Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates in vivo against L1210 leukaemia

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Summary DBA mice were inoculated i.p. with 10⁴ L1210 cells. Animals subsequently treated with daunomycin (single i.p. dose, 0.25-5.0 mg kg⁻¹) all died. The maximum increase in mean survival time observed was ~135%. Animals treated with N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers conjugated to daunomycin (DNM) showed a significant increase in mean survival time when the polymer-drug linkage was biodegradable (i.e., Gly-Phe-Leu-Gly). Such treatment also produced a number of long term survivors (>50 days). In contrast, HPMA copolymer conjugated to DNM via a non-degradable linkage (Gly-Gly) produced no increase in survival time relative to untreated control animals. The effect observed with biodegradable HPMA copolymer-DNM conjugates was dependent on the concentration of conjugated drug administered (optimum >5 mg kg⁻¹); the frequency of administration (multiple doses were more effective than single); the timing of administration (single doses given on days 1 and 3 were most effective); and the site of tumour inoculation and route of drug administration. Biodegradable HPMA copolymer-DNM conjugates administered i.p. were active against L1210 inoculated s.c. at higher doses than required to curb a peritoneal tumour. Under certain experimental conditions polymer-DNM conjugates containing fucosylamine or galactosamine proved more active than conjugates without the carbohydrate moeity. The mechanism of drug-conjugate action in vivo is at present unclear. Radiodiation of polymer showed ~75% of polymer-drug conjugate to be excreted 24 h after i.p. administration. Synthesis of HPMA conjugates containing [³H]DNM showed that polymer containing Gly-Gly-³H]DNM was excreted (60% of radioactivity in the urine, 24 h) in macromolecular form. In contrast polymer containing Gly-Phe-Leu-Gly-[³H]DNM was largely excreted in the form of low molecular weight species.

Use of tailor-made polymeric drug-carriers to achieve tumour-specific drug-targeting is receiving increasing interest (Duncan & Kopeček, 1984). Unlike liposomes and microparticles, whose distribution within the body is severely limited, macromolecular drug-carriers can move from one body compartment to another and target to specific organs following different routes of administration (Duncan et al., 1986; Duncan, 1987; Seymour et al., 1987a). A number of different macromolecules have been proposed as carriers of antitumour agents: dextran-mitomycin C (Kojima et al., 1980; Takakura et al., 1984), poly-L-aspartic acid-daunomycin (Zunino et al., 1984), poly-L-lysine-methotrexate (Ryser & Shen, 1980; Chu & Howell, 1981), bovine or human serum albumin-methotrexate (Chu et al., 1981; Kopeček & Kopečková, 1985), DNA-daunomycin (Deprez-de Campeneere & Trouet, 1980), human serum albumin-daunomycin (Trouet et al., 1982). Many of these drug conjugates are potent cytotoxic agents in vitro and in certain cases have been used to circumvent drug resistance (Ryser & Shen, 1980). Some also display increased therapeutic efficiency in vivo (Trouet et al., 1982).

Soluble synthetic copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) developed as drug-carriers have previously been described (Kopeček & Duncan, 1987a; Kopečková et al., 1985a). They can be synthesized to include peptide side-chains for drug attachment and release (Duncan et al., 1983; Rejmanová et al., 1983) and also for attachment of targeting moieties, e.g., carbohydrates (Duncan et al., 1986) or antibodies (Rihová & Kopeček, 1985). Such copolymers containing daunomycin (DNM) and puromycin were shown to be toxic to mouse and human leukaemia grown in vitro (Duncan et al., 1987) and degree of toxicity found to correlate with biodegradability of the drug-polymer linkage, and to the presence of residues known to promote cellular uptake. Similarly, melphalan-HPMA copolymers were shown to be toxic (although less so) to L1210 in vitro (Ulbirch et al., 1987). In addition, HPMA copolymers containing DNM and anti β antibodies show antibody-dependent toxicity to T lymphocytes in vitro and in vivo (Rihová et al., 1986).

