > 0.1$).

Since the most pronounced differences in drug sensitivity were observed for the fluoropyrimidines, we measured several enzymes required for the metabolism of 5FU (Table II). No significant difference in the activity of OPRT was found, but the activity of thymidylate synthase was significantly higher in the wild-type cell line. The FUP activity was very low and the "channeled" FUP-UK activity was not detectable in the wild type. The transformed cell lines showed a higher activity of these enzymes than the wild type. Because of the pronounced

FIGURE 1 – Percentage of DXR accumulation in cell lines NIH-3T3, H-*ras* and *trk*. The accumulation observed in NIH-3T3 was 132.6 ± 11.5 pmol/ 10^6 cells (mean $\pm$ SE, $n = 3$).

TABLE II – ACTIVITIES OF RATE-LIMITING ENZYMES IN FLUOROPYRIMIDINE INSENSITIVITY

Conversion	NIH-3T3	H- <i>ras</i>	<i>trk</i>
dUMP $\rightarrow$ dTMP	0.52 ± 0.03	0.12 ± 0.03^1	0.14 ± 0.05^2
FU $\rightarrow$ FUMP	1.81 ± 0.14	1.34 ± 0.01	1.71 ± 0.33
FU $\rightarrow$ FUR	<0.1	109 ± 0.01^1	105 ± 12^1
FU $\rightarrow$ FUR $\rightarrow$ FUMP	ND	7.20 ± 0.39^1	7.70 ± 1.02^1
5'dFUR $\rightarrow$ FU			
0.5 mM	$<1,500$	$4,269 \pm 123^2$	$4,919 \pm 942^2$
1 mM	$<2,700$	$14,715 \pm 503^1$	$16,380 \pm 1,966^1$
2 mM	$<5,900$	$43,131 \pm 2,518^1$	$49,284 \pm 7,056^1$

Values (in nmol of product formed per hr per 10^6 cells) are means $\pm$ SE from 3–4 separate experiments. ND, not detectable. The conversion of 5FU to FUMP was measured in the presence of PRPP (OPRT assay), that of 5FU to FUR in the presence of ribose-1-P (UP assay), that of 5FU to FUR and FUMP in the presence of ribose-1-P and ATP (FUP-UK assay). The conversion of dUMP to dTMP was measured at a saturating dUMP concentration.^{1,2}Significantly different from the wild type at the level, ¹ $p < 0.001$; ² $0.01 < p < 0.05$.

difference in sensitivity to 5'dFUR, we also determined the conversion of 5'dFUR to 5FU. No enzyme activity was detectable in the wild type in 2 separate experiments, while the values were low in another experiment. In the transformed cell lines, at least 3–8 times higher enzyme activity was found. Since in other cell lines and tumors the K_m of the enzyme was 1–2 mM (Laurensse *et al.*, 1988; Peters *et al.*, 1989), the activity was measured at different substrate concentrations; however, no reliable K_m values could be calculated. Also, the inclusion of more substrate concentrations could not solve this problem.

Because of the differences in drug sensitivity we also determined the nucleotide pattern in NIH-3T3 and the NIH-3T3 transformants (Table III). The relative nucleotide pattern within each cell line was comparable to that of lines tested in the past, with ATP being highest, and an ATP/ADP ratio varying between 5 and 7. The ADP concentrations were comparable in the 3 cell lines, but in the *trk*-transformed line all triphosphates tended to be lower than in the wild-type and the H-*ras*-transformed line.

