Opinion

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A Rapid Fluorescent *In Vitro* Assay Suitable for Studying the Kinetics of O^6-Alkylguanine Lesion Progression to DNA Inter-Strand Cross-Links and the Kinetics of the Primary Lesion's Repair

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Introduction

In the author's opinion, by far the best assay for MGMT activity in cells is the assay developed by Ishiguro, et al. [1-3]. This assay is based on the transfer of radioactive benzyl residues from [benzene-^{3}H]O^6-benzylguanine to MGMT However, this assay is unsuitable for studying the kinetics of the progression of the primary O^6-alkylguanine lesion to DNA-DNA inter-strand cross-links, and the kinetics of the primary lesion's repair by MGMT, because the rate constants involved in the Ishiguro assay means it takes several hours to determine the MGMT activity, whereas many of the dynamic processes we are interested in occur on the time frames of minutes or even seconds. Thus they could not be readily resolved by the [Benzene-^{3}H]-O^6-benzylguanine method as the reaction kinetics of [Benzene-^{3}H]-O^6-benzylguanine with MGMT would be highly rate limiting in many cases. The rapid fluorescent *in vitro* assay, to study the kinetics of lesion formation and repair, was developed from our DNA cross-linking assay [4]. This assay allows for the determination of the fraction of DNA molecules containing one or more covalent inter-strand cross-links. These cross-linked DNA molecules rapidly renature upon snap cooling following thermal denaturation, because the cross-links hold the complementary DNA strands in close proximity and in register.

Since H33258 forms a highly fluorescent complex with double stranded but not mispaired/denatured DNA, DNA molecules containing cross-links, will yield highly fluorescent complexes following a heat/chill cycle with H33258 dye; whereas DNA molecules devoid of such cross-links do not. Since all assay steps are conducted at neutral pH values, potential problems caused by base catalyzed lesion hydrolysis are avoided [4]. The fluorescent *in vitro* assay outlined in this opinion piece allows for the *in vitro* study of the relative rates of the primary lesions' progression to cross-links and competing repair processes. This rapid assay technique also permits the investigation of the initial DNA alkylation event which is 1st order with respect to the concentration of the bis-sulfonyl hydrazine (BSH), and has $t_{1/2}$ values of <30s [5]. This initial reaction can thus be effectively quenched at any point by a 100-fold rapid dilution (see Figure 1) of the reaction mixture. The proportion of DNA molecules that already contained a guanine O^6-alkylguanine lesion prior to dilution step can then be determined by measuring the fraction of the DNA molecules that are cross-linked as described in [5]. The number of cross-linking moieties produced in the test DNA relates to the cross-linked fraction as follows: The average number of cross-links per DNA molecule (A), assuming a Poisson distribution, equals −ln(1-X), where X = the cross-linked fraction (the value obtained from the assay).

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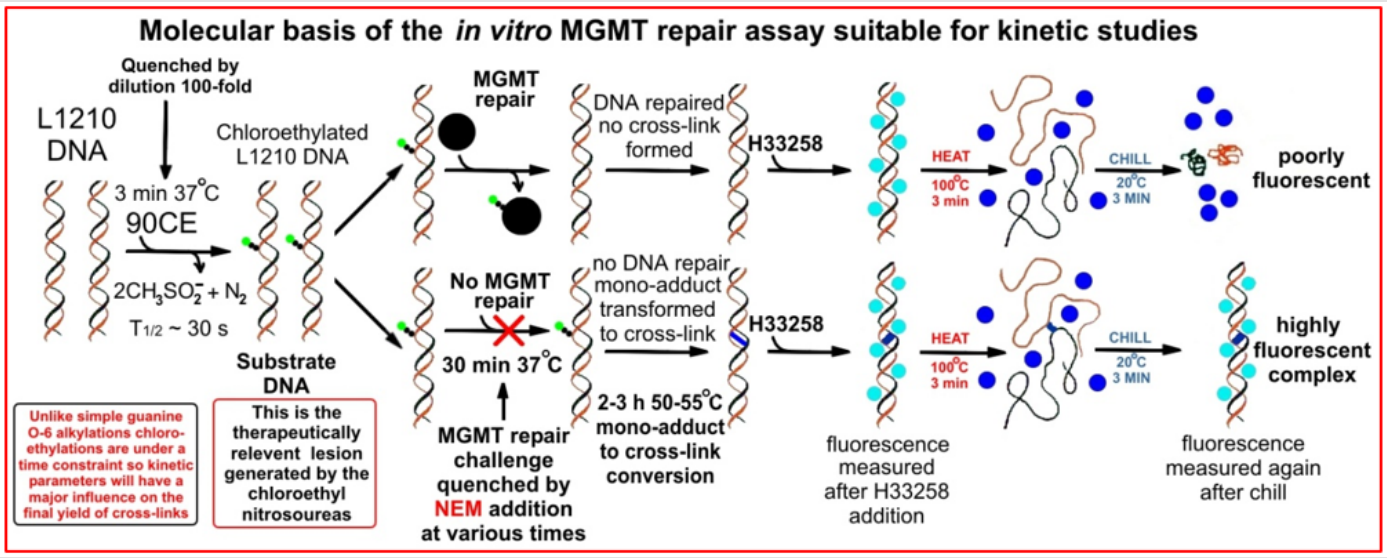