To evaluate their potential for clinical use HPMA copolymer-DNM conjugates have been tested against L1210 leukaemia in DBA mice. Mice bearing L1210 (i.p. or s.c.) were treated intraperitoneally with free DNM or HPMA copolymer-DNM conjugates (chemical structures shown in Figure 1 and Table 1). Animal weight and survival-time was

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monitored. Drug conjugates containing non-degradable drug-polymer linkages (Gly-Gly) or biodegradable linkages (Gly-Phe-Leu-Gly) were synthesized and certain polymer conjugates contained in addition carbohydrate residues. Conjugates containing fucose were prepared as L1210 cells are known to possess a cell surface receptor which recognises fucose (Monsigny et al., 1984). In addition we have shown that incorporation of galactose into HPMA copolymers effectively targets the polymer to hepatocytes in vivo (Duncan et al., 1986) and human hepatoma, HepG2, in vitro (O'Hare et al., in preparation). Therefore the antitumour activity of an HPMA copolymer containing galactosamine was also investigated.

Although this study represents the first investigation of pharmacological activity of HPMA copolymer-DNM, it was also considered important to investigate the body distribution of polymer-drug for comparison with that of free drug. Also to investigate the degradation of the polymer-drug conjugates in vivo. Previous measurement of conjugate stability had been made in vitro. The body distribution of radiolabelled drug ([3H]DNM) and radio-labelled polymeric carrier (125I-labelled or [3H]DNM was followed over 7 days after i.p. administration.

Materials and methods

Chemicals

1-Aminopropan-2-ol, methacryloyclohexyl chloride, glycyglycine, dimethylsulphoxide (DMSO) and 4-nitrophenol were from FLUKA AG, Buchs, Switzerland. Glycylphenylalanine, leucylglycine, phenylalanylleucine, tyrosinamide, L-fucosylamine, D-galactosamine and daunomycin were from Sigma Chemical Co., Poole, Dorset, U.K. [125I]Iodide (preparation IMS.30) was from Amersham International, UK and [3H]DNM (preparation NET-582; 2.2 Ci mmol⁻¹) was from New England Nuclear, Boston, USA. Liquisint was from National Diagnostics, Somerville, New Jersey, USA.

Preparation of polymers

HPMA copolymer-DNM conjugates were prepared as described previously (Kopecek et al., 1985b; Duncan et al., 1987). Briefly, the conjugates were synthesized using a two-step procedure. Polymeric precursors (Table I) were prepared by radical precipitation copolymerization of HPMA, MA-TyrNH₂ (N-methacryloyltyrosinamide) and the respective N-methacryloyloligopeptide p-nitrophenyl (ONp) ester (Kopecek, 1977; Kopecek & Rejmanová, 1979). DNM, galactosamine and fucosylamine were subsequently bound to these polymeric precursors by consecutive aminolysis (Kopecek et al., 1985b; Duncan et al., 1987). The structure and chemical characteristics of the polymers used in this study are shown in Figure 1 and Table I. All HPMA copolymer-DNM conjugates were purified by dissolving the polymer in methanol and applying to a Sephadex LH-20 column (2 × 95 cm, eluant methanol). The high molecular weight fraction was isolated and the methanol evaporated. Polymers were subsequently dissolved in water and freeze-dried. All contained <0.1 relative % of free DNM compared with the amount of bound DNM.

The HPMA copolymers containing [3H]DNM (samples 7 and 8) were prepared as follows:

| Polymer code no. | Precursor used | Side-chain structure | Substitution (mol %) | Drug content (wt %) |
|-----------------|----------------|---------------------|---------------------|---------------------|
| 1               | P-TyrNH₂       | ~1                  |                     |                     |
| 2               | P-TyrNH₂       | ~1                  |                     |                     |
| 3               | P-GlyGlyONp    | 3                   |                     |                     |
| 4               | P-GlyPheLeuGly| 3                   |                     |                     |
| 5               | P-GlyPheLeuGly| 3                  |                     |                     |
| 6               | P-GlyPheLeuGly| 3                   |                     |                     |