DISCUSSION

Marked differences have been observed in several properties of the H-*ras*- or *trk*-transformed cell lines compared to the wild-type fibroblasts. Transformation was associated with either increased or decreased sensitivity to a number of commonly used anti-cancer agents. The long doubling time of

TABLE III – NUCLEOTIDE PATTERN OF NIH-3T3 CELLS AND H-*ras*- AND *trk*-TRANSFORMED VARIANTS

	NIH-3T3	H- <i>ras</i>	<i>trk</i>
ADP	865 ± 190	767 ± 36	686 ± 68
UTP	$2,065 \pm 380$	$2,065 \pm 7$	$1,363 \pm 114^1$
CTP	692 ± 122	580 ± 11	347 ± 51^1
ATP	$4,465 \pm 380$	$5,235 \pm 736$	$3,351 \pm 509$
GTP	$1,242 \pm 202$	$1,063 \pm 40$	786 ± 112
ATP/ADP ratio	5.8 ± 1.3	6.8 ± 0.8	5.0 ± 0.9

Nucleotide concentrations are given as pmol/ 10^6 cells. Values are means $\pm$ SE of 3–4 separate experiments. The ratio was calculated from the separate experiments.¹Significantly different from the *trk*-transformed cell line ($p < 0.05$).

NIH-3T3 agrees well with the relatively low proportion of cells in the S-phase. However, this was not in line with the relatively high activity of thymidylate synthase in these cell lines, although thymidylate synthase has been reported to be associated with the cell cycle, with a positive correlation between the percentage of cells in S-phase and the amount of the enzyme (Cadman and Heimer, 1986). In addition, we did not observe pronounced differences in the nucleotide content; pool sizes of the slowly proliferating NIH-3T3 wild type were similar to those of the H-*ras*-transformed NIH-3T3, but generally larger than those of the *trk*-transformed NIH-3T3.

These properties, however, do not provide an adequate explanation for the differences in drug sensitivity between the 3 cell lines. A significantly increased CDDP resistance was demonstrated for both H-*ras*- and *trk*-transformed NIH-3T3. This effect of transformation by members of the *ras* oncogene family on sensitivity to CDDP has previously been reported by several groups (Sklar, 1988; Isonishi *et al.*, 1991). Resistance to CDDP was observed in cell lines transformed with either c-H-*ras*, v-H-*ras* or mutated *ras* compared to the wild-type NIH-3T3 (Sklar, 1988). A resistance to CDDP was also observed when NIH-3T3 cells were compared to a c-H-*ras* proto-oncogene-transformed NIH-3T3 cell line in which over-expression was caused by linkage to a retroviral ltr, as well as in a cell line in which the *ras*-oncogene was hormone-inducible (Isonishi *et al.*, 1991). However, Niimi *et al.* (1991) did not observe resistance against CDDP in a *ras*-transformed cell line, but a C-*myc* transformed cell line showed a 2-fold resistance. Resistance against CDDP as a result of *trk* transformation has not previously been reported. Decreased CDDP accumulation has been observed in many CDDP-resistant cell lines from a variety of species (Bungo *et al.*, 1990; Andrews and Howell, 1990). A reduction in CDDP influx may be at least partly responsible for resistance of the cell lines, since a decrease in accumulation can be detected as early as 30 sec after the start of drug-exposure (Andrews and Howell, 1990). Although differences in glutathione content were observed in resistant cell lines, this does not seem to contribute to CDDP resistance in *ras*-transformed lines (Isonishi *et al.*, 1991).

The wild-type NIH-3T3 appeared to be more sensitive to DXR than the cell lines transformed with either H-*ras* or *trk*. The same observation was made earlier for c-H-*ras*-transformed murine fibroblasts (Kadoyama *et al.*, 1989). The results of P-glycoprotein staining demonstrated that there was no correlation between the rate of DXR resistance and P-glycoprotein expression, as was also observed in other *ras*-transformed lines (Wishart *et al.*, 1991). In contrast, no cross-resistance against VCR was observed. It appears likely that, in these cell lines, a non-P-glycoprotein-related resistance to DXR is present which is associated with a lower accumulation of DXR. Other mechanisms of resistance might be related to topoisomerase II, although no significant difference in enzyme activity was found in DXR-resistant human leukemic cells (Kato *et al.*, 1990). It is of interest to note that transformation with *ras* did not influence sensitivity to CPT-11,

a topoisomerase inhibitor (Niimi *et al.*, 1991), or to VP-16, another topoisomerase inhibitor. An increased content and expression of topoisomerase II was observed in another *ras*-transformed NIH-3T3 (Woessner *et al.*, 1990), associated with increased sensitivity to VP-16.

