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# A quick method for detecting metabolic cooperation

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Cells in culture are able to exchange nucleotides and other molecules by a process known as metabolic cooperation [1].

This process can be monitored by autoradiography [1–4], as well as by assessment of cell survival after transfer of a toxic compound from sensitive to resistant cells [5]. Autoradiographic and colony counting methods are, however, laborious and time consuming. In the usual cell survival assays such as the one developed by Yotti *et al.* [5], at least a week is required for the few hundred 6-thioguanine resistant (6-TGR*) cells that are plated among the WT cells to grow into colonies which can be counted. Ledbetter and Lubin [6] have examined transfer of potassium between cells resistant and sensitive to ouabain and similarly, Pitts and Shaw [7] measured incorporation of tritiated hypoxanthine by hypoxanthine guanine phosphoribosyl transferase (HGPRT) deficient rodent and HGPRT + human cell co-cultures in the presence of ouabain. In this short report we describe a simple method for detecting gap junctional intercellular communication which makes use of co-cultures of V79 WT and 6-TGR cells. The latter strain lacks HGPRT and thus cannot convert 6-TG into its toxic metabolite. Whereas, WT cells possess the enzyme and are thus killed when 6-TG is taken up and metabolized. WT cells transfer the toxic metabolite to cells with which they are in communication.

## *Materials and Methods*

V79 WT and 6-TGR cells were plated at a 1:1 ratio at a total density of 60,000 cells per 1-cm well in 1 mL of Dulbecco's Modification of Eagles Medium with 10% foetal calf serum, 2 mM glutamine and 50 μg/mL gentamycin. Twenty-four-well plates were used. Cultures were left for 24 hr to adhere and form gap junctions. Media containing 10 μg/mL of 6-TG were then added together with inhibitors or presumptive enhancers of cell communication where appropriate. Since the toxicity of 6-TG is dependent upon DNA synthesis, cultures were again left for 24 hr in order for the toxic effect to be exerted. At this time the 6-TG-containing media were replaced with media containing 0.1 μCi/mL of [$^3$H]thymidine and left for 4 hr in order to assess culture viability. Cells were then washed three times with phosphate-buffered saline before being detached with 1 mL of 0.25% trypsin. The resulting cell suspension was then placed in a 1.5 mL microcentrifuge tube with 0.4 mL of 20% TCA and 0.1 mL of 1 mg/mL calf thymus DNA and left to stand at 4° overnight. The resulting precipitate was spun down, washed twice with ice-cold 5% TCA and once with ice-cold ethanol, dissolved in 0.5 mL of formic acid and counted in 4.5 mL of scintillation fluid for 2 min.

## *Results and Discussion*

Observations of the control co-cultures after 48 hr of growth show near confluence. Treatment with 6-TG for 24 hr, however, leads to a population of cells which are still attached but with crenulated membranes.

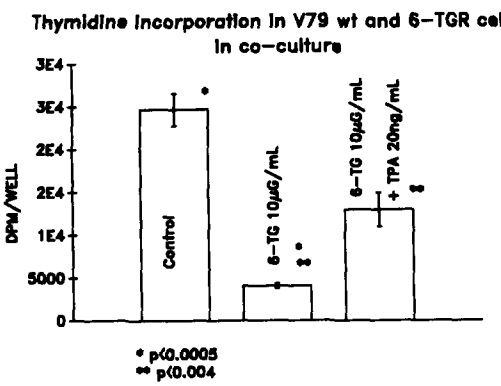


Fig. 1. Cells were grown in co-culture for 24 hr, treated with 6-TG and TPA where appropriate for another 24 hr, exposed to [$^3$H]thymidine for 4 hr, harvested and TCA precipitated overnight. The insoluble fraction was collected and solubilized in formic acid before being counted in a liquid scintillation counter as described in the text. Statistical analyses were performed using two-tailed *t*-test.

Figure 1 shows [$^3$H]thymidine incorporation of the co-cultures in the presence and absence of a concentration of 6-TG which is toxic to WT and also to the 6-TGR cells in co-culture. At a 1:1 ratio of WT to 6-TGR cells, levels of [$^3$H]thymidine incorporation drop to 16.5% of control levels in the presence of 6-TG whereas 6-TGR cells in the absence of WT cells are resistant to 6-TG (not shown). There was almost total killing of WT cells at this dose of 6-TG (not shown). Thus, if communication does not occur between the two cell types only the WT cells are killed and [$^3$H]thymidine incorporation would be about 50% of control values. 6-TG reduced [$^3$H]thymidine incorporation in co-cultures to 9% of control levels ± 5% in seven experiments.