Table I Chemical characteristics of the HPMA copolymers

All copolymers contained TyrNH₂, ~1 mol% to permit radiolabelling. *M₆ of polymeric precursors 1 and 2 were 21,000 and 17,000, respectively; M₆/M₈ was 1.3 in both cases. These values were determined after aminolysis of the polymeric precursors with 1-aminopropan-2-ol using Sepharose 4B/6B (1:1) column chromatography (1.6 × 90 cm). A 0.05 M TRIS buffer containing 0.5 M NaCl (pH 8.0) was used. The column was calibrated using fractions of poly(HPMA); "The amount of bound DNM was estimated spectrophotometrically (ε₆₀₀ = 9.8 × 10³ in H₂O); *Fucosylamine content was extrapolated from the p-nitrophenol release during aminolysis of the polymeric precursor with fucosylamine; *Galactosamine content was estimated as previously described (Plummer et al., 1976; Cheng & Boal, 1978).
Organic compounds were routinely homogenized to a known volume in water or, in the case of stomach and other tissues from the gastrointestinal tract, dissolved in a known volume of 1 M NaOH. All samples, including urine and faeces (dispensed to a known volume) were assayed for radioactivity. The total radioactivity recovered from each organ, urine, faeces and blood (assuming a blood volume equivalent to 5.77 ml/100 g of mouse (Dreyer & Ray, 1910) was calculated and these values summed to give the total radioactivity recovered from the body at each time. This value was also expressed as a percentage of the dose administered.

**Body distribution of [3H]HDN conjugates**

Body distributions were assessed essentially as described above. Under ether anaesthetic, DBA, mice were injected intraperitoneally with [3H]HDN (0.1 ml, ~2.5 μg), sample 7 (0.2 ml, ~260 μg conjugate), or sample 8 (0.2 ml, ~260 μg conjugate). The animals were allowed to recover and maintained in metabolic cages for 1, 5 or 24 h. At the end of the experimental period, a 50 μl blood sample (dispersed in 1 M NaOH, 1.0 ml) was taken, the animal sacrificed and organs (as described above) removed. All samples were prepared as described previously and assayed for radioactivity. Homogenates (0.5 ml) and samples of urine, faeces and blood (0.5 ml) were mixed with 4.5 ml of a complete scintillant cocktail (Liquiscint) and counted for 10 min. Each sample was subsequently spiked with a [3H] standard (~20,000 cpm added to each vial) and re-counted in order to assess the extent of quenching in each sample. The measured radioactivity in each sample was then corrected for the degree of quenching and the body distribution expressed in the same way as described for 125I-labelled polymers.

**Analysis of radioactivity recovered from urine and peritoneal washings using Sephadex G-25 chromatography**

Following certain body distribution experiments radioactivity recovered in the urine or peritoneal washings was subjected to Sephadex G-25 chromatography. Samples were applied to a disposable PD-10 column (Pharmacia) and eluted with 0.05 M sodium acetate (0.5 ml fractions). The columns were calibrated with blue-Dextran, 125I- and [3H]HDN.

**Results**

DBA, mice inoculated i.p. with 10⁶ L1210 cells died consistently after 14-17 days (Figures 2-5; Tables II-IV). Over the experimental period these animals showed a steady weight gain (Figure 2) up to ~140% of the starting weight.
Table II Survival of DBA, mice bearing i.p. L1210 leukaemia following i.p. treatment with DNM

| Treatment (mg kg\(^{-1}\)) | Day of death | Mean survival (\(\pm\) s.e.) | Long term survivors |
|-----------------------------|--------------|------------------------------|---------------------|
| None                        | 15, 15, 16, 16, 17 | 15.8 \(\pm\) 0.4            | 0/5                 |
| DNM 0.25                    | 14, 15, 15, 20, 22 | 17.2 \(\pm\) 1.6 NS         | 0/5                 |
| DNM 0.5                     | 17, 21, 21, 22, 27 | 21.6 \(\pm\) 1.6*           | 0/5                 |
| DNM 0.75                    | 19, 19, 21, 22, 22 | 20.6 \(\pm\) 0.7**          | 0/5                 |
| DNM 2.0                     | 15, 15, 19, 19, 23 | 18.4 \(\pm\) 1.4 NS         | 0/5                 |
| DNM 5.0                     | 10, 10, 10, 13, 13 | 11.2 \(\pm\) 0.7**          | 0/5                 |

*DNM was administered on day 1 after i.p. inoculation of 10\(^5\) L1210 cells.

**P<0.001; \*P<0.01.

Administration of a single, i.p. dose of free DNM (0.25–5.0 mg kg\(^{-1}\)) had a dose-dependent effect indicated by marked weight change (Figure 2). A dose of 2 mg kg\(^{-1}\) produced slight weight loss, 5 mg kg\(^{-1}\) caused rapid weight loss (to 76% of the starting weight), whereas lower doses of drug, 0.75, 0.5 and 0.25 mg kg\(^{-1}\), did not prevent tumour-associated weight gain. Although certain doses did produce a significant increase in the mean survival time (Table II), the maximum increase was limited to \(\sim 135\%\) of that recorded for the untreated control group.