The opposite pattern in sensitivity between 3T3/*H-ras*, 3T3/*trk* and wild-type NIH-3T3 was found for 5'dFUR, in that the wild-type cell line was more resistant than the 2 transformed NIH-3T3 cell lines. A similar pattern was previously described for *ras*-transformed lines (Geng *et al.*, 1991). NIH-3T3 cells with a lower sensitivity for 5'dFUR proved to have insufficient activity of the enzyme required to convert 5'dFUR to 5FU. *H-ras* and *trk* cells that were more sensitive to 5'dFUR showed a higher activity of this enzyme. Such a relation was described earlier (Peters *et al.*, 1986, 1989), and it was postulated that only a very low activity is related to low sensitivity. The efficacy of 5'dFUR is also dependent on its conversion to FUMP by the enzyme OPRT (Pinedo and Peters, 1988). The other 5FU activation pathways appear to play a less important role in the activity of 5'dFUR (Peters *et al.*, 1989). In NIH-3T3 cells only OPRT activity was detectable, and this enzyme probably played a more important role than uridine phosphorylase in the first step of 5FU metabolism in these cells. Although sensitivity to 5FU in transformed cell lines was slightly higher, this is not in relation to the very high difference in phosphorylase activity. Apparently this pathway only contributes to a minor extent to 5FU activation and subsequently to 5FU growth-inhibitory effects. The difference in 5FU sensitivity appears to be related to the difference in activity of thymidylate synthase.

The clear difference in 5'dFUR sensitivity between the wild type and the transformed cell lines could be relevant in further development of the clinical use of this drug. Values for plasma concentrations exceed the IC₅₀ values observed for the transformed cell lines (De Bruijn *et al.*, 1989; Peters *et al.*, 1989). Depending on dose, schedule and the route of administration, a significant anti-tumor effect is found for 5'dFUR, but toxicity of 5'dFUR is sometimes unpredictable and this has hampered

its clinical development (De Bruijn *et al.*, 1989). For CDDP and DXR, transformation by *H-ras* or *trk* resulted in increased resistance. The clinical relevance of this finding is not yet clear, although colon tumors which have a high expression of *ras* (Field and Spandidos, 1990; Vogelstein *et al.*, 1989), show clinical resistance against treatment with CDDP or DXR. Interestingly, colon tumors show sensitivity to 5FU (Pinedo and Peters, 1988) in contrast to other drugs presented in Table I.

Both *H-ras*- and *trk*-transformed lines showed a difference in the drug sensitivity profile compared to the wild-type NIH-3T3 for DXR, CDDP and the fluoropyrimidines, with no significant differences between both transformed lines. However, for the unrelated drugs EO9, an indoloquinone (Roed *et al.*, 1989), MTX and Ara-C, the sensitivity of 3T3/*trk* and NIH-3T3 was similar, but a 2- to 3-fold increase in IC₅₀ was observed in the *ras*-transformed cell line. It is unlikely that the differences are related to a clonogenic variation induced by the selection procedure. NIH-3T3 is a standard oligo-passage cell line used for a number of different studies, while the transformed cell lines were selected for the most malignant phenotype. The only known genotypic differences are those which have been introduced. The differences between the cell lines suggest that it is not the transformed phenotype itself which is responsible for the observed difference in sensitivity, but most likely the molecular mechanisms by which the oncogene induced this transformation. This is supported by data obtained with a hormone-inducible *ras* oncogene (Isonishi *et al.*, 1991). Studies with transformed human cell lines (Wishart *et al.*, 1991) indicate that the results described are not unique to murine fibroblasts. Therefore, malignant activation of an oncogene or a combination of oncogenes in a tumor may be an important determinant of the outcome of chemotherapy.

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