The decrease in [$^3$H]thymidine incorporation in the 6-TG treated co-culture results from communication between WT and 6-TGR cells and transfer of the toxic metabolite. In the presence of TPA, which inhibits gap junctional intercellular communication, there is greater survival of the 6-TGR cells (see Fig. 1). Survival in the presence of TPA is approximately 50% of the control level. This is in agreement with the results expected if most 6-TGR cells survive and all WT cells die.

In summary, we have devised a simple method for the study of gap junctional intercellular communication using WT and 6-TGR co-cultures. This method for detection of metabolic co-operation takes approximately 3 days to carry out. By contrast, the colony counting and autoradiography methods require more than 1 week. In addition, the colony counting method requires the use of large tissue culture plates whereas the present method uses multi-well plates thereby saving on space and cost. It is also possible to perform a battery of tests on gap junctional intercellular communications in one multi-well plate.

* Abbreviations: 6-TG(R), 6-thioguanine (resistant); WT, wild type; HGPRT, hypoxanthine guanine phosphoribosyl transferase; TCA, trichloroacetic acid; TPA, 12-*O*-tetradecanoyl-13-acetate.

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# Anti-neoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody–enzyme conjugate

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Treatment of cancer with anti-neoplastic prodrugs that are specifically activated to cytotoxic agents at the tumor site is a conceptually attractive strategy. For example, a glucuronide prodrug [(*p*-di-2-chloroethylaminophenol-β-D-glucopyranosid) uronic acid, HAMG*] of *p*-hydroxyaniline mustard [*N*,*N*-di-(2-chloroethyl)-4-hydroxyaniline, HAM] cured mice bearing well-established PC5 plasma tumors containing high levels of β-glucuronidase [1–3]. The relatively nontoxic prodrug HAMG was apparently formed *in vivo* in the liver of mice treated with aniline mustard [*N*,*N*-di-(2-chloroethyl)aniline] and subsequently converted to highly cytotoxic *p*-hydroxyaniline mustard by β-glucuronidase at the tumor site [2]. Clinical trials using aniline mustard [4, 5], however, have been disappointing, likely due to insufficient elevations of β-glucuronidase in most human tumors [4].

Inadequate differences in enzyme levels between normal and tumor cells for specific prodrug activation can be alleviated by targeting appropriate enzymes to tumor cells with monoclonal antibodies that bind to tumor-associated antigens [6–8]. Anti-neoplastic prodrugs susceptible to enzymatic activation can then be administered and converted to cytotoxic agents at the tumor site. We report here the development of a glucuronide prodrug/enzyme-antibody system and show that cytotoxic drug can be selectively generated at tumor cells using this strategy.

* Abbreviations: HAM, *p*-hydroxyaniline mustard; HAMG, *p*-hydroxyaniline mustard glucuronide; BHAMG, tetra-*n*-butyl ammonium salt of HAMG; $IC_{50}$, concentration of drug resulting in 50% inhibition of cellular protein synthesis; PBS, phosphate-buffered saline; and SPDP, *N*-succinimidyl-3(2-pyridyldithio)propionate.

## *Materials and Methods*

*Materials and cells.* β-Glucuronidase (EC 3.2.1.31) from *Escherichia coli* and pepsin were purchased from the Sigma Chemical Co., St. Louis, MO. *N*-Succinimidyl-3(2-pyridyldithio)propionate (SPDP) was from Pharmacia LKB Biotechnology, Uppsala, Sweden. [$^3$H]Leucine (50 Ci/mmol) was purchased from ICN Biomedicals Inc., Costa Mesa, CA. COLO 205 human colon and WISH human amnion cell lines were obtained from the American Type Culture Collection, Rockville, MD. Cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin.

*Drug synthesis. p*-Hydroxyaniline mustard and the tetra-*n*-butyl ammonium salt of HAMG (BHAMG) were synthesized as previously described [9]. Structures were confirmed by NMR and melting point determination.

*β-Glucuronidase conjugation to monoclonal antibody.* Mab 12.8 is a murine $IgG_3$ monoclonal antibody that binds strongly to COLO 205 human colon carcinoma cells but does not react with WISH cells. Mab 12.8 also reacts positively with about 40% of human colon carcinoma tissues but has limited reaction with normal human tissues [10]. Mab 12.8 F(ab')$_2$ fragments were generated by pepsin digestion [11].

β-Glucuronidase was linked to Mab 12.8 F(ab')$_2$ via a disulfide bond with the heterobifunctional cross-linking agent SPDP [12]. An average of 2.4 and 2.0 2-pyridyl groups, measured as described [12], were introduced into Mab 12.8 F(ab')$_2$ and β-glucuronidase molecules, respectively. Derivatized enzyme was reduced with 1 mM dithiothreitol, and after removing excess reducing agent by gel filtration, derivitized enzyme and antibody were mixed and incubated overnight at room temperature.