Initial experiments carried out to evaluate the effectiveness of HPMA copolymer-DNM conjugates used a treatment regime comprising three i.p. doses of conjugate given on days 1, 2 and 3 after i.p. inoculation of 10\(^5\) L1210 cells. Survival times (Figure 3 and Table III) and the weight change of animals treated with conjugates (Figure 4) are compared with untreated controls and those treated with free drug (Figure 2).

Figure 3 Effect of HPMA-daunomycin on the survival of DBA, mice inoculated i.p. with 10\(^5\) L1210 cells. L1210 cells were administered on day 0 followed by DNM, or HPMA copolymer-DNM given i.p. on days 1, 2 and 3. Panel (a) shows the survival of untreated mice (——) and mice treated with DNM, 2 mg kg\(^{-1}\) (----) or 5 mg kg\(^{-1}\) (---). The survival of animals treated with conjugate 3 (d), conjugate 4 (b) and conjugate 5 (c) is also shown.

Table III Survival of DBA, mice bearing i.p. L1210 leukaemia following i.p. treatment with HPMA copolymer-DNM conjugates (3 doses)

| Treatment | Mean survival (\(\pm\) s.e.) | Long term survivors |
|-----------|------------------------------|---------------------|
| None      | 14, 15, 16, 17, 18, 19       | 16 \(\pm\) 0.6      | 0/5                 |
| DNM       | 10, 10, 10, 10, 10, 10       | 17.5 \(\pm\) 0.5 NS | 0/5                 |
| P-Gly-Leu-DNM | 24, 38                     | 2.5                 | 0/5                 |
| P-Gly-Leu-Gal-DNM | 21, 18                   | 25                  | 0/5                 |

**P<0.001; \*P<0.01.

DNM (5 mg kg\(^{-1}\)) is given i.p. 24 hr following inoculation. The survival period was terminated at 30 days.
changes of the animals in each group (Figure 4) are shown. Free DNM (3 doses) at a daily dose of 2 or 5 mg kg\(^{-1}\) was unable to prolong survival time; indeed the higher dose significantly decreased life expectancy (Figure 3). Administration of HPMA copolymer-DNM conjugates affected the survival time of L1210-bearing mice, in a manner related to the conjugate composition (Table III). The non-degradable P-Gly-Gly-DNM conjugate (sample 3) caused no significant increase in lifespan. In contrast the biodegradable conjugates containing P-Gly-Phe-Leu-Gly-DNM (samples 4, 5 and 6), produced an increase in the mean lifespan (of those animals that died during the course of the experiment) and, in certain experiments, a number of animals survived.

Weight changes observed in mice treated with either free, or conjugated, DNM were consistent with their measured survival-times (Figure 4). The animals treated with free DNM (3 doses in this case) all lost weight and died early in the experiment. Both those treated with the non-biodegradable conjugate (sample 3), and the untreated control group, gained weight rapidly, and also showed visual evidence of their rapidly growing peritoneal ascites. However, animals treated with biodegradable HPMA copolymer-DNM conjugates (with or without L-fucosylamine) showed little or no weight-change. Those animals dying within the experimental period did eventually succumb to the tumour.

Effect of the timing, and size of the dose administered is shown in Table IV. A single dose of P-Gly-Phe-Leu-Gly-DNM (sample 4) given on day 1 or day 3 was sufficient to increase the mean survival-time considerably. However, when administered on day 5 or day 8 a single dose of sample 4 produced no significant increase in mean survival time. Administration of sample 4 at different doses on day 1 or day 3 (Table IV, experiments 1 and 3) showed doses in the range 5.0-20 mg kg\(^{-1}\) to be similarly effective (with a slight increase in activity as the dose administered increased).