Figure 1.

Thus for a sample of test DNA with, for example, a cross-linked fraction (X) of 0.4, A calculates to be 0.51. The probability that a given DNA molecule has N cross-links equals $e^{-A} \times A^N/N!$; where N! equals factorial N. In this example, 60% of the DNA molecules would have 0 cross-links, 30.6% would have 1 cross-link, 7.8% would have 2, 1.3% would have 3, etc., to give a total of 40% of the DNA molecules containing one or more cross-links, with the average number of cross-links per DNA molecule being 0.51. Thus at low levels of cross-linking (<10%) X ≈ A, as very few molecules contain more than one cross-link. Our research team (now disbanded), designed modular drugs which were composed of domains with specific functions [6,7].

A. A targeting domain (e.g., a trigger for hypoxic region activation [6], or solid tumor targeting via the EPR effect [7].)

B. A linker domain which could also have a fuse function (time delay to allow drug accumulation [7]).

C. A BSH domain which functions as the warhead [6].

To efficiently design the modular nano-drug/particle, it is best to optimize each domain separately. This would require the BSH domain (C) to be optimized to maximize its differential toxicity between MGMT containing cells and those lacking MGMT activity. To do this one would have to maximize the relative repair window (i.e., slow the rate of the transition from the MGMT repairable cross-link precursor to the highly lethal and poorly tolerated DNA-DNA inter-strand cross-link [5]). Using our rapid fluorescent *in vitro* assay with NEM (1µL of a 1M solution in DMSO per 20µL of reaction mixture) quenching (see Figure 1), we were able to show that the cross-link precursor lesions generated by 90CE (1,2-bis(sulfonyl)-1-(2-chloroethyl) hydrazine) were essentially completely removed in ~20 seconds by a small molar excess of MGMT. This is an extreme rate for two reactants (MGMT and lesion) both at miniscule concentrations. In fact, it is greater than the maximum diffusion controlled reaction rate of (10^9 to 10^{10} M^{-1} s^{-1}) at 37 ºC and thus would be impossible for reactants in free solution where the molecules are free to diffuse in 3 dimensions. However, this reaction is occurring on a linear DNA molecule with the lesion in a fixed position like a sitting duck, and the MGMT molecule is free to walk/diffuse in a single dimension along the DNA molecule.

To avoid lethality the cell needs to have sufficient MGMT molecules to remove all the cross-link precursors before a small (<10/cell) but lethal number have progressed to cross-links [5]. If 90CE analogs were synthesized as potential warheads, e.g., by replacement of the chlorine moiety with pseudohalogens, or of the chloroethyl hydrogens by deuteriums, or replacement of the chloroethyl moiety with a chloroisopropyl moiety as described in [8], they would likely change both the rate of the transition of the cross-link precursor to a G-C ethane cross-link (1-(N^3-cytosinyl),-2-(N^1-guaninyl)ethane) and its repair rate by MGMT. Thus, the kinetics of these processes would need to be ascertained to ensure these modifications increased the magnitude of the repair window, and increased the differential toxicity between MGMT expressing and non-expressing cells to BSH warhead. An association between MGMT promoter methylation and tumorigenesis features in patients with ovarian cancer has been reported [9]. Since MGMT promoter methylation results in the silencing of the MGMT gene [3,6,10] it is likely that such tumors would show dramatic responses when treated with the described modular BSH nano-drugs and existing mildly targeted BSHs such as 101m, (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl] hydrazine)

[11,12]. This assay had been previously used to determine the inhibitory activities of 2-nitro-6-benzyloxypurine, an analog of O^6-benzylguanine, in which the essential 2-amino group is replaced by a nitro moiety and its reduction products. 2-Nitro-6-benzyloxypurine was found to be non-inhibitory (>2000-fold weaker than O^6-benzylguanine), whereas it's hydroxylamino and amino reduction products were potent inhibitors [13].

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