**Table IV** Survival of DBA\(_2\) mice bearing i.p. L1210 leukaemia after i.p. treatment with DNM or HPMA copolymer-DNM. Effect of dose and timing of administration

| Experiment | Sample no. | Polymer side-chain | Dose (mg kg\(^{-1}\)) | Day of admin. | Day of death | Mean survival (± s.e.) | Long term survivors |
|------------|------------|---------------------|-----------------------|---------------|--------------|-----------------------|---------------------|
| 1. Administration on days 1 or 3 | None | – | – | 15, 15, 15, 15, 15, 15, 15, 15, 16, 16 | 16.4±0.6 | 0/10 |
| DNM | 4 | P-Gly-Phe-Leu-Gly-DNM | 5.0 | 1 | 15, 15, 15, 15, 16, 23 | 17.0±1.5NS | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 2.5 | 15, 17, 19, 20, 20, 20, 22 | 19.1±0.8* | 0/8 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 5.0 | 21, 24, 28, 28, 30, 39 | 27.8±3.3*** | 1/8 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 7.5 | 21, 23, 24, 24, 26, 27, 43 | 26.8±2.8*** | 1/8 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 10.0 | 3 | 21, 22, 25, 28, 31, 32 | 26.5±1.9*** | 0/6 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 15.0 | 27, 28, 29, 35, 38, 38 | 32.5±2.1** | 2/8 |
| P-Gly-Phe-Leu-Gly-Fucosylamine | 5 | P-Gly-Phe-Leu-Gly-Fucosylamine | 7.5 | 25, 26, 26, 32, 32, 34 | 29.2±1.6*** | 0/6 |
| P-Gly-Phe-Leu-Gly-DNM | 5 | 3 | 28, 31, 33 | 30.7±1.5*** | 4/6 |
| 2. Administration on days 1, 5 or 8 | None | – | – | 15, 15, 17, 18, 18 | 16.6±0.7 | 0/5 |
| DNM | 4 | P-Gly-Phe-Leu-Gly-DNM | 2.0 | 1 | 19, 19, 22, 22, 23 | 21.0±0.8** | 0/5 |
| HPMA | 2.0 | 1 | 12, 17, 18, 18, 23 | 17.6±1.7NS | 0/5 |
| DNM | 4 | P-Gly-Phe-Leu-Gly-DNM | 5.0 | 1 | 19, 20, 22, 22, 25 | 21.6±1.0** | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 5.0 | 15, 17, 17, 18, 18 | 17.0±0.5NS | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 5.0 | 8 | 17, 17, 18, 18, 18 | 17.6±0.2NS | 0/5 |
| 3. Administration on day 3 | None | – | – | 16, 17, 17, 17, 17 | 16.8±0.2 | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 5.0 | 3 | 20, 24, 25, 34 | 25.8±3.0* | 1/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 7.5 | 3 | 16, 19, 19, 23, 25 | 20.4±1.6* | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 10.0 | 3 | 17, 22, 26 | 21.6±2.6* | 2/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 15.0 | 3 | 20, 23, 24, 26, 33 | 25.2±2.2*** | 0/5 |
| P-Gly-Phe-Leu-Gly-DNM | 4 | 20.0 | 3 | 20, 21, 25, 26 | 23.0±1.1*** | 0/5 |

*\(P<0.05\); **\(P<0.01\); ***\(P<0.001\).
In contrast the lower dose of 2.5 mg kg\(^{-1}\) was totally ineffective.

Sample 2 (containing L-fucosylamine) was not obviously more effective than sample 4 when given on day 1. However, when administered on day 3, sample 5 appeared more active producing a larger number of long term survivors. Administration of polymer-bound DNM up to doses of 20 mg kg\(^{-1}\) produced no visual signs of toxicity and measured animal weights in these experiments did not show any signs of sudden weight loss such as those associated with acute DNM toxicity (cf. Figures 2 & 5).

Untreated animals inoculated s.c. with L1210 died with a mean survival time of 16-20 days (Table V). Again free DNM did not improve this situation. Although, polymer-bound DNM (Sample 4) administered i.p. was not effective at 5 mg kg\(^{-1}\) (a concentration shown previously to act against i.p. tumour), concentrations of 10-20 mg kg\(^{-1}\) significantly increased the mean survival time and also give rise to long term survivors.

\(^{125}\)I-Labelled samples 4 and 5, administered i.p. showed different body distributions during the first 24 h (Figures 5 & 6). Both left the peritoneal cavity rapidly, 50% leaving within the first hour. However, the polymer containing L-fucosylamine residues (sample 5) seemed to pass more readily from the bloodstream into the kidney. At 24 h both polymers showed an almost identical body distribution, with 76% of radioactivity recovered from sample 5 in kidney or urine, and likewise 65% of sample 4 (Figure 7). Sephadex G-25 chromatography of urine collected during the 24 h following i.p. administration of \(^{125}\)I-labelled sample 5 showed >60% of radioactivity in urine to be in macro-molecular form (Figure 8). After 7 days almost all of the administered sample 4 and 5 had been excreted.

\(^{125}\)I-Labelling of tyrosinamide residues in the polymer backbone is limited as it permits only tracing of the polymeric carrier. Studies were therefore carried out using \(^{3}H\)DNM and HPMA copolymers containing \(^{3}H\)DNM to monitor drug fate, both in free and conjugated form. After i.p. administration, body distributions were assessed (1, 5 and 24 h) and results obtained are shown in Table VI. Free \(^{3}H\)DNM showed substantial association with the intestine (49% of the radioactivity recovered after the first hour), and subsequently radioactivity appeared in the faeces. Both \(^{3}H\)DNM and sample 7 showed some evidence of liver association, and this was particularly noticeable in the case of D-galactosamine-containing conjugate (sample 7), 32% of the radioactivity recovered being detected in the liver after 1 h and (29%) after 5 h. Sample 8 did not accumulate to any significant extent in the liver.

It is interesting to compare the rate of excretion of free

Table V Survival of DBA\(_2\) mice bearing s.c. L1210 leukaemia following i.p. treatment with DNM or HPMA copolymer-DNM

| Treatment* | Dose (mg kg\(^{-1}\)) | Day of death | Mean survival time (±s.e.) | Long term survivors | Increase in mean survival time (%) |
|------------|-----------------------|--------------|----------------------------|---------------------|----------------------------------|
| No (Expt. 2) | 16, 19, 23, 23 | 20.5±1.1 | 0/6 | - |
| DNM (Expt. 2) | 5, 9, 9, 10, 16 | 12.2±2.9* | 0/5 | 60 |
| Sample 4 (P-Gly-Phe-Leu-Gly-DNM) | 20, 23, 24, 35 | 25.2±2.5NS | 0/5 | 123 |

*DNM and HPMA copolymer-DNM were administered on days 1, 2 and 3 after tumour inoculation; *P<0.05.

Figure 5 Effect of dose on animal weight after i.p. administration of P-Gly-Phe-Leu-Gly-DNM to DBA\(_2\) mice bearing i.p. L1210. L1210 cells were administered i.p. on day 0 and HPMA copolymer-DNM on day 3; no treatment (○); 5 (□); 10 (△); 20 mg kg\(^{-1}\) (□). The percent weight change is shown and also the day of death of the last surviving animal in each group.
ACTIVITY OF HPMA-DAUNOMYCIN CONJUGATES IN VIVO

Figure 6 Body distribution of radioactivity after i.p. injection of $^{125}$I-labelled copolymer 4 to DBA$_2$ mice. The radioactivity recovered in each organ was expressed as a percentage of the total radioactivity recovered from the animal (mean ± s.e. of 3 animals). Radioactivity recovered from the peritoneal cavity (○—○), urine and faeces (■—■), blood (●—●), kidney (▲—▲), gastrointestinal tract (□—□) and liver (△—△) is shown.

Figure 7 Body distribution of radioactivity after i.p. injection of $^{125}$I-labelled copolymer 5. The radioactivity recovered in each organ was expressed as a percentage of the total radioactivity recovered from the animal (mean ± s.e. of 3 animals). Radioactivity recovered in the various organs is indicated according to the key shown in the legend to Figure 6.

and conjugated $[^3]$HJDNM. For free $[^3]$HJDNM, 46% of the radioactivity recovered after 24 h was in the urine and faeces. This compares with 72% of sample 7 and 73% of sample 8. If these results are related to the dose administered, only 10.2% of free $[^3]$HJDNM is excreted within 24 h compared with 29% and 35% of samples 7 and 8 respectively. Sephadex G-25 chromatography of peritoneal samples 1 h after administration showed two peaks corresponding to either low or high molecular weight material and, representing $[^3]$HJDNM and conjugate respectively (Table VII).

After 24 h free $[^3]$HJDNM was detectable in the urine samples of animals given either free drug or sample 7 (Figure 9). Correspondingly little free $[^3]$HJDNM was detected in the urine of animals given sample 8.

Figure 8 Sephadex G-25 elution of mouse urine collected 24 h after i.p. administration of $^{125}$I-labelled copolymer 5 procedure is given in Materials and methods. Elution of dextran blue ($V_o$) and $^{125}$I- is shown.

Figure 9 Sephadex G-25 elution of mouse urine collected 24 h after i.p. administration of (a) $[^3]$HJDNM; (b) sample 7; (c) sample 8. Procedure is given in Materials and methods. Elution of dextran blue ($V_o$) and $[^3]$HJDNM is also shown.
Discussion

Modification of anthracycline antibiotic disposition with the aid of drug-carriers can produce an increase in a drug’s therapeutic index, and in certain cases overcome cell resistance (Yanovich et al., 1984). This has been demonstrated with anthracyclines using a number of different carriers, including liposomes (Fichtler et al., 1984), low density lipoprotein (Yanovich et al., 1984), and DNA or protein carriers (Trouet & Jolles, 1984). In this study we have shown that a soluble synthetic polymer may also have potential as a carrier of anticancer agents. Administration of DNM bound to HPMA copolymer, via a lysosomally degradable tetrapeptide (Gly-Phi-Leu-Gly), increased the mean survival-time (relative to untreated controls) of DBA2 mice bearing L1210 leukaemia and also produced a number of animals surviving long-term (Figure 3 and Tables III & IV). These data are in accord with previous studies describing successful treatment of i.p. tumours using i.p. administration of degradable drug conjugates: non-covalently linked DNA (Deprez-de Campeneere & Trouet, 1980), polyl-aspatic acid and polyl-L-lysine (Zunino et al., 1984), poly-L-aspatic acid (Pratesi et al., 1985) and succinylated fetuin or albumin (Trouet et al., 1982).

There are at least two mechanisms of action of macro-molecular drug conjugates (Duncan, 1987). Conjugation of a drug alters its pharmacokinetics and this simple procedure can exclude drug from the principle sites of toxicity thus improving the therapeutic index considerably. Secondly, conjugation limits capture of drug to the cellular uptake process of pinocytosis, affording potential to target to particular cells using cell-specific surface receptors or antigens to promote capture by receptor-mediated pinocytosis. Internalization of a drug-conjugate in this way ultimately results in its exposure to lysosomal enzymes and therefore lysosomally degradable conjugates can liberate drug intracellularly following uptake. As yet there are no convincing examples of significant, tumour-specific, drug targeting, even with the use of tumour-specific monoclonal antibodies as drug-carriers. Most drug conjugates currently proposed probably exert their effect by modulation of the body distribution, and rate of excretion, of the attached agent.

Throughout this investigation sample 3, P-Gly-Gly-DNM, shown previously (Kopeck et al., 1987a) to be non-biodegradable, was found to be completely inactive. In contrast samples 4, 5 and 6, containing biodegradable drug-carrier linkages, all displayed marked antitumour activity. The stability of sample 8 was confirmed as almost all the radioactivity recovered in the urine after i.p. administration was macromolecular, i.e., polymer-bound DNM. In contrast Sephadex G-25 chromatography of urine following administration of sample 7 showed a large peak of low molecular weight material coeluting with DNM. (Figure 9.) These observations suggest that drug release from HPMA copolymer conjugates by enzymic hydrolysis is a prerequisite for pharmacological activity. Inability of rat plasma and serum to hydrolyse such HPMA copolymers (Rejmanová et al., 1985); and the known ability of lysosomal enzymes to cleave these substrates (Rejmanová et al., 1983; Duncan et al., 1983) suggests that drug release occurs intracellularly. Trouet et al. (1982) showed that albumin-DNM, and albumin-Ala-Leu-DNM, were much less active against L1210 in vivo than albumin-Ala-Leu-Ala-DNM, an observation that was attributed to resistance of the former to hydrolysis by lysosomal enzymes. However, ability of anthracycline
ANTIBIOTICS TO INDUCE TOXICITY VIA INTERACTION WITH THE CELL SURFACE SHOULD NOT BE OVERLOOKED. Tokes et al. (1982) and Tritton & Lee (1982) attached Adriamycin to polyglutaraldehyde microspheres, and agarose respectively, and were able to show that non-penetrating drug formulations can be toxic to tumour cells in vitro, including L1210 leukaemia. Previous in vitro studies with HPMA copolymer conjugates (Duncan et al., 1987) showed differential toxicity of polymer conjugates containing stable or biodegradable DNMPolymer linkages to L1210 cells. The non-degradable conjugates were slightly toxic, but much less so than biodegradable ones, a factor attributed to cell-surface activity. In these in vivo studies lack of activity of non-degradable sample 3 implies that the observed antitumour activity of biodegradable drug-conjugate is not simply caused by cell surface interaction.

Inoculation of tumour intraperitoneally, followed by i.p. treatment may apparently confine the tumour and treatment to one body compartment, thus biasing the investigation in favour of tumour targeting, or tumour killing. However, polymer-DNM administered i.p. was also shown to be active against s.c. implanted tumour (Table V) and the ability of the polymeric conjugate to act at a site remote from the point of administration is consistent with the short half-life of radiolabelled HPMA copolymer-DNM conjugates in the peritoneal cavity (Figures 6 & 7), demonstrated previously (Scriver et al., 1970), to be independent of polymer-molecular weight over the range 10,000-800,000. HPMA copolymer-drug conjugates, either 125I-labelled or containing [3H]DNCM were eliminated from the body more readily than [3H]DNCM (Table VI; Figures 6 & 7). Taking into account total recovery of radioactivity in respect of administered dose, the rate of excretion of [3H]DNCM was more than three times greater when attached to polymer. Attachment of DNM to polymer prevents random intracellular access and therefore rapid binding of drug to intracellular constituents such as DNA, and as the molecular weight of the polymer conjugate is sufficiently small to pass across the kidney glomerulus any conjugate resident in, or passing into, the circulation was quickly removed. This may in part explain the decreased toxicity of polymer-bound drug. (Doses of polymer-bound drug up to 20 mg/kg being administered without any obvious ill effect.) The fact that polymer-bound anthracyclines show less toxicity to human lymphocytes and mouse spleen cells in vitro (Rihová, unpublished) and are probably less cardiotoxic will also contribute to the overall reduction in toxicity of conjugated drug. It is noteworthy that the more rapid excretion of a dose of polymer-drug is still accompanied by a measurable therapeutic response.

The HPMA copolymer bearing [3H]DNCM and galactosamine (sample 7) showed greater association with the liver (Table VI) than polymer without the sugar, almost certainly due to receptor-mediated pinocytic uptake by hepatocytes (Ashwell & Harford, 1982; Duncan et al., 1986). DNCM subsequently released from this intracellular depot was effective against L1210 inoculated i.p. (Table III). This is perhaps surprising as the liver is known to be the primary site of DNCM metabolism (Craddock et al., 1973) and it could be predicted that any DNCM released from hepatocyte lysosomes might be inactivated. This is clearly not the case and these observations suggest that targeted HPMA copolymers bearing anthracyclines have potential, following i.v. bolus administration, as a controlled release depot in the liver. Anthracyline released could be used to treat primary (known also to retain the galactose-recognising receptor, Schwartz et al., 1982) and secondary liver cancer. Two lethal diseases. HPMA copolymer-DNCM conjugates administered i.v. to mice were not non-specifically hepatotoxic as measured by mouse weight and their plasma transaminase/alkaline phosphatase levels (McCormick et al., 1987).

Substitution of conjugates with the other carbohydrate residue fucosylamine, did not increase the mean survival of animals in comparison with treatment using non-carbohydrate conjugate (Table IV), the only exception being after administration of single doses (7.5 mg/kg−1) on day 3. The explanation of this observation is not clear, but could indicate greater expression of the fucose receptor during the rapid growth phase of the developing ascites.

Recent experiments have shown that HPMA copolymer-adriamycin conjugates are more effective than the described DNCM conjugates in prolonging life of DBA mice inoculated with L1210 cells (Kopeček & Duncan, 1987b). After i.v. administration they do not manifest toxicity until a dose of 75 mg kg−1. As this anthracycline is one of the most important clinical agents, such HPMA copolymer conjugates appears to have real therapeutic potential. Unlike many of the other drug-carriers evaluated in this context this polymer and its conjugates are not immunogenic (Rihová et al., 1984) and so can be administered repeatedly. Use of techniques in polymer chemistry to synthesise drug conjugates prevents either denaturation of the carrier or inactivation of drug.

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